Ramón Aparicio · John Harwood *Editors*

Handbook of Olive Oil

Analysis and Properties

Second Edition

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ISBN 978-1-4614-7776-1 ISBN 978-1-4614-7777-8 (eBook)
DOI 10.1007/978-1-4614-7777-8
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2013943591

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Preface

Olive oil, which is perhaps the oldest reported crop in history, is the major edible oil of the Mediterranean countries and an important commodity in other countries such as New Zealand, Chile, and the USA. Olive oil is much prized for its nutritional benefits, but the highest-quality olive oil, which is obtained from fresh and healthy fruits without solvent extraction, is also appreciated because of its delicate and unique flavor.

In this book, we provide a wealth of detail about all aspects of olive oil in some 20 chapters. After an introduction to olive oil by the International Olive Council, the book focuses the attention on the new agricultural practices that have spread to olive orchards all over the world and increased olive oil yield per hectare with no loss of sensory and nutritional properties. This revolution in agricultural techniques is partly due to Mediterranean crossbreeding programs to obtain new cultivars that combine an optimal adaptation to high-density plantations and irrigation regimes with a juvenile behavior and resistance to diseases. This section of the book is followed by an updated chapter about olive oil processing. Today, modern olive mills extract virgin olive oil (VOO) by means of centrifugation systems because this allows high-quality oils to be obtained with less production cost and energy consumption. This environmentally friendly olive oil production, when combined with the simultaneous availability of olive by-products, is demanded by a society that is conscious of the need for environmental protection. Few recent agricultural and industrial advances would not have been possible without a better knowledge of biochemical, genetic, and molecular aspects of the olives that are analyzed in the following two chapters. The consequence is that VOO's unique chemical composition is made not only of well-balanced fatty acids but also of a plethora of minor compounds, which allows chemical characterization and explains the huge variety of aromas, tastes, and colors that VOO can offer to consumers. Prior to describing the sensory assessment of VOO and the most updated developments in traceability,

the book analyzes, with practical examples and descriptive tables, the instrumental advances for the identification and quantification of VOO chemical composition by means of separative and non-separative techniques (e.g. chromatography vs. spectroscopy).

Despite the numerous in-house and official methods described in the previous chapters, which make olive oil one of the most strictly regulated food products, it is still a preferred target for defrauders because of its high price and reputation among consumers. In the current context of the olive oil authenticity, to which a chapter is devoted, the purpose is to detect whether an olive oil is genuine not only to confirm that it has not been spiked with cheaper edible oils, according to established limits for analytical parameters, but also to validate that the geographical origin and botanical variety are as described on the label.

The demand for olive oil has increased significantly due to the discovery that olive oil can be used as an "everyday" cooking oil by consumers. Thus, understanding consumer attitudes to olive oil is crucial for the success of this market in the future. The acceptability depends on sensory properties, but it is also affected by cognitive, social, and cultural variables. The chapter about consumers is intended to introduce the most appropriate methods to understand how they perceive olive oil, and gives clues to revise opinions and consumption strategies for market prospects in the next decade.

The chemical characteristics of olive oil, one of the main ingredients of the Mediterranean diet, also support the unquestionable healthy food claim as a consequence of the balance between mono- and polyunsaturated fatty acids and the bioactivity of minor compounds such as phenols, squalene, and triterpenic alcohols. Two chapters point out facts and perspectives regarding the protective effect of olive oil intake on cardiovascular diseases and other pathologies, although further research is needed within the emergent discipline of nutrigenomics that may help in explaining those properties that give health benefits.

The penultimate chapter describes different steps of the refining process for making olive oil fit for consumption. The refining process allows the removal of color, odor, or flavor of those oils that are unacceptable to consumers or the removal of chemical compounds that might affect olive oil stability. There is a need to devise possible alternative uses of the by-products obtained from the refining process. Global trends show that green products and technologies are needed and more efforts should be carried out in that direction.

The book ends with a description of the chemical composition of virgin olive oils from diverse cultivars that are harvested all over the world. The intention is to provide the average values of the main chemical compounds of olive oils from diverse olive oil–productive regions and countries and also to point out possible changes in the chemical composition of the same varietal olive oil when cultivated in different geographical regions.

We have strived, through the recruitment of world experts in the field, to ensure that the book is authoritative and up to date. The importance of edible oils

throughout the world is predicted to increase steadily, and olive oil is no exception. Moreover, with the increasing perception of developed nations regarding the high quality of olive oil as well as its nutritional desirability, there undoubtedly will be more interest in its analysis. We hope that this book will prove to have widespread utility for such analyses and will contribute to a popular appreciation of the unique properties of olive oil.

Cardiff, UK — John Harwood
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Chapter 1
Introduction

Jean-Louis Barjol

Contents

1.1 Worldwide Economic Importance of Olive Oils

In the last 50 years, animal fats in all their guises have seen a rapid decrease in their relative share of world demand for fats and oils. Like other vegetable oils, olive oil has capitalised on this development, its production trebling from 1.032 million tonnes in 1958/1959 to around 3 million tonnes in 2009/2010, and a prevision of 3.4 million tonnes in 2011/2012. It now accounts for approximately 3 % of the world's output of vegetable fats and oils. However, this rise in production has not been smooth. Production increased gradually until 1994/1995, when it levelled out for three seasons at just over 1.8 million tonnes. At times, the crop bearing pattern of the olive in alternate years has had a heavy impact on production. For instance, the very large harvest of 1963/1964 was sandwiched between two poor harvests in 1962/1963

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties*,
DOI 10.1007/978-1-4614-7777-8_1, © Springer Science+Business Media New York 2013

and 1964/1965. Conversely, the very low level of production in 1981/1982 was flanked by two good harvests (1980/1981 and 1982/1983). The same thing occurred during the period between 1987/1988 and 1991/1992. After dipping slightly in 1995/1996, world production moved up a gear in 1996/1997, when it passed the 2.5 million tonne mark for the first time ever. From then onwards, production resumed its upward rate of growth, albeit in fits and starts, until it topped three million tonnes in 2004/2005 and 2005/2006, and again in 2009/2010. Looking ahead to the future, production looks poised to continue upwards at an annual rate of close to 3 % to meet demand from consumers motivated more and more by the nutritional assets and flavour of olive oil.

1.2 Distribution of World Production of Olive Oils

Production is distributed in three groups of countries: the 27-member EU, which dominates world production, accounting for an average 76 % share over the past 50 years; the 16 other current IOC member countries (Albania, Algeria, Argentina, Croatia, Egypt, Iran, Iraq, Israel, Jordan, Lebanon, Libya, Montenegro, Morocco, Syria, Tunisia and Turkey) with an average 22 %; and the rest of the world, with 2 %.

A more in-depth analysis of the situation inside the EU reveals that over 50 years, two countries have lost in significance to two others. Italy has seen its share of EU production shrink by an average of more than 10 points from around 40 % to 30 % by the end of the period. The same trend can be seen for Portugal, whose share has fallen from 7.3 % in the first decade to no more than 1.6 % in the most recent 10-year period. Moving in the opposite direction, Greece's share of EU production has climbed from 15 %to 20 %, although this has been dampened in recent years. Spain stands out as the most dynamic country over the 50 years, boosting its share of EU production from 43.2 % in the first decade to 51.9 % in the last. Even so, Italy and Spain remained more or less on a par for a long time. It was not until the mid-1990s that Spain clearly raced ahead, gaining an average extra 10-point share of EU production. In 2009/2010, this group of EU producing countries produced 2.255 million tonnes. Spain accounted for 1.401 million tonnes of this volume.

Outside the EU, but among the other current IOC members, Tunisia overshadows the other countries, although its slice of production is sagging in terms of 10-year averages (down by 7 points). The same applies to the production of the group of other IOC member countries comprising Argentina, Egypt, Israel, Jordan, Lebanon and Libya, which has decreased by 10 points albeit with a sudden spurt in the last decade, mainly due to the good performance of Jordan and Argentina. This ground has clearly been taken up by Syria (up by more than 17 points), as well as by Turkey, whose production has climbed by 41.2 % over the 50-year period, ranking it fifth in 2009/2010 behind Spain, Italy, Greece and Syria, and Morocco, whose production doubled in 2009/2010 to reach 160,000 t.

Taken as a whole, these countries produced 452,300 t of olive oil on average in the last decade. This breakdown of production shares should not make us overlook the fact that within the space of 52 years, the output of all the current IOC member countries (excluding the EU-27) has risen from 215,000 to 722,000 t in 2009/2010.

Outside the IOC member countries the other non-IOC countries of significance on the olive oil scene are Palestine, followed by Australia and Chile. Two other countries – Saudi Arabia and the USA – also produce olive oil, although not yet on a relevant scale.

1.3 Distribution of Worldwide Consumption of Olive Oils

This upward trend of production has been accompanied by a similar trend in consumption. The most striking characteristic is the sharp growth in the consumption share of the non-IOC member countries, which accounted for more than 20 % of world consumption in 2009/2010. In this group, the USA has experienced the most spectacular consumption growth in the last 20 years. Since its level of production is small, the USA has gradually become the leading world importer of olive oil. Although the EU share of world consumption has narrowed, consumption has recorded regular growth in Italy and Spain. The same thing has occurred in other EU non-producing countries. Amongst the other IOC member countries, consumption growth over the past 50 years has been most significant in Syria, Turkey and Morocco, and to a lesser extent in Algeria. In most of the other countries it has declined over the past 20 years, whereas in Tunisia it has held steady, although with occasional small oscillations. In terms of per capita consumption, Syria is ranked first in the group of non-EU member countries of the EU, followed at quite a distance by Algeria.

This review of past trends can only encourage the IOC to keep up its promotional efforts to find new catalysts for consumption growth in non-producing countries, following in the tracks of the USA. These efforts need to be accompanied by a policy to support consumption in traditional markets, which continue to be the main outlet for olive oil but where per capita consumption levels are still low except in the top trio of Greece, Spain and Italy.

1.4 International Trade in Olive Oils

From 1981/1982 onwards (with the notable exception of 1984/1985) the profile of EU exports became less jerky than previously. It traces a curve of regular growth marked, however, by a sudden change in scale in 1996/1997. Tunisia is the world's second exporter, behind the EU. The stable volumes exported in the 1970s and 1980s have increased considerably since then but swing extensively from year to year. Third place in the world export ranking goes to Turkey, whose exports have

exceeded 50,000 t since the 1990s, although its performance is still uneven. Most recently, the emergence of Syria as a regular exporter is also noteworthy, as is that of Morocco in 2009/2010.

The USA has spearheaded the striking exponential growth of imports in the last 20 years. The EU emerges as the world's second biggest importer, although its purchases fell in the 1980s and only regained their 1970s levels in the 2000s. The third biggest importer in the world is the group of non-producing countries (mainly Brazil, Canada and Japan), where imports started to move up in the 2000s. Despite producing olive oil, Libya was a major importer for 30 years, but now its imports are virtually nil.

In recent years the trend of world imports has been upwards, but tonnages remain very limited, standing at around 650,000 t in 2009/2010. The USA, EU-27, Brazil, Canada, Australia and Japan account for approximately 80 % of world imports.

1.5 Chemical and Organoleptic Composition of Olive Oil

Between 98 % and 99 % of olive oil is made up of triglycerides (triacylglycerols) (Chap. 6). It also contains free fatty acids, the proportion of which is variable and depends on the extent of triglyceride hydrolysis. But the fatty acid composition of olive oil varies according to variety, climatic conditions and production location (Chap. 12). The IOC trade standards for olive oil and olive-pomace oil and the Codex Alimentarius food standards have fixed the values for the distribution of the fatty acids in olive oil (Tables 16.6 and 16.7). Olive oil contains a high proportion of monounsaturated fatty acids. The most common percentage breakdown by type of fatty acid is saturated fatty acids (14 %), monounsaturated fatty acids (72 %) and polyunsaturated fatty acids (14 %).

The minor constituents of olive oil are characteristic indicators of its physico-chemical authenticity and lend it its distinctive sensory and biological properties:

- Squalene accounts for a large proportion of the terpenic hydrocarbons in olive oil (300–700 ppm), while beta-carotene, the biological precursor of vitamin A, is found in very small quantities (a few ppm);
- Triterpene alcohols (24-methylene-cycloarthenol together with cycloarthenol, and α- and β-amyrin) are particularly important from a biological point of view. Erythrodiol is also important, but from the analytical angle in detecting olive-pomace oil;
- Sterols are the analytical fingerprint that enables olive oil to be identified, notably because its apparent β-sitosterol content accounts for less than 93 % of total sterols. The IOC trade standard sets the limits for sterol composition (Table 1.1) as a percentage of total sterols;
- Tocopherols, notably α-tocopherol or vitamin E (150–300 ppm);
- Phenolic compounds with marked antioxidant properties, some of which are part of the distinctive flavour of olive oil;
- One hundred or so aromatic compounds, whose chemical composition varies according to the variety of olive, pedoclimatic conditions and quality of the oil.

Table 1.1 Limits for sterol composition (as a percentage of total sterols) according to IOC Trade Standard

Sterols	Limits (%)	Sterols	Limits
Cholesterol	≤0.5	Stigmasterol	<campesterol
Brassicasterol	≤0.1[a]	Δ7-stigmasterol	≤0.5 %
Campesterol	≤4.0	Apparent β-sitosterol	≥93.0 %[b]

[a]≤0.2 % for olive-pomace oils

[b]Apparent β-sitosterol comprises β-sitosterol, Δ^5-avenasterol, $\Delta^{5,23}$-stigmastadienol, clerosterol, sitostanol, Δ^{5-24}-stigmastadienol

1.6 The International Olive Council and Olive Oil Standardisation

The IOC is the intergovernmental organisation responsible for administering the International Agreement on Olive Oil and Table Olives, which has been negotiated at United Nations commodity conferences. Olive oil is the only commodity in the fats and oils sector to have its own international accord.

The international agreement is open to signature by members of the United Nations. At present, the Council has the following members: Albania, Algeria, Argentina, Croatia, Egypt, the EU (Austria, Belgium, Bulgaria, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, United Kingdom), Iran, Iraq, Israel, Jordan, Lebanon, Libya, Montenegro, Morocco, Syria, Tunisia and Turkey. In addition, the council has co-operative and working ties with a large number of UN organisations or agencies, as well as with other intergovernmental, governmental and non-governmental organisations.

The international agreement lays down the policy that members should take on the standardisation of the market for olive oil, olive-pomace oil and table olives. Amongst other things, this involves adopting international rules to determine the quality of the products on sale and to monitor international trading. By signing the agreement, members undertake to make whatever arrangements are necessary, in the manner required by their legislation, to ensure the application of a number of principles and measures. These refer to the designations (grade names) and definitions of olive oils and olive-pomace oils, the use of the designation ‘olive oil’, geographical indications, and the designations and definitions of table olives.

The application of these principles and provisions is compulsory in international trade and recommended in domestic trade. The members undertake to prohibit the use in their territories, for the purposes of international trade, of any designations that run counter to these principles. The Council takes whatever measures it considers necessary to curb unfair international competition, including by non-member states or their citizens. These principles and provisions are embodied in two standards: the trade standard applying to olive oils and olive-pomace oils and the trade standard applying to table olives. A list follows of the designations and definitions of olive oils and olive-pomace oils.

Olive oil is the oil obtained solely from the fruit of the olive tree (*Olea europaea* L.), to the exclusion of oils obtained using solvents or re-esterification processes and of any mixture with oils of other kinds. It is marketed in accordance with the following designations and definitions.

- *Virgin olive oil* (VOO) is the oil obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions, particularly thermal conditions, which do not lead to alterations in the oil, and which has not undergone any treatment other than washing, decantation, centrifugation and filtration. Virgin olive oil fit for consumption as is includes the following:
  - *Extra virgin olive oil* (EVOO) is a VOO which has a free acidity, expressed as oleic acid, of not more than 0.8 g per 100 g and whose organoleptic characteristics correspond to those fixed for this category in the trade standard.
  - *Virgin olive oil* is a VOO which has a free acidity, expressed as oleic acid, of not more than 2 g per 100 g and whose organoleptic characteristics correspond to those fixed for this category in the trade standard.
  - *Ordinary virgin olive oil* is a VOO which has a free acidity, expressed as oleic acid, of not more than 3.3 g per 100 g and whose organoleptic characteristics correspond to those fixed for this category in the trade standard.
  - *Virgin olive oil not fit for consumption* as is, known as *lampante virgin olive oil*, is a VOO which has a free acidity, expressed as oleic acid, of more than 3.3 g per 100 g or whose organoleptic characteristics correspond to those fixed for this category in the trade standard. It is intended for refining or for technical purposes.
- *Refined olive oil* is the olive oil obtained from VOOs by refining methods which do not lead to alterations in the initial glyceridic structure.
- *Olive oil* is the oil consisting of a blend of refined olive oil and VOO fit for consumption as is.
- *Olive-pomace oil* is the general term for the oil obtained by treating olive pomace with solvents, to the exclusion of oils obtained by re-esterification processes and of any mixture with oils of other kinds. Olive pomace is the residual paste left over from the production of VOO. It still contains a variable proportion of oil (5–10 %) depending on the system used to produce the VOO. It is marketed in accordance with the following designations and definitions:
  - *Crude olive-pomace oil* is intended either for refining with a view to being used in food for human consumption or for technical purposes.
  - *Refined olive-pomace oil* is the oil obtained from crude olive-pomace oil by refining methods which do not lead to alterations in the initial glyceridic structure.
  - *Olive-pomace oil* is a specific term for the oil comprising a blend of refined olive-pomace oil and VOO fit for consumption as is. This blend may not be called ‘olive oil’.

The IOC trade standard lays down minimum purity and quality criteria for each grade of olive oil and olive-pomace oil that is traded. It also lays down rules for

hygiene, packing and labelling and recommends methods of analysis for determining the various analytical parameters.

The methods recommended in the trade standard have been devised in one of three ways. Some have been developed by the ISO or IUPAC, and their applicability to olive oil has been checked by the group of chemists representing the laboratories and institutes that collaborate with the IOC in studying methods of olive oil and olive-pomace oil analysis. Other methods have been developed by national bodies and been approved by the IOC because their applicability to olive oil has been proven. Yet others have been drawn up in collaborative trials led by the IOC and approved after it has been demonstrated that their repeatability and reproducibility are acceptable. The latter is the case with the method for the organoleptic assessment of VOO and the accompanying standards on sensory analysis, general basic vocabulary, tasting glass, general methodology for the organoleptic assessment of VOO, and the guide for the selection, training and monitoring of skilled VOO tasters.

The purity criteria stipulated in the trade standard are designed to detect other kinds of oils in olive oil and olive-pomace oil:

- Seed oils are detected in olive oil and olive-pomace oil by calculating the difference between real HPLC-analysed ECN (equivalent carbon number) 42 triglyceride content and theoretical content assessed according to a computer program based on the content of C16 and C18 fatty acids in the oil when analysed by gas-chromatography (GC).
- Other kinds of oils are identified in olive oil by determining the sterol composition and content as well as the fatty acid composition.
- Refined vegetable oils and desterolised seed oils are detected by determining the stigmastadiene content and the R1 sterene ratio (stigmasta-3,5-diene/campesta-3,5 diene); R1 is the ratio between stigmasta-3, 5-diene and campesta-3, 5-diene. These are sterenes (hydrocarbons derived from sterols during the refining or desterolisation processes applied to vegetable oils). It can be applied when stigmastadiene content is more than 4 ppm in order to detect the presence of desterolised seed oils in refined olive oil, refined olive-pomace oil and mixes of these two oils with VOO.
- Olive-pomace oil is detected in olive oil by determining the content of C40–46 waxes and the content of erythrodiol + uvaol.
- Refined oils are detected by determining the *trans* fatty acid content.
- Re-esterified oils are detected by determining the content of 2-monoglyceryl palmitate.

The quality criteria specified in the trade standard are intended to classify the oil into each of the various designations or grades, as shown in Table 1.2.

Where additives are concerned, the trade standard fixes a maximum for refined olive oil, olive oil, refined olive-pomace oil and olive-pomace oil. This limit is 200 mg/kg of total alpha-tocopherol in the final product. Only alpha-tocopherol is authorised for these grades in order to replace the natural tocopherols eliminated during refining, but no additive is authorised for VOOs or crude olive-pomace oil. The trade standard also specifies limits for the following contaminants: moisture and volatile matter, insoluble impurities, trace metals (iron and copper) and halogenated solvents.

Table 1.2 Quality criteria

	Extra virgin olive oil	Virgin olive oil	Ordinary virgin olive oil	Lampante virgin olive oil[a]	Refined olive oil	Olive oil	Crude olive-pomace oil	Refined olive-pomace oil	Olive pomace oil
4.1. *Organoleptic characteristics*									
– Odour and taste					Acceptable	Good		Acceptable	Good
– Odour and taste (on a continuous scale):									
• Median of defect	Me = 0	0 < Me ≤ 3.5	3.5 < Me ≤ 6.0[b]	Me > 6.0					
• Median of fruity attribute	Me > 0	Me > 0							
– Colour					Light yellow	Light, yellow to green		Light, yellow to brownish yellow	Light, yellow to green
– Aspect at 20°C for 24 h					Limpid	Limpid		Limpid	Limpid
4.2. *Free acidity*									
% m/m expressed in oleic acid	≤0.8	≤2.0	≤3.3	>3.3	≤0.3	≤1.0	No limit	≤0.3	≤1.0
4.3. *Peroxide value*									
In milleq. peroxide oxygen per kg/oil	≤20	≤20	≤20	No limit	≤5	≤15	No limit	≤5	≤15
4.4. *Absorbency in ultraviolet* $(K^{1\%})^{1\,cm}$									
270 nm (cyclohexane)/268 nm (iso-octane)	≤0.22	≤0.25	≤0.30		≤1.10	≤0.90		≤2.00	≤1.70
– Δ K	≤0.01	≤0.01	≤0.01		≤0.16	≤0.15		≤0.20	≤0.18
– 232 nm[c]	≤2.50[d]	≤2.60[d]							

4.5. *Moisture and volatile matter* (% m/m)	≤0.2	≤0.2	≤0.2	≤0.3	≤0.1	≤0.1	≤1.5	≤0.1	≤0.1
4.6. *Insoluble impurities in light petroleum* (% m/m)	≤ 0.1	≤ 0.1	≤ 0.1	≤ 0.2	≤ 0.05	≤ 0.05		≤ 0.05	≤ 0.05
4.7. *Flash point*	–	–	–	–	–	–	≥120 °C	–	–
4.8. *Trace metals* (mg/kg)									
Iron	≤3.0	≤3.0	≤3.0	≤3.0	≤3.0	≤3.0		≤3.0	≤3.0
Copper	≤0.1	≤0.1	≤0.1	≤0.1	≤0.1	≤0.1		≤0.1	≤0.1
4.9. Fatty acid methyl esters (FAMEs) and fatty acid ethyl esters (FAEEs)[e]	see note[e]								
4.10. *Phenol content*									

The limits established for each criterion and designation include the precision values of the attendant recommended method

[a]It is not obligatory for the criteria in 4.1, 4.2 and 4.3 to be concurrent; one is sufficient

[b]Or when the median of the defect is less than or equal to 3.5 and the median of the fruity attribute is equal to zero

[c]This determination is solely for application by commercial partners on an optional basis

[d]Commercial partners in the country of retail sale may require compliance with these limits when the oil is made available to the end consumer

[e]$\sum$FAME + FAEE ≤ 75 mg/kg or ($\sum$FAME + FAEE > 75 mg/kg and ≤ 150 mg/kg and FAEE/FAME ratio ≤ 1.5) for EVOO. No official limits are set for phenolics, although a validated method is available for their determination

1.7 Role of International Olive Council

Using every means and encouragement in its power, the Council is responsible for encouraging any action conducive to the harmonious development of the world olive economy in the areas of production, consumption and international trade, bearing in mind the way in which they are interrelated. It is a forum for discussion and reflection and for reconciling the various interests in the sector in order to:

- Modernise olive growing, olive oil technology and table olive processing by encouraging research and development to improve product quality, to lower cost prices, and to improve the environmental impact of the olive and olive oil industry;
- Expand international trade, to raise consumption of olive oil and table olives, and to prevent and monitor any unfair competition;
- Ensure regular international trading in olive oils and table olives by taking measures to strike a balance between production and consumption and to harmonise national laws.

1.8 Nutritional and Health Aspects of Olive Oil. Past, Present and Future of Research

Collecting and circulating scientific research findings on the health-related properties of olive oil provides key support for the campaigns to promote and expand worldwide consumption of olive oil.

Olive oil is an integral part of the cultural and culinary heritage of the Mediterranean countries. It is a product that is set apart by its undeniable organoleptic qualities as well as by its nutritional and therapeutic properties, which are being increasingly corroborated by science.

The objectives written into the International Olive Oil and Table Olive Agreement include implementing actions and measures to highlight the biological value of olive oil and table olives.

The beginnings of the current interest in the Mediterranean diet, characterised by a low content of saturated fatty acids (animal fats and palm and coconut oil) and by monounsaturate-rich olive oil as its main source of fat, can be traced back to Ancel Keys of the University of Minnesota. In 1952, Keys was struck by the low incidence of coronary heart disease in Naples (Keys et al. 1954), which led him to believe there was a link between dietary fat consumption, elevated cholesterol levels and the risk of mortality from cardiovascular disease.

The first systematic attempt to study monounsaturate-rich diets was made in 1953 on the island of Crete, but it was limited by the shortage of clinical and food composition data. The first studies conducted in the 1950s and 1960s measured total cholesterol levels as opposed to the levels of cholesterol transported by low-density (LDL) and high-density (HDL) lipoproteins. This led to the conclusion that the

replacement of saturated fatty acids by monounsaturates did not affect serum cholesterol levels and that the substitution of polyunsaturates for saturated fatty acids lowered cholesterol levels (Keys 1970). However, later advances in the determination of blood lipid profiles prompted reassessment of these results.

These observations about saturated-fat consumption culminated in the body of research known as the Seven Countries Study (Keys 1980), which provided major epidemiological evidence of the effects of fats and fatty acids on serum cholesterol levels. By comparing the diet of population groups in countries such as Greece, Italy, Yugoslavia, Finland, Japan, the Netherlands and the USA, this was the first international prospective study of its kind and a scientific cornerstone on the health advantages of the Mediterranean diet. It was the first reference of the beneficial effects of olive oil and demonstrated that the incidence of cardiovascular disease amongst middle-aged men on Crete was lower than expected and directly proportional to their total cholesterol levels. Primarily owing to their high consumption of olive oil, their traditional diet supplied them with a high content of total fat (40% of total daily energy) but a low content of saturated fatty acids. This low incidence of vascular disease was linked to the potentially beneficial effects of monounsaturate-rich diets on lipoprotein profiles, which led to the belief that the kind of fat is what matters, not the quantity. Subsequent studies comparing different population groups provided further confirmation of the benefits of the olive-oil-rich Mediterranean diet (Kouris-Blazos et al. 1999; Kafatos et al. 1999; Predimed 2008–2009; Sorriguer et al. 2009).

Schaefer et al. (1981) demonstrated that high-polyunsaturate diets (seed oils) significantly lowered HDL cholesterol. Mattson and Grundy (1985) reported that olive oil lowered serum cholesterol to the same extent as polyunsaturated fats without lowering HDL cholesterol, which plays a protective, anti-atherogenic role by encouraging the elimination of LDL cholesterol (Gordon et al. 1989).

Sirtori et al. (1986) demonstrated that, besides its effect on cholesterol and atherosclerosis, olive oil also has a preventive effect on thrombosis and platelet aggregation, whereas polyunsaturated fat has no such effect. Baggio et al. (1988) demonstrated that olive oil could be used as an alternative to polyunsaturates for controlling hypercholesterolaemia. There is growing awareness that regular intake of large quantities of n-6 (omega-6) polyunsaturated fatty acids may not be healthy since it may cause increased risk of certain non-cardiovascular diseases. Such intake has been reported to encourage cancer formation in experimental animals, to alter cell membrane composition, to lower HDL levels and, possibly, to heighten the risk of gallstones (Mattson and Grundy 1985). Conversely, high intakes of olive oil are not harmful; they lower serum LDL-cholesterol levels but do not lower HDL levels, which they may even raise (Mattson and Grundy 1985; Keys 1970).

Numerous teams of researchers in Europe, Australia and North America have further documented these results. At the same time, they discovered additional advantages of olive oil such as its antioxidant effects stemming from the minor components present in its unsaponifiable fraction [e.g. vitamins, phenolics (hydroxytyrosol), flavonoids) (Cicerale et al. 2009a; Dessi et al. 2009; Katan et al. 2001; Reaven et al. 1993; Ruiz-Gutiérrez et al. 1996; Visoli and Galli 2003; Galli et al. 2000; Manna et al. 2000; Visioli et al. 2002; Zappia et al. 2000). The polyphenols in VOO may

function in vivo as protective antioxidants by exerting a protective effect against LDL oxidation; it is well known that oxidised LDLs are atherogenic, whereas normal ones are not (Katan et al. 1995; Covas et al. 2000; Fito et al. 2000).

Other oils have high monounsaturate content but contain smaller quantities of these polyphenols. There are oils that are rich in oleic acid, such as rapeseed and safflower oil and a special variety of sunflower oil, but they all necessarily must be refined before being eaten. As a result, they do not taste as pleasant as olive oil and are used less often for dressings. Olive oil (virgin) is the only oil that can be eaten as soon as it is extracted without refining or industrial processing, which helps it to retain innumerable substances, antioxidants and vitamins that add to its nutritional value. The monounsaturated fatty acids in olive oil make it more resistant to heat than the polyunsaturated fatty acids, which degrade more easily because they have additional double bonds in their molecules. Consequently, olive oil can be reused for frying without its fatty acids undergoing hydrogenation or isomerisation processes that cancel out its beneficial effects on lipid metabolism. It is the lightest and tastiest fat for frying (Choe and Min 2007).

Psaltopoulou et al. (2004) have studied blood pressure and observed that it was significantly lower when olive oil was consumed regularly, thus confirming the data reported by other researchers (Williams et al. 1987). Ferrara et al. (2000) reported that the use of olive oil decreased the dosage of drugs required to lower blood pressure.

An olive-oil-rich diet is not only a good alternative in the treatment of diabetes mellitus (Bonanome et al. 1991); it can also prevent or slow down the onset of the disease. It prevents insulin resistance and its possible harmful consequences (Tierney and Roche 2007) and raises HDL cholesterol and lowers blood triglycerides (Lerman-Garber et al. 1994). In addition, it ensures better control of glucose in the blood (Garg 1998) and lowers blood pressure (Rasmussen et al. 1993). Olive oil significantly improves cell glucose utilisation and lowers triglycerides, and it is more pleasing to the palate than a high-carbohydrate diet containing the same amount of fibre.

Epidemiological studies also suggest that olive oil has a protective effect against some types of malignant tumours (e.g. breast, prostate, endometrial, digestive tract) (Trichopoulou et al. 1995, 1997, 2000, 2003; La Vecchia et al. 2000; Menendez et al. 2009). It adds to the palatability of vegetables, pulses and greens, whose beneficial effects in the prevention of cancer have been amply proven (Willett and Trichopoulos 1996; WCRF 1997).

Olive oil has been shown to strengthen the immune system against external attacks caused by micro-organisms such as bacteria and viruses (Peck et al. 1996, 2000). A research team at the University of Athens led by Athena Linos found that regular olive oil consumption reduces the risk of developing rheumatoid arthritis. Although the mechanism is not yet clear, the authors state that antioxidants are believed to be behind this beneficial effect (Spinella et al. 2009). Olive oil is rich in vitamin E, which plays a positive biological role in removing free radicals, which are the molecules involved in certain chronic diseases and in the ageing process. Hence, olive oil is believed to play a part in increasing life expectancy.

Owing to its high vitamin E and antioxidant content, olive oil has a protective, toning effect on the skin, which is why it is believed to be especially suited for preventing the appearance of skin lesions (Viola et al. 2009).

Olive oil appears to play a favourable part in bone calcification, and high consumption improves bone mineralisation (Laval-Jeantet et al. 1987) and is believed to prevent osteoporosis. According to recent research (Solfrizzia et al. 2005), it prevents the cognitive loss associated with age, some types of dementia and neurodegenerative diseases such as Alzheimer's disease (Klein et al. 2009) or multiple sclerosis (Materljan et al. 2009).

Olive oil is the fat that is best digested and absorbed; it has excellent properties in this respect and a mild laxative effect that helps to combat constipation (Yadollahi et al. 2011). In confirmation of this, olive oil promotes the discharge of bile from the system and emptying of the gallbladder, and Grataroli et al. (1985) agreed regarding its utility in treating gallbladder disease and considered it a 'food medicine'. To conclude, owing to its effective action on the tonus and activity of the gallbladder, olive oil is conducive to lipid digestion because it is emulsified by the bile and prevents the appearance of cholesterol gallstones.

Olive oil is a very healthy nutrient that has a high calorie value, which might make one think that its consumption encourages obesity. Experience shows, however, that Mediterranean peoples, who consume the most olive oil, suffer less from obesity than do peoples in English-speaking countries. It has been proven that, when compared with a low-fat diet, an olive oil diet leads to greater and more lasting weight loss. It is tolerated better because it tastes good and makes eating vegetables more enjoyable (Sacks 2002; Estruch et al. 2009; Sorriguer et al. 2009). Aspects of nutrition are covered further in Chaps. 17 and 18.

When carrying out its work to encourage and finance scientific research into the medical and nutritional properties of olive oil, the IOC has tried to adapt to and move with the medical community and the times. The gateway to researching the further effects of olive oil is still open to new discoveries providing scientific corroboration of the healthy properties it is believed to have.

1.9 Other Effects of Olive Oil

Olive oil has many attributes, a large number of which were initially sensed empirically. Many of today's diseases have to contend with an enemy in this 'liquid gold', especially VOO.

Recent research conducted by the University of Granada and the Instituto Carlos III Charles III Institute in Madrid discovered that maslinic acid, which is extracted from the olive, might act as an inhibitor of the AIDS virus. Maslinic acid hinders the spread of HIV by inhibiting the action of an enzyme (serine-protease) that uses the virus to burst the cell walls in which it is established and to spread to new cells. This acid may also have a positive effect in the control of malaria by acting as an anti-inflammatory agent, neuronal activator or liver protector.

An olive oil component, dubbed oleocanthal, which inhibits cyclo-oxygenase (COX) like ibuprofen, provides relief from pain and inflammation. It was estimated that 50 g of extra VOO, equivalent to the daily intake in the Mediterranean diet, were equivalent to 9 mg of ibuprofen. However, other researchers (Beauchamp et al. 2005; Cicerale et al. 2009b), working on the basis of the stinging properties shared by extra VOO with ibuprofen, wondered whether these two compounds might also share the capacity to relieve inflammation.

Olive oil helps to absorb certain substances that are beneficial for the body such as the omega-3 fatty acids in fish or lycopene in tomatoes. When it is eaten with these foods, it is twice as beneficial because it improves their bioavailability. It also exerts a synergistic effect with garlic and onions. In addition, the vitamin C in oranges has a complementary effect with the vitamins E and A in olive oil. The World Health Organisation recommends a breakfast of milk + orange juice + a slice of toast with olive oil for a healthy start to the day.

Olive oil, especially VOO, produces a lining on the stomach owing to the fat-soluble vitamins it contains (E and A). It is also an ideal carrier for omeprazole in the body, the drug used to treat gastroduodenal ulcers and hiatal hernias.

In skin cosmetics olive oil is being used increasingly in the manufacture of soaps, creams and other products and in the treatment of skin disorders (e.g. dermatitis, acne, psoriasis) and burns.

Studies released by the University of Sydney and the Eye Research Centre Australia recently showed that the acid- and antioxidant-rich composition of extra VOO prevents macular degeneration of the eye, the second most common cause of blindness in the elderly after diabetes (Chong et al. 2009).

The US Food & Drug Administration (FDA) recommends consuming 23 g of olive oil daily.

1.10 Looking Ahead

The dissemination of what is known about the healthy effects of eating olive oil has been decisively effective in increasing consumption. That is why it is so worthwhile to publicise this knowledge through information and promotional events.

The IOC tracks, compiles, processes, updates and circulates international studies and research findings on the nutritional and health-promoting aspects of olive oil. This updated source of scientific information is then used as the basis for the ongoing production and review of promotional messages and the production of scientific information.

Another challenge the IOC has set itself is to interweave all the aspects of the product. The aim is to customise scientific information to the idiosyncrasies of each country. When designing the focus of campaigns, the IOC takes into account the prevalence of specific diseases and the proven effect of olive oil in preventing those diseases which are of extensive concern to the population. For instance, faced with the serious problem of child and adult obesity in developed countries, promotion

centres on the use of olive oil as the choice fat for preventing obesity and its associated risk factors.

The objective pursued by the IOC has been – and always will be – to provide extensive, confirmed information on the acknowledged sensory, gastronomic, nutritional and health attributes of olive oil. That information must be true, based on scientific evidence and readily accessible to the layperson.

References

Baggio G, Pagnan A, Muraca M, Martini S, Opportuno A, Bonanome A et al (1988) Olive oil enriched diet: effect on serum lipoprotein and biliary cholesterol saturation. Am J Clin Nutr 47:960–964

Beauchamp GK, Keast RSJ, Morel D, Lin J, Pika J, Han Q, Lee C, Smith AB, Breslin PAS (2005) Phytochemistry: ibuprofen-like activity in extra-virgin olive oil. Nature 437(7055):45–46

Bonanome A, Visona A, Lusiani L, Beltramello G, Confortin L et al (1991) Carbohydrate and lipid metabolism in patients with non-insulin-dependent diabetes mellitus: effects of a low-fat, high-carbohydrate diet vs a diet high in monounsaturated fatty acids. Am J Clin Nutr 54:586–590

Choe E, Min DB (2007) Chemistry of deep-fat frying oils. J Food Sci 72:77–86

Chong EW et al (2009) Fat consumption and its association with age-related macular degeneration. Arch Ophthalmol 127(5):674–680

Cicerale S, Conlan XA, Sinclair AJ, Keast RSJ (2009a) Chemistry and health of olive oil phenolics. Crit Rev Food Sci Nutr 49:218–236

Cicerale S, Conlan XA, Barnett NW, Sinclair AJ, Keast RS (2009b) Influence of heat on biological activity and concentration of oleocanthal – a natural anti-inflammatory agent in virgin olive oil. J Agric Food Chem 57:1326–1330

Covas MI et al (2000) Virgin olive oil phenolic compounds: binding to human low density lipoprotein (LDL) and effect on LDL oxidation. Int J Clin Pharm Res 20:49–54

Dessi M et al (2009) Extra virgin olive oil phenolics: absorption, metabolism and biological activities in the GI tract. Toxicol Ind Health 25(4–5):301–310

Estruch R et al (2009) A 3 years follow-up of a Mediterranean diet rich in virgin olive is associated with high plasma antioxidant capacity and reduced body weight gain. Eur J Clin Nutr 63:1213–1219

Ferrara LA, Raimondi S, d'Episcopo L, Guida L, Dello Russo A, Marotta T (2000) Olive oil and reduced need for antihypertensive medications. Arch Intern Med 160:837–842

Fito M, Covas MI, Lamuela-Raventos RM, Vila J, de la Torre C, Marrugat J (2000) Olive oil and inhibition of low density lipoprotein oxidation. Role of phenolic compounds. Med Clin 115(5):166–169

Galli G et al (2000) Evidence of postprandial absorption of olive oil phenols in humans. Nutr Metab Cardiovasc Dis 10:111–120

Garg A (1998) High-monounsaturated-fat diets for patients with diabetes mellitus: a meta-analysis. Am J Clin Nutr 67:577S–582S

Gordon DJ et al (1989) High density lipoprotein cholesterol and cardiovascular disease: four prospective American studies. Circulation 79:8–15

Grataroli R, Charbonnier M, Nalbone G, Lairon D, Chabert C et al (1985) Hydrolysis of intralipid by pancreatic lipase and phospholipase A2-gel filtration studies. Lipids 20(11):765–772

Kafatos A et al (1999) Physical activity and nutrition in older adults. Nutrition society. Public Health Nutr 2(3a):429–436

Katan MB et al (1995) Dietary oils, serum lipoproteins, and coronary heart disease. Am J Clin Nutr 61:S1368–S1373

Katan MB et al (2001) Effect of phenol-rich extra virgin olive oil on markers of oxidation in healthy volunteers. Eur J Clin Nutr 55:334–341
Keys A (1970) Coronary heart disease in seven countries (American Heart Association, monograph 29). Circulation 41:S1
Keys A (1980) Seven countries: a multivariate analysis of death and coronary heart disease. Harvard University Press, Cambridge, MA
Keys A, Fidanza F, Scardi V, Bergami G, Keys MH, DiLorenzo F (1954) Studies on serum cholesterol and other characteristics on clinically healthy men in Naples. Arch Intern Med 93:328
Klein WL et al (2009) Alzheimer's-associated Abeta oligomers show altered structure, immunoreactivity and synaptotoxicity with low doses of oleocanthal. Toxicol Appl Pharmacol 240:189–197
Kouris-Blazos A et al (1999) Are the advantages of the Mediterranean diet transferable to other populations? A cohort study in Melbourne, Australia. Br J Nutr 82:57–61
La Vecchia C et al (2000) Food groups and risk of squamous cell esophageal cancer in northern Italy. Int J Cancer 87:289–294
Laval-Jeantet AM, Miravet L, Bergot C, de Vernejoul MC, Kuntz D, Laval-Jeantet M (1987) Tomodensitométrie vertébrale quantitative. Résultats sur 105 femmes consultant pour ostéoporose. J Radiol 68:495–502
Lerman-Garber I, Ichazo-Cerro S, Zamora-González J, Cardoso-Saldaña G, Posadas-Romero C (1994) Effect of a high-monoin-saturated fat diet enriched with avocado in NIDDM. Diabetes Care 17:311–315
Manna C, Galletti P, Maisto G, Cucciolla V, D'Angelo S, Zappia V (2000) Transport mechanism and metabolism of olive oil hydroxytyrosol in Caco-2 cell. FEBS Lett 470:341–344
Materljan E et al (2009) Multiple sclerosis and cancers in Croatia. A possible protective role of the Mediterranean Diet. Coll Antropol 33(2):539–545
Mattson FH, Grundy SM (1985) Comparison of effects of dietary saturated, monounsaturated and polyunsaturated fatty acids on plasma lipids and lipoproteins in man. J Lipid Res 26:194–202
Menendez JA et al (2009) Extra virgin olive oil polyphenols inhibit HER2 (erbB-2)-induced malignant transformation in human breast epithelial cells. Int J Oncol 44(1):43–51
Peck M et al (1996) Association of murine splenocyte CD3 Complex to the cytoskeleton: absence of modulation by exogenous fatty acids. Cell Biol Int 20(8):531–537
Peck M et al (2000) High-fat diets suppress CD3 and CD25 expression on the surface of murine lymphocytes. Nutrition 16:278–283
Predimed (2008–2009) Effect of a Mediterranean diet supplemented with virgin olive oil or nuts on a metabolic syndrome status. Arch Intern Med 168:2449–2458, J Nutr 139(1):128–34 (2009), Nutr Metab Cardiovasc Di 19(8):563–570 (2009)
Psaltopoulou T, Naska A, Orfanos P, Trichopoulos D, Mountokalakis T, Trichopoulou A (2004) Olive oil, the Mediterranean diet, and arterial blood pressure: the Greek European Prospective Investigation into Cancer and Nutrition (EPIC) study. Am J Clin Nutr 80:1012–1018
Rasmussen OL, Thonsen C, Hasen KW, Veslerlund M, Winther E et al (1993) Effects on blood pressure, glucose, and lipid levels of a high-monounsaturated fat diet compared with a high-carbohydrate diet in NIDDM subjects. Diabetes Care 16(12):1565–1571
Reaven PD, Parthasarathy S, Grasse BJ, Miller E, Steinberg D, Witztum JL (1993) Effects of oleate-rich and linoleate-rich diets on the susceptibility of low-density lipoprotein to oxidative modification in mildly hypercholesterolaemic subjects. J Clin Invest 91:668–676
Ruiz-Gutierrez V, Suriana FJ, Guerrero A, Cert AM, Villar J (1996) Plasma lipids, erythrocyte membrane lipids and blood pressure of hypertensive women after ingestion of dietary oleic acid from two different sources. J Hyperten 14:1483–1490
Sacks FM (2002) Dietary fat, the Mediterranean diet, and health: reports from scientific exchanges, 1998 and, 2000. Introduction. Am J Med 113(Suppl 9B):1S–4S
Schaefer EJ, Levy RI, Ernst ND et al (1981) The effects of low cholesterol, high polyunsaturated fat and low fat diets on plasma lipids and lipoprotein cholesterol levels in normal and hypercholesterolemic subjects. Am J Clin Nutr 34:1758–1763

Sirtori CR, Tremoli E, Gatti E et al (1986) Controlled evaluation of fat intake in the Mediterranean diet: comparative activities of olive oil and corn oil on plasma lipids and platelets in high-risk patients. Am J Clin Nutr 44:635–642
Solfrizzia V et al (2005) Dietary fatty acids intake: possible role in cognitive decline and dementia. Exp Gerontol 40:257–270
Sorriguer F et al (2009) Intake and home use of olive oil or mixed oils in relation to healthy lifestyles in a Mediterranean population. Findings from the prospective Pizarra study. Br J Nutr 14:1–9
Spinella P et al (2009) The Mediterranean diet model in inflammatory rheumatic diseases. Reumatismo 61(1):10–14
Tierney AC, Roche HM (2007) The potential role of olive oil-derived MUFA in insulin sensitivity. Mol Nutr Food Res 51:1235–1248
Trichopoulos D et al (1997) Olive oil and human cancer: an assessment of the evidence. Prev Med 26:181–190
Trichopoulos D et al (2000) Cancer and Mediterranean dietary traditions. Cancer Epidemiol Biomarkers Prev 9:869–873
Trichopoulou A, Kouris-Blazos A, Wahlqvist ML et al (1995) Diet and overall survival in elderly people. BMJ 311:1457–1460
Trichopoulou A, Costacou T, Bamia C, Trichopoulos D (2003) Adherence to a Mediterranean diet and survival in a Greek population. N Engl J Med 348:2599–2608
Viola P et al (2009) Virgin olive oil as a fundamental nutritional component and skin protector. Clin Dermatol 27(2):159–165
Visioli F, Galli C, Galli G, Caruso D (2002) Biological activities and metabolic fate of olive oil phenols. Eur J Lipid Sci Technol 104:677–684
Visoli F, Galli C (2003) Biological properties of olive oil phenolic antioxidants in liposomes. Chem Phys Lipids 124:23–56
Willett WC, Trichopoulos D (1996) Summary of the evidence: nutrition and cancer. Cancer Causes Control 7:178–180
Williams PT et al (1987) Associations of dietary fat, regional adiposity, and blood pressure in men. JAMA 257:3251–3256
World Cancer Research Fund (WCRF), American Institute for Cancer Prevention (1997) Food, nutrition and the prevention of cancer: a global perspective. World Cancer Research Fund and American Institute for Cancer Prevention, Washington, DC
Yadollahi PMS, Khormaei F, Shokrpour N (2011) The relationship between pregnant women's lifestyle and common gastrointestinal disorders. Eur J Sci Res 60:359–364
Zappia V et al (2000) Hydroxytyrosol, a natural molecule occurring in olive oil, induces cytochrome C-dependent apoptosis. Biochem Biophys Res Commun 278:733–739

Chapter 2
Growing Olives for Oil

Paul Vossen

Contents

2.1 Introduction

The primary goal of olive oil orcharding is to produce large quantities of very high-quality olive oil efficiently, economically, and consistently. This means having an annual production of between 1,000 and 2,000 kg of oil per hectare that receives

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties*,
DOI 10.1007/978-1-4614-7777-8_2, © Springer Science+Business Media New York 2013

recognition from oil judges, buyers, and consumers, with the costs of production being well below the going price for the specific target market. The farmer must have the right climate then manipulate the choice of cultivars, tree spacing, pruning, fruit thinning, irrigation, fertilizing, pest control, harvest timing, and harvest method in order to maximize quality and keep costs low. When making these decisions, having a good knowledge of the production and quality characteristics of each cultivar is important.

To a large extent the cultivar will dictate fruit yield, oil yield, the fatty acid profile, and color and flavor characteristics based on the profile and concentration of pigments, phenols, volatiles, and other phytochemicals present. Ideally each cultivar's genetic capacity would be matched to the climate, general environment, and specific farm site in order to maximize production and quality. The chosen cultivars also need to appeal to the ultimate buyer, so that demand and price are sufficient to make a profit. Then the agriculturist must regulate the crop load every year to have similar sized fruit all with good sun exposure to assure getting a good crop of oil with a typology that is in demand and familiar to buyers based on past flavor profiles. Irrigation and, to a lesser extent tree nutrition, must be carefully managed to get good vegetative shoot growth for next year's crop and reduce alternate bearing, but without excessive growth that shades out the tree and requires excessive pruning. Irrigation should also be carefully administered to avoid giving the trees too much water, which can dilute out the oil's flavor, or having excessively dry conditions, which can stress trees and produce extremely bitter oils. Insect and disease pests must be monitored and controlled in order to avoid fruit damage that could alter the flavor of the oil and create defects. Harvest timing that takes into consideration economics and the efficiency of fruit removal in the orchard without significantly damaging the fruit and lowering quality, as well as selecting the most appropriate stage of fruit maturity, is extremely important. The ripeness of the olive has a huge effect on the ultimate flavor of the oil, its keeping quality, processing extraction efficiency, and health benefits. Lastly, the harvested fruit must be handled carefully and then transported and processed without delay.

More than 1,275 autochthonous olive cultivars have been identified and characterized from a wild ancestor, which is still subject to debate. Its origin is from somewhere in Mesopotamia around 5,000 years ago. It is well known that Phoenicians and Greeks spread the tree to Western regions. Through the centuries, farmers selected cultivars for their best characteristics and either propagated those trees directly or grafted over wild olive trees to superior selections (Bartolini et al. 1998). Olives were often planted on hillsides where annual grains might be grown but where irrigation water was not available for the production of more water-demanding tree crops or vegetables. Trees were dry-farmed and depended on seasonal rainfall and the water the soil held. They were planted at wide spacings to take advantage of larger quantities of stored soil moisture per tree. The soils were cultivated to a dust mulch to remove any vegetation that might compete for water. The trees received minimal pest control, and only natural animal-manure-based fertilizers were used. Cultural practices depended extensively on hand labor, especially for harvest, and yields were very low compared to modern standards.

Expansion in olive orchard plantings around the Mediterranean Sea over the last 150 years included the adoption of growing practices using tractor power instead of horses, low-cost conventional fertilizers, and broad-spectrum pesticides for controlling insects, diseases, and weeds. In the last 40 years new practices have been continuously introduced that have brought us drip irrigation, mechanical harvest, and continuous-flow processing systems. With new varieties, irrigation, good pest control, harvest and handling techniques that do not damage the fruit, quick processing after harvest, time- and temperature-controlled extraction within a few hours of crushing the fruit, and stainless steel storage, a completely new "fresh" olive oil product has emerged. This is really the basis for high-quality olive oil today. Now many producers are trying to differentiate their oils in the marketplace based on flavor characteristics or by promoting their oil's healthful components such as its content of monounsaturated fatty acids and polyphenols. This chapter introduces the current production situation around the world, then provides research-based information regarding the agronomic factors that can influence an oil's quality characteristics such as cultivar, climate, soil, irrigation, fruit ripeness, and other farming practices.

2.2 World Olive Oil Production and Consumption

Olive oil is a very complex food that is influenced by thousands of years of history and culture in southern Europe, North Africa, and the Middle East, an area that produces over 95 % of the world's olive oil. More recently, several new producer countries, outside the Mediterranean region, have begun to produce olive oil for their domestic and export markets. In the modern era of olive oil production starting in the mid-1970s, the world produced approximately 1.2 million t of olive oil per year on approximately 4 million ha of land. Since then, almost 6 million additional ha have been planted worldwide to reach an average production of 2.74 million t of oil over the last 6 years. The range of production from year to year varies according to the natural alternate bearing cycle of the olive tree and with weather patterns that influence fruit set and crop load (Fig. 2.1). In 2010–2011, world production reached 3 million t and is expected to range from approximately 2.8 to 3.3 million t of oil over the next few years. Typically, consumption just slightly exceeds production, and the difference is made up from carryover from the previous year (Fig. 2.2).

Most of the world's orchards that were developed in the 1970s to the 1990s were planted at very wide tree spacings of over 10 m apart in both directions, and the orchards were only dry-farmed (rain-fed). Some orchards, however, were planted to higher densities with tree spacings of approximately 6 × 3 or 7 × 4 or 8 × 4 m apart, and many of these orchards were irrigated if water was available. They were quite profitable because they were higher yielding and mechanically harvested with trunk shakers to lower costs. In the mid-1990s some orchards were planted to even higher densities with tree spacings of 4 × 1.5 m apart with very early bearing cultivars and harvested with fast and efficient over-the-row

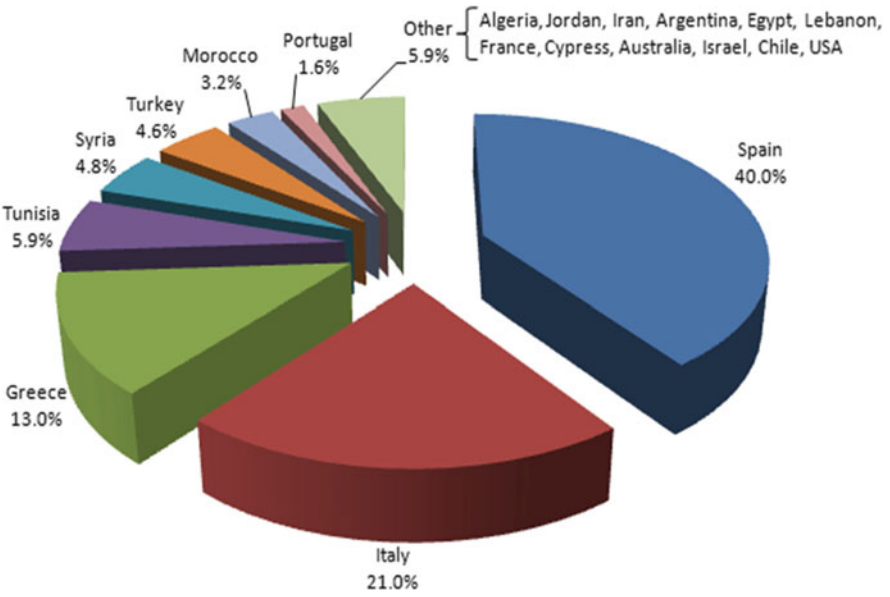

Fig. 2.1 World production of olive oil by country

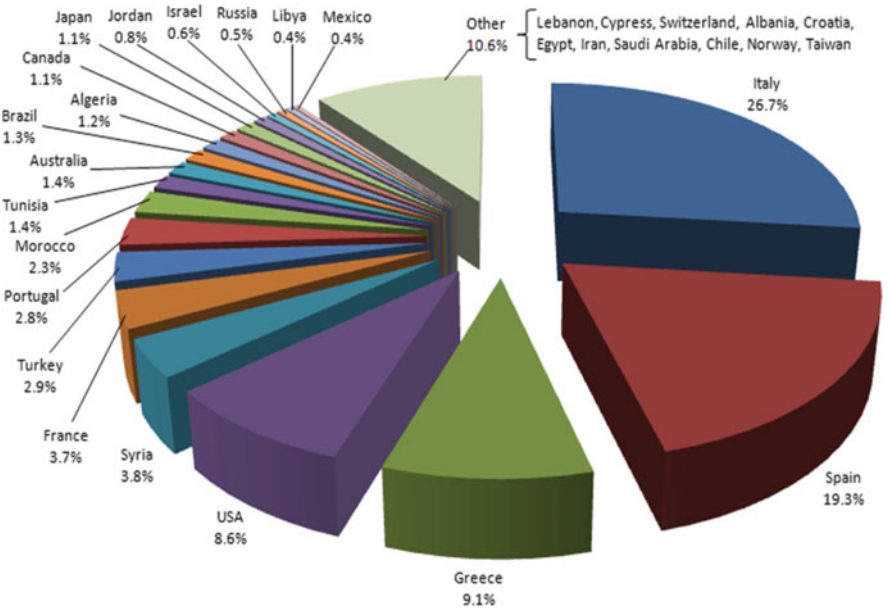

Fig. 2.2 Worldwide consumption of olive oil by country

grape-type harvesters. All of these orchards were irrigated and provided a quicker return on investment. Today there are almost 10 million ha of olives with 9.0 million (90 %) at traditional spacings (80–200 trees/ha); 900,000 ha (9 %) planted at medium- or high-density spacings (200–600 trees/ha); and 80,000 ha (<1 %) planted at super-high-density spacings (>600 trees/ha).

Average yields per hectare around the world under dry-farmed (rain-fed) orchard conditions and wide spacings range from 0.5 to 4 t of fruit/ha. The huge variability in production is due to the olive tree's strong tendency toward alternate bearing (large crop every other year) and a strong dependence on seasonal rainfall and stored soil moisture to generate adequate shoot growth for the next year's production. Many of these orchards are on steep terrain and have large tree structures that cannot be harvested mechanically with trunk or canopy shakers, making them very marginal economically. The more modern, medium-density to high-density to super-high-density orchards that are irrigated can produce from 5 to 12 t of fruit/ha.

- ***Spain*** is the world's largest olive oil producer country with approximately 2.4 million ha (28 %). Approximately half of Spain's production is consumed domestically, and the other half is exported. Continuous expansion of the country's orchards has occurred since 1986 when Spain entered the EU. New orchards are being trained into modern systems that can be harvested mechanically. Spain annually produces 38–45 % of the world's olive oil (1.2–1.5 million t), primarily from 24 cultivars dominated by Picual, Hojiblanca, Arbequina, Cornicabra, and Empeltre. Spain has just over 2,000 oil-processing mills, most of them quite large. Southern Spain's Andalusia region has 63 % of the country's olives and produces 83 % of the oil. In most of the olive-growing regions rainfall ranges from 300 to 600 mm per year (Barranco and Rallo 2000), and when supplemented with 300–400 mm of irrigation, results are quite good for production (Gomez del Campo and Barranco 2009; IOC 2011b).

  A recent economic study compared the costs of production in Spain between low-yielding, widely spaced orchards that were either mechanized or nonmechanized and more modern orchards with either medium-high-density or super-high-density spacings that were being mechanically harvested. The results indicated a range of costs from $1,330 to $3,270/ha, with the more densely planted orchards costing the most to manage. The costs per kilogram of oil, however, ranged from $1.82 to $4.28/kg, with the most efficient systems being the medium-density one of 200–600 trees/ha (6 × 3 to 6 × 6 m spacing), which was harvested by trunk shakers, and the super-high-density systems with 1,000–2,000 trees/ha harvested with over-the-row grape harvesters (Table 2.1). Spain is the largest volume producer and tends to set world prices. Most of its dry-farmed (rain-fed) land with low yields is no longer economical to farm without an EU subsidy. Spain has many large farms that have enough volume to be solid economical units, but the average farm size is just over 5 ha (Cubero and Penço 2010).
- ***Italy*** is the second largest producer of olive oil with an annual production of approximately 0.5–0.6 million t (21 % of the world's production) on approximately 1.1 million ha (13 %) primarily from the cultivars Coratina, Carolea,

Table 2.1 Comparative cost in US dollars ($) by country for medium-high-density and super-high-density orchards in 2007–2011

Country	Land[a]	Establishment[a]	Production[a]	Production[b]
Australia: Med-high-density	$1,450	$26,722[c]	$4,495	$1.92
Chile: Super-high-density	$2,000–8,500	$8,000	$1,500–2,492	$1.33–2.20
Italy[d]: Med-density	$25,000–50,000	$10,472–12,583	$3,543–7,278	$2.23–4.58
Spain[e]: Med-high-density	$25,000–50,000	$5,000–9,000	$1,330–3,270	$1.82–4.74
USA[f]: Super-high-density	$12,355–24,700	$20,933	$1,330–3,270	$1.65–3.00

Source: Baldari et al. (2009), CORFO (2008), Cruzat and Barrios (2009), Cubero and Penço (2010), IOC (2011a), Ravetti (2011), Roselli and De Gennaro (2011), Vossen (2007), Vossen et al. (2011)
[a]Cost/ha
[b]Cost/kg oil
[c]Includes purchase of water rights
[d]Calabria
[e]Andalusia
[f]California, Central Valley

Ogliarola, Frantoio, and Leccino. Italy consumes over 175,000 t of oil more than it produces, plus it is a large exporter of oil, hence it is the largest importing country. Approximately half of the 1.2 million olive farms are less than 1 ha in size, and consequently the costs of production are quite high and often much of the work is done by family members. Italy has over 6,000 mills, most of which are also quite small or artisanal. A cost study completed in 2009 in Calabria indicated that the total cost to produce olives in medium-density systems was $3,543–7,278/ha with a yield of 1,588 kg of oil/ha based on a mechanically harvested, irrigated orchard with 300–400 trees/ha (Table 2.1) (Baldari et al. 2009; IOC 2011b).

- ***Greece*** is the third largest producer of olive oil in the world with approximately 0.75 million ha and an annual average production of approximately 0.36 million t of oil (13 %), which is approximately 100,000 t more than it consumes. Oil production is dominated by the Koroneiki cultivar and some Mastoides for a pollen source. The Peloponnesus and island of Crete are the dominant growing areas. Most of the orchards have been divided up into very small parcels less than 2 ha in size. Greece has 3,248 oil-processing mills, mostly of modern continuous-flow systems (IOC 2011b).
- ***Syria*** annually produces 54,000–160,000 (average 113,000) t of olive oil on 478,000 ha from small farms that are mostly less than 5 ha in size. There are an additional 100,000 ha devoted to table olives. The primary oil varieties are Zaiti, Sorani, Khodieri, Doebli, Dan, and Jlot. Syria has 922 oil-processing mills, of which half are modern centrifugal extraction systems and half are traditional presses (IOC 2011b).
- ***Tunisia*** has 1.67 million ha of olives for oil in 270,000 farms (72 % are less than 10 ha) with two primary cultivars, Chetoui and Chemlali. Most of the orchards have only 50–60 trees/ha with an average yield of 0.8 t/ha. Total production is approximately 150,000 t annually. There are 1,674 processing mills for oil (IOC 2011b).

- ***Turkey*** produces approximately 145,000 t of olive oil on 500,000 ha of land primarily from the Ayvalik cultivar. Yields per hectare are quite low due to alternate bearing in dry-farmed (rain-fed) conditions (IOC 2011b).
- ***Morocco*** has 600,000 ha of olives in 400,000 farms producing approximately 60,000–108,000 t of oil each year primarily from the Moroccan Picholine cultivar. Many new orchards are being planted with varieties from Europe. There are 350 modern processing plants and 16,000 traditional presses (IOC 2011b).
- ***Portugal*** has 430,000 ha devoted to olive production with farms ranging in size from 0.5 ha to over 200 ha, with an average size of 6.1 ha. Annual oil production is approximately 50,000–80,000 t primarily from the Galega Vulgar cultivar. Many new orchards have been planted recently to Spanish cultivars where irrigation water is available. Portugal has 1,472 processing mills (IOC 2011b).
- ***Algeria*** has approximately 300,000 ha of olives of which 80 % is for oil. Oil production ranges from approximately 50,000 to 70,000 t, with orchards typically yielding 2 t of fruit/ha. The main oil variety is Chemlal. There are 1,650 mills, of which 165 have modern continuous-flow processing machinery (IOC 2011b).
- ***Jordan*** notes that olives are the number one crop with just over 120,000 ha under production. More orchards are being planted, and production has been approximately 100,000 t of fruit yielding approximately 20,000 t of oil. The primary variety grown is Rasi'i (IOC 2011b).
- ***Iran*** has just over 100,000 ha of olives with a production of approximately 61,000 t of fruit. The average yield is low for dry-farmed (rain-fed) orchards, but some irrigated orchards produce 13–20 t/ha. The two principal cultivars grown are Zard and Roghani (IOC 2011b).
- ***Egypt*** has approximately 50,000 ha of olives, 75 % of which are grown for table fruit, so oil production is only approximately 14,000 t of olive oil per year (IOC 2011b).
- ***Lebanon*** has 30,000 ha of olives, and the average farm size is 0.3 ha. Production ranges from 80,000 to 180,000 t per year due to alternate bearing. The main cultivars produced are Souri, Beladi, and Ayrouni. There are approximately 500 oil-processing mills (IOC 2011b).
- ***Israel*** has 21,000 ha of olives, 89 % for oil producing 24,000 t of olives yielding approximately 4,500 t of olive oil in approximately 100 processing mills, mainly from the Souri and Barnea cultivars (IOC 2011b).
- ***France*** has approximately 35,000 ha of olives producing 2,000–4,500 t of olive oil annually from orchards with an average yield of 0.7 t/ha of fruit (IOC 2011b).
- ***Australia*** has approximately 30,000 ha of olives producing 18,000 t of oil from 110 processing plants. Mean fruit yield is 10 t/ha, ranging from 8 to 16. Production is primarily from medium-density orchards and the following cultivars: Barnea: 27 %, Frantoio: 23 %, Picual: 15 %, Manzanillo: 10 %, Coratina: 8 %, Arbequina: 7 %, others: 10 %. Large farms harvest the fruit with giant over-the-row, custom-built olive harvesters (Colossus). Expansion is limited due to the scarcity and high cost of irrigation water. Average production costs for a medium-high-density orchard are approximately $4,500/ha or approximately $1.92/kg of oil (Table 2.1) (IOC 2011b; Ravetti 2011).

- ***Argentina*** has approximately 100,000 ha of olives producing 30,000 t of olive oil (IOC 2011b) from 83 processing mills. Approximately half of the production is for table olives. The primary oil cultivar grown is Arbequina, followed by many other minor varieties. Argentina also has its own indigenous Arauco cultivar that is used for both table fruit and oil production.
- ***Chile*** has recently planted approximately 20,000 ha of olives for oil, with approximately 50 % being Arbequina followed by Frantoio, Arbosana, Picual, and Leccino. Current oil production is approximately 8,000 t, which is projected to double in the next 5 years as the orchards come into bearing. Production costs for a super-high-density system range from $1,500 to $2,492/ha, or approximately $1.33–2.20/kg oil (Table 2.1) (CORFO 2008; Cruzat and Barrios 2009; IOC 2011b).
- ***US*** olive oil production is 99 % from California. Currently California has approximately 700 farmers growing 11,000 ha of olives for oil, producing approximately 4,000 t of oil, which represents only approximately 2 % of total US consumption. Production has doubled in the last 2 years and will continue to increase significantly as new orchards come into full bearing. Average fruit yields are approximately 10 t/ha. Over 70 % of the production is from the cultivar Arbequina, followed in descending order by Arbosana, Koroneiki, Frantoio, and Leccino. The USA is the fourth largest olive-oil-consuming country, importing approximately 238,000 t per year. Production costs for the super-high-density system grown in the Central Valley range from $3,311 to $6,020/ha, or $1.65 to $3.00/kg oil; differences are mostly due to orchard size (Table 2.1) (IOC 2011b; Vossen et al. 2007).

2.3 Orchard Systems

The use of drip irrigation and mechanical harvesters has led to the redesign of olive orchards over the last 30 years. Dry-farmed (rain-fed), hand-harvested orchards with a traditional spacing of over 8 m between trees are no longer popular anywhere in the world where new orchards are being planted. Modern olive oil producers are planting trees at higher densities with supplemental irrigation to boost productivity and harvesting those trees with trunk shakers or very large over-the-row harvesters. The trees are normally allowed to grow approximately 4 m tall and 3–4 m wide. Any cultivar can be grown and managed in the medium- or high-density-system orchards, and with track-mounted trunk shakers they can be planted on sloped terrain that will not accommodate large, top-heavy, over-the-row harvester machines.

The super-high-density system is designed around specific precocious cultivars that come into bearing very early and reduce the overall vegetative growth and size of the trees, allowing for rapid mechanized harvesting with modified grape harvesters, but the land must be almost flat. The system requires more intensive pruning, irrigation, and fertility management in order to keep the trees productive yet small enough to accommodate the grape-type harvesters (approximately 3 m tall and

Fig. 2.3 Classic olive tree orchards in Tunisia (*Source*: Author)

2 m wide). The more trees that are planted per hectare, the faster the orchard comes into full bearing, which makes this system popular for investors and nurseries selling trees (Civantos and Pastor 1996; Pastor 1994; Tous et al. 1999a).

2.3.1 Traditional

The old, traditional olive production system in dry-farmed (rain-fed) areas around the Mediterranean range in tree spacings from 8 to 18 m apart (30–156 trees/h). Their yields are very low (0.5–4 t/h), with a long delay before full production (15–40 years) and severe alternate bearing. Harvesting of trees is inefficient and expensive because it is almost always done by hand or by beating the fruit off with long poles onto nets. Mechanical harvesting with trunk shakers is difficult or impossible. The positive characteristics of these orchards is that they are very beautiful and majestic, occupying a historical and cultural space where few other crops can be grown, almost like living museums. They also provide a wide range of interesting oil flavors as they represent ancient cultivars that are most likely no longer being planted (Civantos 2001; Rallo et al. 2005) (Fig. 2.3).

2.3.2 Medium and High Density

The medium- and high-density production system was developed in the 1980s so that producers could have a more rapid return on investment compared to traditional systems. Many orchards have been planted at different spacing combinations, usually with the in-row spacing closer than the between-row spacing to create a hedgerow with tree densities of approximately 250–555 trees/ha. With narrower spacings the trees are being trained to a modified palmette by allowing free branch

Fig. 2.4 Machineries for harvesting young olives in orchards with different densities of olive trees. (*Source*: Author)

development within the row, but removing large branches if they grow perpendicular to the hedgerow. Some evenly spaced orchards (same distance between trees in all directions), planted 7–8 m apart, are being trained to the open-center form. The overall benefits have been significant increases in yields per acre, usually double to triple what had been achieved previously with traditionally spaced orchards. The orchards also come into full production sooner, suffer less alternate bearing problems, and are more efficient to harvest (Pastor 1994; Tous et al. 1999a).

In medium- to high-density plantings, the fruit is primarily harvested with trunk shakers that use wrap-around, have slanted side-by-side catch frames, or use nets placed on the ground to capture the fruit. Late-harvest and salvage operations shake the fruit directly onto the ground and pick it up with large sweepers, thereby reducing harvest costs even further (Fig. 2.4). This system has wide adaptability and will accommodate almost any olive variety, soil type, terrain, or training system. On steep terrain where tractors and mechanical shakers cannot function, the trees can be harvested by hand or with various assisted hand-harvesting methods. Some large orchards with flat ground are being harvested with very large over-the-row canopy shakers that surround the whole tree. They move down the rows without stopping, with vibrating rods that knock the fruit down onto belts that carry it up and over to a trailer moving in an adjacent row (Civantos 1998; Ravetti and Robb 2010; Vossen 2007; Zion et al. 2011).

Well-managed medium- and high-density orchards in Argentina, Australia, Chile, Italy, Portugal, and Spain have been planted at spacings of 4 × 8, 7 × 4, or 7 × 5 m (Fig. 2.5). They come into bearing in the fourth year, reaching full production at 6–7 years old. The establishment costs are lower than a super-high-density system, but early yields are not as good because the trees are more vigorous, go through a juvenile nonproductive phase, and must grow to fill a larger space between trees in order to capture the maximum amount of sunlight. Full production, however, at maturity has been observed to be slightly higher (0.5 t/ha) than with the super-high-density system because the trees are taller and capture more sunlight. An advantage of this system is that there is no limit to the cultivars that can be grown, which

Fig. 2.5 Medium-density olive tree (at 4 x 8) of var. Arbequina in Argentina (*Source*: Author)

expands the number of oil styles and flavors that can be targeted to specific markets. Mechanical harvesting with very large over-the-row harvesters such as the Colossus as well as side-by-side trunk shakers work quite well but are slower and less efficient than more rapid smaller over-the-row grape-type harvesters. The long-term viability of these orchards has been well established at over 40 years. Compared to the super-high-density system, the medium- to high-density orchards cost approximately half to establish and approximately half as much to prune. The medium- and high-density-system orchards take approximately 2–4 more years to come into full bearing and cost approximately 2–3 times more to harvest with trunk shaker harvesters or large over-the-row harvesters compared to grape-type harvesters (Table 2.2) (Cubero and Penço 2010; Ravetti and Robb 2010; Vossen et al. 2007, 2011; Vossen 2002).

2.3.3 Super-High-Density

Successful super-high-density olive orchards were pioneered in Spain in the mid-1990s and have now reached over 80,000 ha worldwide with many new plantings still going on. This system uses specific precocious varieties planted at tree spacings of approximately 1.0–1.5 m within the row to approximately 3–5 m between rows for approximately 1,655–2,990 trees/ha. The most typical spacing is 4 × 1.5 m (Fig. 2.6). The ultimate size of the trees is approximately 3 m tall and 2 m wide. The three best known varieties for super-high-density systems, observed to date, are Arbequina, Arbosana, and Koroneiki. They grow in an upright fashion, are initially trained in a mini central leader form, and are then allowed to develop more of a palmette shape, creating a narrow hedgerow. These cultivars are very precocious, tend to produce a good crop every year, start bearing at an early age, and have

Table 2.2 Main characteristics of the orchards according to the density of their olive trees

Medium- to high-density	Super-high-density
Typical spacing (2.4 × 4.8 m to 3.3 × 6.7 m)	Typical spacing (1.2 × 3.66 m to 1.5 × 3.96 m)
247–840 trees/ha	1,655–2,990 trees/ha
Spacing matched to variety, site, and management	Spacing matched to variety, site, and management
Trees okay on deep soils with plenty of rain	Excess vigor on deep soils with plenty of rain
Begins bearing in fourth to fifth year	Begins bearing in third year
Full production by sixth to tenth year	Full Production by fourth to fifth year
Full production of 11–13 t/ha/year) (~20 % more)	Full production of 9–11 t/ha (20 % less)
Harvest by hand, shakers, or very large machines	Harvest with over-the-row grape harvesters
Harvest cost is $120–500 per ton	Harvest cost is $40 per ton (1/3 less)
Highest cost is harvest, ~50 % of production	Highest cost is pruning, ~25 % of production
Medium establishment costs (1/2 less)	High establishment costs (2 × more)
Periodic pruning for light management (1/2 cost)	Specific pruning for light management (2 × cost)
4 m high × 3 m wide – open vase to palmette	3 m high × 2 m wide – monocone to palmette
Controlled deficit irrigation is helpful	Must control irrigation to keep trees small
Medium-intensity fertility management	Very high-intensity fertility management
Can use any variety (potentially higher value oil)	Must plant specific compact varieties
Can plant on any terrain	Must plant on flat or gentle rolling terrain
Can delay processing with undamaged fruit	Must process fruit very quickly due to bruising
Can harvest in almost any weather	Heavy harvest equipment sinks in wet ground
Foliar diseases are more easily managed	Foliar diseases must be intensively managed
Proven system that has worked for 40 years	New system with only 14 years of experience

Source: Roselli and De Gennaro (2011), Vossen et al. (2007)

Fig. 2.6 Super-high-intensity olive tree orchard in California (USA) (*Source*: Author)

excellent oil quality characteristics. However, these are not dwarf varieties and must be managed with deficit irrigation, timely fertilization, and very specific pruning techniques to keep them productive and small enough to accommodate the grape-type harvesters (Tous et al. 1999a, c; Tous et al. 2003; Vossen 2007).

Fig. 2.7 Harvesting young olive trees (*Source*: Author)

The Arbequina variety has been the most widely planted variety for several years in both high-density and especially in super-high-density systems. The variety has several clones, one of which is the I-18, which in some trials came into bearing earlier and produced heavier yields. The Arbosana variety has fruit that looks very much like Arbequina, but it matures approximately 3 weeks later and has 25 % less vigor than Arbequina. Koroneiki is the primary oil variety of Greece and has annual heavy cropping and precocious bearing. It has approximately the same vigor as Arbequina but smaller fruit size and requires greater force for fruit removal.

Well-managed super-high-density orchards will bear some fruit in the second year, but they are normally spray thinned off with 1-Naphtaleneacetic acid (NAA) hormones to encourage greater growth and productivity of the third year. Full production can be reached in the fourth or fifth year. Early fruit yields are quite good but eventually taper off to approximately 9–12 t/ha. It has been observed that production levels of approximately 9 t/ha can be maintained with this system. The long-term productive capacity of the super-high-density orchard system is not known. The main problem is maintaining light exposure to the trees' interior canopies. This system has been quite successful for investors who want a quick return on their investment. The high level of production early in the orchard's life, almost complete mechanization (Fig. 2.7), and production of consumer-friendly sweet oil (primarily from Arbequina) have made this system very popular worldwide. The super-high-density system requires fairly flat ground and requires the ability to control the trees' vigor through pruning, fertility management, and controlled deficit irrigation. A larger capital investment is needed due to the extra trees, trellis, and more closely spaced irrigation system. The super-high-density system also requires a high degree of technical skill by the farm manager because of the requirement to manage the size, light exposure, and increased disease susceptibility of the closely spaced trees (Table 2.2) (Vossen et al. 2007; Vossen 2007).

Table 2.1 shows estimated production costs in Italy and Spain compared with three new-world production areas, Australia, Chile, and California. The analysis is from a mix

of super-high-density and medium-high-density orchard systems. These costs are based on published production cost studies in each country. Land values are quite variable from country to country and within country. Differences in establishment and production costs are reflective of each country's labor rates, equipment costs, cultural practices, and oil yields per hectare.

2.4 Cultivar Influence on Oil Composition and Properties

The amount of variability seen in the composition and properties of different olive oils is a complex interaction between environment (climate, temperature, rainfall, and soil water status), agronomic factors (crop load, shading, leaf-to-fruit ratio, pruning, fertility, irrigation, and fruit maturity stage), and the specific cultivar (genetics). These variables influence chemical compounds that explain olive oil color (pigments) (Chap. 7), aroma (volatiles) (Chap. 8), and taste (phenols) (Chap. 9) and determine olive oil authenticity, traceability, and characterization (e.g., fatty acids, sterols, and hydrocarbons) (Chaps. 6, 12, and 16) through biochemical pathways and chemical reactions. Defect flavors in olive oil, for example, can come from pest damage or mishandling of fruit during and after harvest that lead to fermentations, but they can also occur due to poor oil-processing and storage techniques. Lower-quality defective oils have a high free fatty acid percentage, peroxide value, or UV absorbance reading.

Cultivar characteristics can vary by tree vigor, productivity, precocity (Fig. 2.8), alternate bearing, cold hardiness, flowering and maturity dates, susceptibility to certain diseases, fruit size, pit-to-pulp ratio, oil content, profiles of major and minor chemical compounds, and sensory characteristics. A review of the scientific literature from research in Spain, Italy, France, Morocco, Tunisia, Australia, and the USA shows that there are strong similarities in how most cultivars perform no matter where they are grown, but that some oil characteristics can vary significantly. Comparative varietal studies have shown that some cultivars are consistently better

Fig. 2.8 Precocity of *var.* Arbosana i-43 (*Source*: Author)

Fig. 2.9 Olives from different cultivars

producers than others and that many resident cultivars growing in specific geographical locations may be there only because other cultivars have not been adequately evaluated and subsequently adopted over time.

The choice of which cultivar to grow is one of the most important decisions in growing any horticultural crop, but it is especially important for olives that live a long time and take several years to come into full bearing. Historically, those long-lived trees gave us many traditions and a passion for certain oil styles that were produced and enjoyed by our ancestors. Farmer-induced selection occurred over thousands of years, weeding out the poor producing cultivars with fewer favorable characteristics. Much of that selection, however, was very local, without the benefit of having included other cultivar selections from other regions or countries. Consequently, the real choices of cultivars were quite limited. There are over 1,200 documented olive cultivars in the world (Bartolini et al. 1998), but only approximately 30 represent the vast majority of the production (Vossen 2007) (Fig. 2.9). These cultivars dominate in specific growing areas due to their superior characteristics.

Thanks to a number of comparative cultivar trials in Spain, Italy, Greece, France, Morocco, Argentina, and Australia that evaluated cultivars from all over the world, under local conditions, producers can now make rational decisions about which cultivars to plant. Orchard profitability is most often influenced by how the cultivar yields and if it comes into bearing early (Vossen et al. 2007, 2011). Yield is based on the raw tonnage of fresh fruit produced, but also the oil content of the cultivar-processing techniques being used. Cultivar characteristics such as cold hardiness, pollination, and disease resistance might also be deciding factors (Vossen 2007) (Table 2.3). Oil flavor and oxidative stability are becoming important quality characteristics used to characterize virgin olive oils (VOOs). Market demand for specific oil styles and cultivars with certain characteristics can influence the choice of which cultivar to plant and grow (Romero et al. 2005). Finally, the differences in the production costs from one cultivar to another can be extremely important since olives are a low-margin crop and money spent on cultural costs can be difficult to recover (D'Imperio et al. 2007; Sweeney 2005; Vossen 2007).

Table 2.3 Primary world olive tree cultivars, including several California table olive varieties for comparison

Cultivar and origin	Oil (%)	Cold hardiness	Fruit size	Phenol content	Pollenizer varieties[a]
Arbequina, Spain	22–27	Hardy	Small	Low	Arbosana – Koroneiki
Aglandau, France	23–27	Hardy	Medium	Medium	Bouteillan – Picholine
Ascolano, Italy	15–22	Hardy	Large	Medium	Manzanillo – Mission
Barnea, Israel	16–26	Sensitive	Medium	Medium	Self – Manzanillo – Picholine
Barouni[b], Italy	13–18	Hardy	Large	Medium	Manzanillo – Ascolano – Mission
Bosana, Italy	18–28	Hardy	Medium	High	Tondo de Cagliari – Pizzé Carroga
Bouteillan, France	20–25	Hardy	Medium	Medium	Aglandau – Melanger Verdale
Chemlali, Tunisia	26–28	Hardy	Very small	High	Chetoui
Coratina, Italy	23–27	Hardy	Medium	Very high	Cellina di Nardo – Ogliarola
Cornicabra, Spain	2–27	Hardy	Medium	Very high	Picual – Manzanilla Cacerena
Empeltre, Spain	18–25	Sensitive	Medium	Medium	Arbequina – Morrut
Frantoio, Italy	23–26	Sensitive	Medium	Medium-High	Pendolino – Moraiolo – Leccino
Farga, Spain	23–27	Hardy	Medium	Medium	Arbequina
Hojiblanca, Spain	18–26	Hardy	Large	Medium	Picual – Picudo
Kalamon, Greece	15–25	Moderate	Large	Medium	Mastoides
Koroneiki, Greece	24–28	Sensitive	Very small	Very high	Mastoides
Leccino, Italy	22–27	Hardy	Medium	Medium	Frantoio – Pendolino – Moraiolo
Manzanillo[c], Spain	15–26	Sensitive	Large	High	Sevillano – Ascolano
Maurino, Italy	20–25	Hardy	Medium	High	Lazzero – Grappolo
Mission[c], USA	19–24	Hardy	Medium	High	Sevillano – Ascolano
Moraiolo, Italy	18–28	Sensitive	Small	Very high	Pendolino – Maurino
Pendolino, Italy	20–25	Hardy	Medium	Medium	Moraiolo – Frantoio – Leccino
Picudo, Spain	22–24	Hardy	Large	Low	Picual – Hojiblanca
Picual, Spain	24–27	Hardy	Medium	Very high	Hojiblanca – Picudo
Picholine, France	22–25	Moderate	Medium	High	Bouteillan – Aglandau
Sevillano[b], Spain	12–17	Hardy	Very large	Low	Manzanillo – Mission – Ascolano
Taggiasca, Italy	22–27	Sensitive	Medium	Low	Pendolino

Source: Barranco and Rallo (2000), Cimato et al. (1996), Griggs et al. (1975), Rallo et al. (2005), Note: Most olive varieties are somewhat self-incompatible. Cultivars will usually set a better crop with cross-pollination, especially under adverse weather conditions

[a]Leccino, Pendolino, Moraiolo, and Maurino are self-sterile and require a pollen source from another variety

[b]Barouni and Sevillano are not compatible cross pollenizers for each other

[c]Manzanillo and Mission are not compatible cross pollenizers for each other

Table 2.4 Superior cultivars in terms of productivity, disease resistance, cold hardiness, and oil yield

Productivity	Disease resistance	Cold hardiness	Oil yield
Arbequina	Arbosana	Arbequina	Arbequina
Arbosana	Blanqueta	Ascolano	Arbosana
Barnea	Bouteillan	Chemlali	Coratina
Blanqueta	Frantoio	Cornicaba	Empeltre
Coratina	Koroneiki	Hojiblanca	Frantoio
Hojiblanca	Leccino	Leccino	Koroneiki
Koroneiki		Mission	Hojiblanca
Leccino		Picual	Leccino
Manzanilla		Sevillano	Mission
Picual			Picual
			Picudo
			Taggiasca

Source: Vossen (2007)
Note: Cultivars have been ranked in alphabetic order

2.4.1 Cultivar: Vigor, Precocity, Alternate Bearing, and Yield

The productivity of a cultivar is strongly correlated with tree vigor, which is usually measured in shoot length, trunk diameter, and total tree size or volume. Strongly vigorous trees tend to have long shoot growth and a long nonproductive juvenile period with no flowering or fruit set for the first few years. More precocious cultivars have a tendency to flower and bear fruit at an early age. They are usually less vigorous because heavy early cropping moderates shoot growth. Cultivars with more or less vigor are typically matched to the specific orchard site, based on climate, soil conditions, irrigation water availability, harvest method, and desired tree spacing. Alternate bearing is the tendency to produce a good crop every other year and is a prevalent characteristic in olives. It is strongly cultivar dependent but can be influenced by cultural practices. Table 2.4 shows superior cultivars in terms of productivity, disease resistance, cold hardiness, and oil yield.

Researchers in northern and southern Spain have been comparing their local olive cultivars with introduced cultivars from Italy, Greece, France, Tunisia, Turkey, and Morocco (Caballero et al. 2005). When comparing some Italian and Spanish cultivars grown at 204–286 trees/ha, they found large differences in tree volumes (vigor), with some trees being two to three times larger than others over a 9- to 10-year period (Del Río et al. 2005; Tous et al. 2005a). In Italy a comparison between several local cultivars found similarities in overall growth and number of branches, but not in tree height or trunk diameter, which only varied by approximately 25–30 % (Caballero et al. 2005; Cimato 2001; Del Río et al. 2005; Tous et al. 2005a).

Many studies have measured the number of years it takes for various cultivars to come into bearing, the yields from the first few years, yields once the trees were mature, and yield efficiency (kilograms of fruit per cubic meter of tree volume). In Cataluña, the cultivars Arbequina, Arbosana, Blanqueta, and Palomar started bearing fruit in the second year, while all the other cultivars took at least a year or two longer, and some did not start fruiting until 5–6 years old. The yield efficiency for Arbequina, Arbosana, and Joanenca were extremely high (4.0–4.8 kg fruit/m^3 of tree volume) compared to most other cultivars that were much lower (0.4–3.2 kg fruit/m^3). Those same cultivars also had high yields the first 3 years of production and maintained those higher yields into the seventh to tenth years. In Andalusia, the yield efficiencies ranged from 0.84 for Gordal to 5.36 kg fruit/m^3 of tree volume for Manzanillo. The Italian cultivar Leccino had very good yields the first 3 years of production and into the seventh to tenth years (Del Río et al. 2005; Tous et al. 2005a). Some researchers in Northern Spain found that selected clones of Arbequina, I-18 for example, were superior in production to standard Arbequina (Tous et al. 1999b), while clonal comparisons in southern Spain indicated that there were no significant differences observed between various clonal selections of Arbequina (De la Rosa et al. 2007).

In Australia, researchers also found Arbequina and Leccino to come into bearing early, along with Picual, Barnea, Hojiblanca, Koroneiki, and Manzanillo, which produced the highest average yield the first 3 years. The Italian cultivars Leccino and Frantoio did not come into bearing early, but Leccino had the second highest production after Hojiblanca, which was followed by the third highest producing cultivar, Koroneiki, in the fifth year after planting (Sweeney 2005). Field observation and research in Morocco evaluating the clonal selections Haouzia and Menara, which were selections of standard Picholine Marocaine, indicated superior production in yield, alternate bearing, and oil content in comparison to introduced cultivars from Spain, Italy, Greece, and France (Boulouha 1995; Idrissi and Ouazzani 2003; Sweeney 2005).

Table 2.5 is a summary of several research trials conducted in different countries with orchards planted to medium-, high-, and super-high-density spacings with different cultivars. The data show that the three primary cultivars used in the super-high-density system (Arbequina, Arbosana, and Koroneiki) planted at 1,975 trees/ha provided the most rapid return on investment. In most cases these orchards produce more tons of fruit per hectare in the third year alone compared to the cumulative production from 7–8 years with more vigorous cultivars planted in the medium- or high-density systems. The old wide-spacing production system planted at 204 trees/ha only produces approximately half of what the orchards produce at 555 trees/ha and only a third to a quarter of the production of trees planted at 1,975 trees/ha, after 10 years. At approximately 10 years of age, when the trees reach or approach full size in the medium-density system (555 trees/ha), orchard yields are comparable at a density of 1,975 trees/ha; it just takes approximately 2–3 years longer. Both systems can produce consistent yields of 9–11 t/ha and very high-quality olive oils.

Table 2.5 Summary of early olive yield and oil yield (tons/ha) – first 10 years from various orchards in several countries based on cultivar, tree spacing, and oil content (% wet basis) 204 trees/ha (7 × 7 m); 555 trees/ha (3 × 6 m); and 1,975 trees/ha (1.35 × 3.75 m)

	Trees/ha	Arbequina	Arbosana	Koroneiki	Leccino	Frantoio	Coratina	Picual	Manzanilla
Age	Oil content	15–25 %	20–22 %	18–25 %	18–24 %	23–28 %	23–28 %	23–28 %	13–24%
Year 2	204	0	0	0.4	0	0	0	0	0
	555	0.1	0.2	2.0	0	0	0	0	0
	1,975	0.2	0.4	3.6	–	–	–	–	–
Year 3	204	1.7	1.9	2.7	0	0	0	0	0
	555	4.5	4.8	10.4	0	0	0	0	0
	1,975	16.6	17.2	20.7	–	–	–	–	–
Year 4	204	1.8	1.0	3.3	0.1	0	0	0.2	0.1
	555	5.5	2.7	4.8	0.5	0	0	0.6	0.3
	1,975	17.2	9.0	6.1	–	–	–	–	–
Year 5	204	2.1	1.9	4.9	0.3	0.1	0.1	2.2	0.6
	555	6.3	5.2	6.7	0.7	0.4	0.3	5.1	1.1
	1,975	21.5	19.4	12.5	–	–	–	–	–
Year 6	204	3.0	3.0	2.8	4.0	1.0	0.9	3.9	2.0
	555	8.1	6.3	10.0	8.3	2.8	2.2	9.7	5.3
	1,975	6.3	7.4	4.5	–	–	–	–	–
Year 7	204	4.3	6.5	2.8	5.5	1.6	2.0	4.7	2.6
	555	11.6	9.8	14.2	5.9	4.3	4.5	5.9	5.5
	1,975	15.5	11.1	20.6	–	–	–	–	
Year 8	204	8.0	7.4	1.2	10.5	1.2	2.9	5.9	5.5
	555	21.6	9.4	3.0	15.0	3.3	6.7	10.9	11.2
	1,975	14.4	9.5	7.8	–	–	–	–	–
Year 9	204	9.9	6.2	9.0	6.5	4.3	7.5	11.2	8.0
	555	10.0	9.6	12.1	3.1	11.6	9.0	8.6	18.2
	1,975	10.9	10.4	15.9	–	–	–	–	–

(continued)

Table 2.5 (continued)

Age	Trees/ha Oil content	Arbequina 15–25 %	Arbosana 20–22 %	Koroneiki 18–25 %	Leccino 18–24 %	Frantoio 23–28 %	Coratina 23–28 %	Picual 23–28 %	Manzanilla 13–24%
Year 10	204	16.0	11.8	5.2	12.7	6.1	6.7	6.5	6.8
	555	17.9	16.0	10.9	11.2	4.2	11.2	6.8	9.0
	1,975	18.3	17.5	11.5	–	–	–	–	–
Yield	204	*46.8*	*39.7*	*32.3*	*39.6*	*14.3*	*20.1*	*34.6*	*25.6*
Cumulative	555	*85.6*	*64.0*	*74.1*	*44.7*	*26.6*	*33.9*	*53.0*	*50.6*
Total	1,975	*120.9*	*101.9*	*103.2*	–	–	–	–	–
Yield	204	*11.3*	*8.5*	*5.1*	*11.5*	*3.9*	*5.7*	*7.9*	*6.8*
Average	555	*16.5*	*11.7*	*8.7*	*10.8*	*6.4*	*9.0*	*8.7*	*12.8*
8–10th years	1,975	*14.5*	*12.5*	*11.7*	–	–	–	–	–
Oil Yield	204	*9.36*	*8.34*	*6.94*	*8.31*	*3.65*	*5.12*	*8.82*	*4.73*
Cumulative	555	*17.12*	*13.44*	*15.91*	*9.38*	*6.78*	*8.64*	*13.51*	*9.36*
Total	1,975	*24.18*	*21.39*	*22.18*	–	–	–	–	–

Source: Rojas (2007), De la Rosa et al. (2007), Ouazzani (2005), Del Río et al. (2005), Tous et al. (1999b, 2003), Vossen et al. (2007), Vossen (2007)

Table 2.6 Average oil content (% dry weight) by cultivar and geographical location

	Spain	Spain	Italy	Australia
Cultivar	Catalonia	Andalusia	Apulia	South
Arbequina	50.5–52.9	55.1	55.8	61.9
Picual	50.1	43.1	–	52.1
Hojiblanca	44.3	39.8	–	40.4
Frantoio	–	41.6	45.3	57.4
Leccino	47.1	37.6–49.0	46.8	47.6
Coratina	–	53.6	47.6	57.1
Koroneiki	49.3	55.7	57.9	47.8

Source: Camposeo (Personal communication), Ramos and Santos (2010), De la Rosa et al. (2007), Rallo et al. (2005), Hermoso-Fernández et al. (1998), De Felice and Gomes (1983)

2.4.2 Cultivar: Fruit Oil Content and Extractability

Oil accumulation in the olive fruit progresses rapidly from the pit-hardening stage linearly and very rapidly for approximately 9–17 weeks during the summer peaking as the fruit begins to turn color from green to yellow or purple (Chap. 4). The accumulation pattern varies by cultivar, tree water status, and climate. Oil yield predictions are difficult because there are so many variables, but genetics has a greater influence than environmental factors on oil content (dry weight basis) and pit-to-pulp ratio. Oil content on a dry weight basis has been shown to vary in Spanish and Italian germplasm evaluation trials with ranges from 21 % to 60 % for 104 different cultivars in Andalusia; a range of 30 % to 53 % for 35 cultivars in Catalonia; and from 21 % to 51 % for 39 cultivars in Italy (Table 2.6). In Australia, oil content on a dry weight basis ranged from 40.4 % for Hojiblanca to 61.9 % for Arbequina, which was the highest of all the cultivars tested. The quantity of oil in the fruit can also vary from year to year due to tree vigor, crop load, fruit maturity, and fruit moisture content.

The industrial yield or extractability from a modern processing plant depends very heavily on cultivar, fruit maturity, fruit moisture content, and the processing system (Hermoso-Fernández et al. 1998) (Chap. 3). A low degree of correlation exists between fruit oil content (dry weight basis) and oil extracted because some genotypes show a much greater extractability than others (Lavee and Wodner 1991; Rallo et al. 2005). In other words, some cultivars give up their oil quite easily. Other cultivars, however, are known to have a much lower extractability and produce emulsions and difficult pastes. For example, the Gordal Sevillano variety in California contains approximately 48 % oil on a dry weight basis but often only yields approximately 50–80 kg of oil per ton (5–8 %). Leccino, conversely, has a lower oil content of approximately 38 % on a dry weight basis but typically yields 140–180 kg per ton of fruit (14–18 %) (Table 2.7) (Sweeney 2005; Vossen unpublished data).

Table 2.7 Approximate oil yield from 1 t of olives produced in California orchards with different oil content and extractability (% wet weight basis)

Water status	Ripeness	Olive cultivar	Oil yield
Watered	Over green	Gordal (Sevillano)	50 kg/t (5 %)
	Ripe	Sevillano	60 kg/t (6 %)
	Green	Ascolano	
	Very ripe	Sevillano	80 kg/t (8 %)
	Ripe	Ascolano	
Overwatered	Very ripe	Arbequina or Manzanillo	100 kg/t (10 %)
Deficit-irrigated	Green	Ascolano	
Overwatered	Ripe	Arbequina or Manzanillo	140 kg/t (14 %)
Overwatered	Green	Frantoio or Leccino	
	Very ripe	Arbequina or Manzanillo	160 kg/t (16 %)
Over watered	Green	Mission	
Overwatered	Ripe	Frantoio or Leccino	
	Ripe	Frantoio or Leccino	170 kg/t (17 %)
	Green	Mission	
	Deficit-irrigated	Arbequina or Manzanillo	
Overwatered	Ripe	Mission	180 kg/t (18 %)
Deficit-irrigated	Ripe	Frantoio or Leccino	
	Ripe	Mission or Picual	190 kg/t (19 %)
Deficit-irrigated	Very ripe	Mission or Picual	210 kg/t (21 %)

Source: (Vossen, unpublished data); Coit (1909)

2.4.3 Cultivar: Chemical Composition

The triglyceride structure of olive oil is an important factor in oil stability and human health. It varies considerably by cultivar as to the percentage of different fatty acid types. Specific fatty acid profiles have been determined for most of the common cultivars in traditional growing regions, and they have been suggested as one of the standards for determining whether an oil meets the profile for Protected Designation of Origins (PDO). The content of sterols in olive oil is also highly correlated with cultivar and can even be used to help identify cultivars, and it is important in IOC standards, too (Chap. 16). The fatty acid fraction accounts for more than 98 % of an oil's components based primarily on six major fatty acids. In the germplasm bank in Córdoba (Spain) comparing 73 of the world's most common cultivars, the variability based on genetic differences for this large number of cultivars, palmitic fatty acid percent ranged from 8.49 to 16.46, palmitoleic from 0.41 to 2.26, stearic from 1.46 to 3.79, oleic from 56.12 to 78.34, linoleic from 4.44 to 13.34, and α-linolenic from 0.63 to 1.19. Similar results showing variability between seven of the most common cultivars were also observed in comparative trials in Argentina, Australia, and Italy. Table 2.8 shows the average values of main fatty acids, which were quantified in seven olive oil varieties from orchards located around the world (Lombardo et al. 2008; Mannina et al. 2003; Zarrouk et al. 2009).

Table 2.8 Major fatty acids of virgin olive oils from seven different cultivars grown in several growing regions

	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Linolenic
	7.5–20.0[a]	0.3–3.5[a]	0.5–5.0[a]	55.0–83.0[a]	3.5–21.0[a]	1.0[a,b]
Arbequina						
AR-CAT	**20.66**	**3.69**	1.53	**53.39**	18.72	**1.16**
AU-SA	13.00	1.49	1.69	70.69	11.43	0.60
AU-NSW	19.70	3.50	1.20	**54.50**	19.40	0.70
AU-TAS	10.40	0.80	1.90	81.00	4.40	0.60
SP-AND	15.40	1.90	1.60	65.83	13.06	0.80
FRA	11.30	0.76	2.40	68.26	8.30	0.70
MOR	10.06	0.68	2.66	70.85	14.00	0.96
Picual						
AR-CAT	18.20	3.10	2.20	57.80	16.40	**1.10**
AU-SA	11.76	0.90	2.53	76.99	4.82	0.75
AU-NSW	15.30	2.20	1.80	72.60	6.40	0.90
AU-TAS	9.60	0.60	2.40	**83.40**	2.60	0.60
SP-AND	10.16	1.08	3.79	78.34	4.44	0.89
Hojiblanca						
AU-SA	9.47	0.59	2.81	76.93	7.97	0.84
SP-AND	9.79	0.79	2.96	74.61	8.82	**1.04**
Frantoio						
AR-CAT	17.19	1.65	1.63	63.55	14.03	**1.23**
AU-SA	11.78	1.03	1.99	72.88	10.46	0.69
SP-AND	12.45	1.23	1.89	73.91	8.43	0.86
IT-APU	12.34	1.01	1.65	75.77	8.04	0.55
Leccino						
AR-CAT	17.39	1.16	1.71	68.45	9.19	**1.43**
AU-SA	12.50	1.23	1.62	77.57	5.80	0.73
SP-AND	14.13	1.43	1.89	70.82	9.95	0.85
IT-APU	13.23	1.25	1.53	77.96	4.54	0.68
Coratina						
AR-CAT	16.29	0.67	1.77	71.50	7.99	0.27
AU-SA	11.49	1.28	1.96	74.60	7.48	0.72
IT-APU	12.36	0.51	2.10	75.43	7.94	0.72
Koroneiki						
AU-SA	10.09	0.80	2.53	79.16	4.98	0.66
SP-AND	11.32	0.90	2.24	77.66	5.57	0.74

Source: Ceci and Carelli (2007), Cimato et al. (1996), El Antari et al. (2003), Mailer et al. (2010), Mailer (2005), Mannina et al. (2001), Moutier et al. (2004), Paz Aguilera et al. (2005), Ouazzani (2005), Pinelli et al. (2003); Ripa et al. (2008), Sweeney (2005), Tous et al. (2005), Uceda et al. (2005)
Note: *AR-CAT* Argentina, Catamarca, *AU-SA* Australia, South Australia, *AU-NSW* Australia, New South Wales, *AU-TAS* Australia, Tasmania, *SP-AND* Spain Andalusia, *IT-APU* Italy, Apulia, *FRA* France, Unknown, *MOR* Morocco, Unknown
[a]IOC and EU Standard limits – values outside limits are in bold
[b]0.9 is the limit value inside EU

Table 2.9 Phenol content (mg/kg) of selected cultivars growing in different regions of world

Cultivar	Phenols
Chetoui (Andalusia)	1,240
Picual (Andalusia)	664
Picual (Catalonia)	509
Picual (Chile)	402
Manzanilla (Andalusia)	461
Manzanilla (Catalonia)	321
Koroneiki (Andalusia)	411
Koroneiki (Chile)	318
Koroneiki (Tunisia)	236
Leccino (Chile)	357
Leccino (Tuscany)	146–354
Leccino (Marche)	308
Leccino (Andalusia)	302
Hojiblanca (Catalonia)	273
Hojiblanca (Andalusia)	187
Arbequina (Aragon)	347
Arbequina (Chile)	309
Arbequina (Catalonia)	201
Arbequina (Tunisia)	196
Arbequina (Andalusia)	182

Source: García-González et al. (2010a), Tura et al. (2007), Uceda et al. (2005), Tous et al. (2005), Pannelli et al. (2001)

Beyond the triglyceride fraction, olive oil contains minor compounds, most of them are unique to olive oil or occur more abundantly in olive oil since they are derived from a fruit instead of a seed. Many of these naturally occurring phytochemicals have been shown to have positive human health benefits in reducing obesity, diabetes, certain types of cancer, arteriosclerosis, and other anti-inflammatory effects while not being associated with significant increased body weight (Chaps. 17 and 18). Other phytochemical compounds, such as phenols, provide resistance to oxidation (stability) and contribute to taste descriptors, such as bitterness, astringency, and pungency. The concentration and mix of the multitude of fresh fruit compounds found in olive oil are influenced by several factors. Cultivar is the most important followed by fruit maturity and processing techniques, as long as the olives are in excellent condition (not damaged in any way) and processed using good manufacturing practices (Chap. 3) (Kalua et al. 2007). Thus, the range in total polyphenol content in olive oil is from 182 to 1,240 ppm by cultivar (Table 2.9) from fresh extra virgin oils produced in various countries around the world. Even though the analytical techniques used differ somewhat, the general rankings of the varieties by total polyphenol content is consistent. Each olive variety has a suite of different concentrations of phenols and other phytochemical compounds that make each genotype unique, even though external variables (e.g., climate, irrigation, and harvesting) can modulate its final chemical profile. A study of phenolic compounds of Italian VOOs pointed out that five different cultivars showed

concentration differences of twofold to tenfold. The flavor profile of VOO comes from volatile and phenolic compounds, which are highly influenced by cultivar (Romani et al. 1999). Thus, for example, a trial in Italy evaluated ten cultivars, from 489 oil samples over 4 years, and found that variability was much more influenced by cultivar than by crop year. This study linked specific sensory attributes to each cultivar with an IOC-recognized panel, although it evaluated sensory attributes that go far beyond the official standard for classifying VOOs. Bosana oil was very high in fruitiness, bitterness, and pungency; intermediate in grass, fresh almond, and artichoke; and low in tomato and aromatic herbs. Leccino was high to intermediate in fruitiness; intermediate in bitterness, pungency, and fresh almond; and low in grass, artichoke, tomato, and herbal character. Ravece was very high in fruitiness, bitterness, and pungency; intermediate in grass, artichoke, and tomato; and low in fresh almond and aromatic herb flavors.

Many of the volatile compounds responsible for olive oil aroma and flavor come from enzymatic pathways inside olives during processing (Chap. 8). Studies have been undertaken to determine the mechanisms and influences on these reactions, and it was found that the genetic effect (cultivar) is one of the most important (Chap. 4). Specific volatile compounds and their corresponding odor and taste have been correlated with groups of cultivars and individual cultivars so that they can be distinguished by the huge range in concentrations from one to another (Chap. 12). These compounds are being used to distinguish cultivars for use as genetic material in breeding programs, by farmers to add superior oil quality traits (García-González et al. 2010b), to determine the validity of protected designations of geographical origin, and to identify cultivars for DOP standards (García-González et al. 2011).

In evaluating 39 cultivars from all over the world grown in one location at the Spanish germplasm repository in Andalusia (Luna et al. 2006), it was found that total volatiles ranged from 9.83 to 35.0 mg/kg. Total C_6 "green" compounds ranged from 2.52 to 18.1 mg/kg, and most cultivars could be grouped into four sensory groups. Total hydrocarbons ranged from 1.8 to 8.7 mg/kg; these compounds were found to have little sensory significance. Total aldehydes ranged from 0.82 to 17.1 mg/kg and were primarily responsible for green fruity and sweet sensory notes. Total alcohols ranged from 1.00 to 5.93 mg/kg and were connected to fruity, soft green, and aromatic notes. Total ketones ranged from 0.67 to 2.34 mg/kg and were associated with fruity, pungent, and ethereal sensory notes. Total esters ranged from 0.23 to 1.50 mg/kg and were linked to fruity sensory characters. In Australia researchers were able to discriminate six cultivars and four different maturity stages by identifying and correlating all of the volatile and phenolic compounds within the oils (Aparicio and Luna 2002; Luna et al. 2006; Kalua et al. 2005).

2.5 Agricultural Practices that Influence Oil Quality

There is a complex interaction between genetics and environmental factors (climate, growing region, soil, plant nutrition, climate, year, water regime factors, fruit maturity, pest damage, and crop load) in the degree to which each affects oil

characteristics. In the last few years the use of multivariate, principal component, and discriminant statistical analysis has helped separate the intensity of effects related to genotype (cultivar) and various environmental influences (Fiorino and Alessandri 1996).

2.5.1 Climate and Elevation

Within similar climates and growing conditions, it has been determined that there are almost no differences in the fatty acid profile in oils produced from the same cultivar anywhere in the world. In some cases, however, large differences have been observed between regions with some climatic differences (Aparicio et al. 1994) or altitudes (Ferreiro and Aparicio 1992). For example, in latitudes that are close to the equator with very long days, hot summers, and warm temperatures during harvest (e.g., Catamarca, Argentina), palmitic, palmitoleic, linoleic, and α-linolenic fatty acids tend to be much higher. In general, warmer growing conditions lead to oils with lower oleic fatty acid and higher contents of palmitic, linoleic, and, sometimes, α-linolenic fatty acids. The region of Tasmania in Australia, with its very cool climate, is perhaps the other extreme, and in that growing area the oils have a very different fatty acid profile. Tasmania's oils consistently had higher oleic and stearic fatty acid levels. Table 2.8 shows the normal variability in seven of the world's most common olive varieties grown in different parts of the world. Note how some oils from the very hot climate in Catamarca, Argentina, could not meet several of the IOC standards (Allalout et al. 2009).

Differences in total phenol content can be due to temperature (degree day accumulation) – the warmer the temperature and the higher the altitude of the orchard, the lower the phenolic content of the oil – though some studies have shown little or no differences (Mousa and Gerasopoulos 1996). Greek oils produced in warmer coastal areas were less fruity, bitter, or pungent compared to when the trees were grown at higher elevations with greater diurnal variation and especially lower nighttime temperatures (Osman et al. 1994). An ample range of variation in the total content of phenols (55.4–615.5 ppm) was pointed out in an analysis of 4 cultivars and 17 orchards all over Italy (Esti et al. 1998). The differences, based on both growing region and cultivar, have been seen in the comparative levels of different types of antioxidant compounds (Paz Aguilera et al. 2005; Mousa and Gerasopoulos 1996; Ranalli et al. 1999; Ripa et al. 2008; Servili et al. 2004; Tous et al. 1997, 1999a; Tura et al. 2008)

2.5.2 Soil and Tree Nutrition

Soil type has no clear effect on olive oil composition other than an inherent ability to hold more or less water available to the trees based on rooting depth. Plant nutrition, applied through conventional fertilizers, especially nitrogen and phosphorous,

has been shown to affect the fatty acids in olive oil. It has been demonstrated that when the levels of phosphorous in the fruit are increased to optimum levels, oleic and α-linolenic fatty acids increase, but when nitrogen levels increase, beyond deficit levels to optimum levels, oleic fatty acid decreases. Oleic levels continue to decrease when even more N is applied. Beyond adequate to excess levels of N, linoleic increases, too, which lowers oil stability and the ratio of mono- to polyunsaturated fatty acids. Excess N fertilization has also been show to lower total polyphenols in olive oil. Limiting nutrients (nitrogen – N, phosphorous – P, and potassium – K), but especially nitrogen, can have a negative effect on olive tree vigor. Interestingly, little to no negative growth response is seen if N-P-K is adequate, which is relatively easy and inexpensive to achieve with the use of modern fertilizers. Most studies comparing organic (ecological) versus nonorganic (nonecological) cultivation methods have shown no significant differences in oil composition and properties probably because of the high degree of variability due to fruit maturation, pest damage, water status, genetics, etc. (Ayton et al. 2006; D'Imperio et al. 2007; Dag et al. 2009; Fernández-Escobar et al. 2006, 2009; Inglese et al. 1996; Mannina et al. 2003; Morales-Sillero et al. 2007; Ninfali et al. 2008; Padula et al. 2008; Ranalli and Contento 2010; Skevin et al. 2003; Uceda 2006).

2.5.3 Irrigation and Rainfall

Water status has one of the greatest effects on olive tree vigor and production. Olive trees grown all over the world in Mediterranean-type climates rely on dry growing season conditions that assure fruit pollination, fruit set, a minimum of foliar diseases, and the development of typical fruit characteristics associated with each cultivar. Drought stress, however, limits shoot growth and next season's cropping potential plus fruit size and oil production. When favorable rainfall patterns occur or irrigation is applied to olive trees, fruit yield can be dramatically increased. Several studies have documented greater shoot growth, trunk diameter, and higher yields when irrigation water is applied to drought-stressed trees.

Drought stress during the earlier fruit development stage in the spring, when cell division and initial mesocarp cells are forming, is regarded as the most detrimental to fruit size and oil accumulation. Alternate bearing is worsened when there is an intense competition for water and assimilates especially during this early stage of growth. With a heavy crop, mesocarp size is reduced and there is a slower oil accumulation rate. In the middle fruit growth stage during midsummer, when temperatures are very hot, olive trees are more resistant to water stress. In the later growth stage, when primarily cell enlargement occurs, irrigation can play a key role in fruit size and oil accumulation. Irrigation that relieves drought stress can significantly increase fruit oil content along with overall fruit yield. Drought-stressed trees tend to mature their fruit earlier than irrigated trees.

The amount of water necessary to maximize production depends primarily on climatic demand and the age of the trees. Young developing orchards are typically

Table 2.10 Polyphenol content and oxidative stability of Arbequina oils produced in 2002 and 2003 with different levels of tree irrigation in California

Irrigation level	Polyphenols (mg/L caffeic) 2002	Polyphenols (mg/L caffeic) 2003	Hours O_2 Stab. 120°C 2002	Hours O_2 Stab. 120°C 2003
Low (10–34 % ET)	275.3–432.4[a]	85.0–175.3[a]	8.8–11.4[a]	31.2–40.5[a]
Medium (35–59 % ET)	102.9–165.2[b]	70.0–73.7[b]	4.8–5.4[b]	28.0–30.3[a,b]
High (>60 % ET)	53.3–98.2[c]	73.0–97.3[b]	3.2–4.6[b]	23.6–28.1[b]

Source: Berenguer et al. (2006)
Note: Different letters indicate that the values are significantly different at $\alpha = 0.05$

irrigated to full capacity in order to eliminate stress and maximize shoot growth in order to fill the orchard space as quickly as possible. Several studies on mature orchards have shown that the greatest efficiency of water use is at approximately 40–70 % of full evapotranspiration demand. When water is applied beyond this level, growth and production are only slightly improved. Applying almost any amount less than 40 % as supplemental irrigation, however, can have a significant effect on growth, fruit production, and total oil yield (Cimato et al. 1996; Grattan et al. 2006; Lavee and Wodner 2004; Ouazzani 2005; Sweeney 2005; Tous et al. 2005b; Uceda et al. 2005).

The fruit's water content influences the percentage of oil relative to moisture; drier fruit will have a higher percentage of oil by weight. Oil is easier to extract from low-moisture fruit; conversely, it is more difficult to extract from fruit that has been overirrigated or has received rain before harvest, or if the fruit is washed (wet) just prior to crushing. Fruit with high moisture content often forms an emulsion (watery-oily gel) in the paste that cannot be easily separated, so some escapes with the fruit-water or pomace solids. Therefore, most producers strive to produce olives with around 50 % moisture or slightly less by deficit irrigation. Autumn rainfall, however, often rewets the roots and allows the fruit to absorb water, which can increase the percentage of water in the fruit and cause extractability problems.

The effect of olive tree water status on fatty acid composition is inconsistent. Several studies have shown differences for one year and not the next or are in direct conflict with results obtained by other research. In some cases, however, clear changes have been seen, showing oils produced from irrigated trees with higher oleic (monounsaturated) and lower polyunsaturated and saturated fatty acids (Ben-Gal et al. 2011; Berenguer et al. 2006; Ceci and Carelli 2007; Faci et al. 2002; Gomez-del-Campo 2010; Grattan et al. 2006; Gucci et al. 2004; Mailer et al. 2010: Mailer 2005; Pérez-López et al. 2007; Rapoport et al. 2004; Ripa et al. 2008; Servili et al. 2007; Stefanoudaki et al. 2009).

The main effect on oil quality from giving trees more irrigation water is on the concentration of phytochemicals in the oil. Both, the synthesis of polyphenols and other compounds decreases, plus concentration levels are also diluted out in fruit with higher-than-normal moisture content. An experiment in California provides an example of how irrigation level can influence both the total polyphenol content and oxidative stability (Table 2.10) (Berenguer et al. 2006). This experiment

demonstrated that there can be three to four times more or less total polyphenols or bitterness in a single cultivar (Arbequina) based on the amount of irrigation water the trees received. The heavily irrigated trees produced oils that were almost bland with lower fruity, herbaceous, and floral flavors. Very similar results were obtained in Spain with the Cornicabra variety, where drought-stressed trees and deficit-irrigated trees produced oils with sensory attributes that were almost always greater than more heavily irrigated trees (Gomez-Rico et al. 2007). Work in Italy with Leccino showed a reduction in total polyphenols by approximately half and significant differences in the phenolic composition of the oils as the trees were given more water under severe deficit, deficit, and full irrigation regimes (Servili et al. 2007). In Portugal, where the cultivar Cordovil was treated with four different irrigation regimes, the oils produced from the trees receiving less irrigation water were fruitier and graded higher. The application of saline water to olives shows inconsistent or no effects on fatty acid profile, peroxide value, or free fatty acids, but does increase antioxidant polyphenol levels (Grattan et al. 2006; Ramos and Santos 2010; Wiesman et al. 2004).

2.5.4 Fruit Maturity

The effect of fruit maturity on olive oil sterol content is very significant. As a fruit matures, total sterols decrease, in one study from 2,850 to 1,644 g/kg. The effect of fruit ripening on the fatty acids of olive oils is less clear. In some comparative studies no significant differences were noted in fatty acid makeup as fruit ripened, but most studies have shown that changes do occur. One trial in Australia showed that, as fruit ripens, the palmitic acid level declines and that of linoleic acid increases; the oil becomes more polyunsaturated and therefore less stable. In another trial in Spain, the fatty acid profile changed as fruit ripened, but differently according to cultivar. As Picudo, Chetoui, and Picholine fruits matured, the quantity of monounsaturated fatty acids increased while the saturated and polyunsaturated fatty acids decreased, but with Picual and Arbequina the opposite occurred. It has also been shown, for example, that Barnea can be harvested very ripe because it tends to accumulate oil in the late stages of maturity with no significant changes in oil quality. Conversely, Souri should be harvested early because as it ripens, oleic fatty acid declines and linoleic increases, free fatty acids increase, and the fruit falls off the tree, plus polyphenols levels and oxidative stability fall sharply (Dag et al. 2011; Famiani et al. 2002; Gómez-González et al. 2011; Lazzez et al. 2008; Mailer et al. 2010; Varzakas et al. 2010).

Oil polyphenol content and oxidative stability are very strongly tied to fruit maturity. As olive fruits ripen and begin to turn color (from yellow to red-purple to black), the polyphenol content begins to decrease (Chap. 9), the chlorophyll content (green color) decreases, and the other pigments start to increase (Chap. 7). As the fruit flesh colors fully all the way to the pit, the polyphenol content declines very rapidly over approximately 2–5 weeks by approximately half to one-third in most

varieties. One study in Italy showed a decrease in total polyphenols from 535 mg/kg for green/yellow fruit to 396 mg/kg for partially purple fruit to 311 mg/kg for fully ripe black fruit. Correspondingly, many other components change; the green herbal (tomato leaf, artichoke, grass, green apple, cinnamon), tannic, astringent, bitter, and pungent flavors soften. The volatiles responsible for ripe fruit flavors like nuttiness, butteryness, floral character, and some subtle undertones associated with ripe fruits (peach, apricot, berries, tropical fruits, vanilla, and citrus) become more dominant. The decrease in oleuropein and increase in elenolic acid glucoside and 3,4-dihydroxyphenylethanol can indicate olive fruit maturation. Little scientific evidence exists to suggest that olive oil volatile composition could be used to discriminate between growing regions (Anastasopoulos et al. 2011; Angerosa et al. 2004; Ayton et al. 2006; Caponio et al. 2001; De la Rosa et al. 2008; Esti et al. 1998; Skevin et al. 2003).

2.5.5 Pest Damage and Freeze Injury

Fruit damage from pests can have a huge negative impact on olive oil quality, especially flavor. Oil obtained from olives damaged by anthracnose (*Gleosporium olivarum* Alm.) show significant increases in aldehydes such as heptanal, octanal, and nonanal, which are off-flavors that can be attributed to oxidation and decomposition reactions. Olive fruit fly (OLF) (*Bacrocera oleae*) is another pest that must be controlled in order to prevent fruit damage and obtain high-quality oil. In the case of olive-fly-damaged fruit, volatiles such as carbonyl compounds and alcohols intensify, but in addition phenolics decrease, lowering oxidative stability, plus as the fruit ripens, free fatty acids and peroxide values increase. One of the methods to reduce the negative effects of OLF on oil quality is to harvest early before fruit softens and rots from fungal and bacterial contamination. Fruit deterioration is also less in cultivars that have a high polyphenol content that resists oxidation and microorganism attack. Varietal differences are important in that OLF damage is lower in small fruited varieties with a small flesh-to-pit ratio. OLF seems to have no significant effect on oil fatty acid profile. Freeze injury to olives prior to harvest can also reduce oil quality by lowering chlorophyll and carotenoid contents, reducing oil stability, bitterness, and pungency and increasing the levels of vanillin and vanilic acid causing the oil to taste sweet, woody, and bland (Gómez-Caravaca et al. 2008; Koprivnjak et al. 2010; Mraicha et al. 2010; Morelló et al. 2003; Runcio et al. 2008; Tamendjari et al. 2009; Vossen unpublished data).

2.5.6 Crop Load and Pruning

Sugars produced in olive leaves are transported to the fruit and converted into oil. Therefore, the ratio of leaves to fruit can dramatically affect fruit oil content. In years with heavy crop loads, total oil production is higher, but individual fruits have a lower oil content compared to years with light crops. In the autumn, the rate

of sugar production and conversion to oil decreases dramatically because days are getting shorter and cooler and fruit abscission and ripening begin to take place. Fruit on trees with a heavy crop load will mature later than if there is a light crop. Delaying harvest until the fruit is ripe assures the highest yield of oil, though that will change the flavor characteristics. Leaving the fruit on the tree, however, past the color change period does not significantly increase the amount of oil produced in an orchard. Palmitic and linoleic fatty acids decrease with a heavy crop load in most cases, but crop load has no effect on free fatty acids, peroxide value, or sensory characteristics. Training system and pruning appear to have no effect on oil quality (Gucci et al. 2007; Paliotti et al. 1999).

2.6 Conclusions and Future Trends

Researchers and agriculturists are responding to the economic needs of producers by creating new cultivars that are less vegetative and more precocious and have great oil characteristics. These new cultivars will be grown in places where supplemental irrigation is available and costs can be kept low with mechanical pruning and continuous over-the-row harvesters. Orchards that are not viable economically due to inefficiency of scale, low yields, or because they cannot be mechanized likely will have to be abandoned or converted to grow other crops. New orchards will be almost completely mechanized. The best cultivars will have a high content of oil and will produce well in a wide array of growing climates. Consumers will appreciate their high content of monounsaturated fatty acids and long shelf life plus the various different combinations of polyphenol intensities and complex volatiles that provide many different styles and flavors. These new cultivars, along with existing top selections, will continue to require evaluation in new growing regions as the cultivar environment interaction is quantified. New cultivars in new regions will require traceability characterization for fatty acid profile, phenol types, and volatiles to determine authenticity. As specific cultivars are tied to production systems that rely more and more on labor-reducing harvest systems, the trees will have to be manipulated in architecture to accommodate the machines. The machines themselves will need to be evaluated in each growing system. Tree manipulation through mechanical pruning will need to be evaluated for size, light exposure, productivity, oil characteristics, and cost. The orchard trees will require training and pruning to maximize light interception and maintain high productivity. Each cultivar will need to be evaluated in each location for growth habit, vigor, precocity, productivity, oil content, fatty acid profile, polyphenol content, and flavor.

Olive oil needs to be produced with little or no negative environmental impact. Ecological or organic growing methods need to be evaluated and compared to conventional systems. Olives are a crop that lends itself well to organic production methods with comparably few insect and disease problems. Olives can also survive and even thrive on lower water and nutrient inputs than many other tree crops, so they will be popular to grow. With existing problems, growers must reduce the use

of conventional pesticides and evaluate the effectiveness of more natural products or methods to control olive fly, anthracnose, foliar diseases, weeds, and other olive orchard pests. There is a great demand to plant olive trees in soils that have been infested with Verticillium wilt fungus (*Verticillium dahlia*). Controls may come from resistant cultivars, resistant rootstocks, or natural methods that eliminate the fungus from attacking the trees. Orchards will need to be grown without cultivating the soil or controlling weeds with herbicides, so that soil erosion can be reduced. All of these cultural practices should be evaluated for their compatibility with local growing conditions and cultivars. Water use efficiency in olive orchards is extremely important as our freshwater resources become scarcer or water just becomes more expensive. Olives are already more salt tolerant than many other crops, so the use of saline water will likely increase. Research needs to be conducted on deficit irrigation strategies and on the effects of using salty water or treated municipal wastewater on tree yield and oil quality. Climate change will have a big impact on olive growing, from predicted changes in water demand and availability to shifts in temperatures that may allow olives to be grown in areas that were previously too cold.

The future of the olive oil industry is bright. Of course, during economic downturns the worldwide demand for olive oil, prices, and profitability declines, leading to less investment in new orchards or new processing plants. Slower growth for a period is normal and should temper the very rapid expansion in new plantings and mills seen during the past decade. The trend in olive oil consumption, however, has been rising for many years, and predictions are that consumers will continue to buy more and more olive oil, especially as economies improve. A good sign is that demand for olive oil in China, India, Brazil, and other emerging countries is expanding rapidly. As our population in the Western world ages, olive oil will become an even more sought after commodity for health reasons, especially as research continues to reveal the positive effects of using olive oil in preventing human diseases (Chaps. 17 and 18). As population grows and resources diminish, demand will likely increase for organic or ecologically produced olive oil. As consumers have become more aware of the special flavors and real quality differences associated with foods, the trend has been toward better quality, higher prices, and increased consumption. We now have a multitude of gourmet wines known by variety, growing region, and production method. The same is happening with microbrewed beers, coffee, specialty cheeses, grass-fed beef, heirloom tomatoes, and fancy salad mixes. Olive oil is soon to follow. As consumers learn how to differentiate between fresh, high-quality oils and use them to enhance the flavors of their food, demand, quality, and prices will increase for high-quality oils. Chefs worldwide are just starting to learn about cooking dishes with different styles of olive oil and how those new flavors can be attractive to their restaurant clients. Many consumers new to olive oil tend to favor light flavored oils without a lot of bitterness or pungency that have nutty, floral, and buttery characteristics. As olive oil consumers become more familiar with olive oil, they tend to prefer stronger, more herbaceous, or green fruity oils that are quite bitter and pungent. When they find out that those oil styles also contain more of the healthy minor components, then demand for those oil types will increase significantly. Meanwhile, there is huge demand for mild fresh oils that

appeal to new consumers. Buyers will eventually look for olive oil as a fresh fruit product with great anticipation for the new releases every 6 months alternating from the southern and northern Hemispheres.

References

Allalout A, Krichene D, Methenni K, Taamalli A, Oueslati I (2009) Characterization of virgin olive oil from super intensive Spanish and Greek varieties grown in northern Tunisia. Scientia Hortic-Amst 120:77–83

Anastasopoulos E, Kalogeropoulos N, Kaliora AC, Kountouri A, Andrikopoulos NK (2011) The influence of ripening and crop year on quality indices, polyphenols, terpenic acids, squalene, fatty acid profile, and sterols in virgin olive oil (Koroneiki cv.) produced by organic versus non-organic cultivation method. Int J Food Sci Technol 46:170–178

Angerosa F, Servili M, Selvaggini R, Taticchi A, Esposto S et al (2004) Volatile compounds in virgin olive oil: occurrence and their relationship with the quality. J Chromatogr A 1054:17–31

Aparicio R, Luna G (2002) Characterisation of monovarietal virgin olive oils. Eur J Lipid Sci Technol 104:614–627

Aparicio R, Ferreiro L, Alonso V (1994) Effect of climate on the chemical composition of virgin olive oil. Anal Chim Acta 292:235–241

Ayton J, Mailer RJ, Haigh A, Tronson D, Conlan D (2006) Quality and oxidative stability of Australian olive oil according to harvest date and irrigation. J Food Lipids 14:138–156

Baldari M, Gulisano G, Nesci FS, Strano A (2009) Aspeti economici ed organizzativi delle imprese olivcole ed olearie in Calabria. Congress on "Ricerca ed Innovazione per l'Olivicoltura Meridionale (RIOM)". CRA-OLI 11–12 Giugno, pp 303–315

Barranco D, Rallo L (2000) Olive cultivars in Spain. Hort Technol 10:107–110

Bartolini G, Prevost G, Messeri C, Carignani G, Menini UG (1998) Olive germplasm: cultivars and word-wide collections. FAO, Rome

Ben-Gal A, Yermiyahu U, Zipori I, Presnov E, Hanoch E et al (2011) The influence of bearing cycles on olive oil production response to irrigation. Irrig Sci 29:253–363

Berenguer MJ, Vossen PM, Grattan SR, Connel JH, Polito VS (2006) Tree irrigation levels for optimum chemical and sensory properties of olive oil. Hort Sci 41:427–432

Boulouha B (1995) Contribution to the improvement of productivity and regularity of bearing in the 'Picholine Marocaine' olive (*Olea europaea*). Olivae 58:54–57

Caballero JM, Del Rio C, Navarro C, García-Fernández MD, Morales J et al (2005) Ensayos Comparativos de Variedades. In: Rallo L, Barranco D, Caballero JM, Del Río C, Martín A et al (eds) Variedades de olivo en España. Ediciones Mundi-Prensa, Madrid

Caponio F, Gomes T, Pasqualone A (2001) Phenolic compounds in virgin olive oils: influence of the degree of ripenss on organoleptic characteristics and shelf-life. Eur Food Res Technol 212:329–333

Ceci LN, Carelli AA (2007) Characterizatino of monovarietal argentinian olive oils from new productive zones. J Am Oil Chem Soc 84:1125–1136

Cimato A (2001) Risorse genetiche in Olivicoltura. Actes du Seminare International "Multiplication et certification des plants d'olivier", Meknes, 2–7 Aprile, pp 195–210

Cimato A, Baldini A, Caselli S, Marronci M, Marzi L (1996) Observations on tuscan olive germplasm. 3: analytical and sensory characteristics of single-cultivar olive oils. Olivae 62:46–51

Civantos L (1998) The olive tree, the oil, the olive. ADICOM, Madrid

Civantos L (2001) La olivicultra en el mundo y en España. In: Barranco D, Fernández-Escobar R, Rallo L (eds) El Cultivo del Olivo, 4th edn. Ediciones Mundi-Prensa, Madrid, pp 17–34

Civantos L, Pastor M (1996) Production techniques. In: World olive encyclopedia. International Olive Council, Madrid, pp 147–194

Coit JE (1909) Olive culture and oil manufacture in the arid southwest, vol 62, University of Arizona Agricultural Experiment Station Bulletin. University of Arizona, Tucson
CORFO (2008) Olive oil in chile, Chilean Economic Development Agency. http://www.invest-chile.com
Cruzat GR, Barrios E (2009) Resultados y lecciones en producción integrada para obtención de aceite de oliva en pequeña unidad productiva. Proyecto de innovación en la VI Region. http://goo.gl/aAMra
Cubero S, Penço JM (2010) Aproximación a los costes del cultivo del olivo. In: Seminario Asociación Española de Municipios del Olivo (AEMO), Córdoba. http://www.aemo.es/get.php?pathext=descargas/Costes_AEMO.pdf
D'Imperio M, Dugo G, Alfa M, Mannina L, Segre AL (2007) Statistical analysis on Sicilian olive oils. Food Chem 102:956–965
Dag A, Ben-David E, Kerem Z, Ben-Gal A, Erel R et al (2009) Olive oil composition as a function of nitrogen, phosphorus and potassium plant nutrition. J Sci Food Agric 89:1871–1878
Dag A, Kerem Z, Yogev N, Zipori I, Lavee S et al (2011) Influence of time of harvest and maturity index on olive oil yield and quality. Sci Hortic 127:358–366
De Felice M, Gomes T (1983) Valutazione dei contenuti in olio nelle olive. Proposta di un metodo rapido. Scienza e Tecnica Agraria 33:21–29
De la Rosa R, León L, Guerrero N, Rallo L, Barranco D (2007) Preliminary results of an olive cultivar trial at high density. Aust J Agric Res 58:392–395
De la Rosa R, Leon L, Moreno I, Barranco D, Rallo L (2008) Ripening time and fruit characteristics of advanced olive selections for oil production. Aust J Agric Res 59:46–51
Del Río C, Caballero JM, García-Fernández MD (2005) Vigor. In: Rallo L, Barranco D, Caballero JM, Del Rio C, Martín A et al (eds) Variedades de olivo en España. Ediciones Mundi-Prensa, Madrid
El Antari A, El Moudni A, Ajana H (2003) Comparison of the quality and fatty acid composition of the olive oil obtained from Mediterranean cultivars cultivated in Morocco. Olivae 95:26–31
Esti M, Cinquanta L, La Notte E (1998) Phenolic compounds in different olive varieties. J Agric Food Chem 46:32–35
Faci JM, Berenguer MJ, Espada JL, Garcia S (2002) Effect of variable water irragation supply to olive (*Olea Europaea L.*) cv. Arbequina in Aragon (Spain). II Extra virgin oil quality parameters. Acta Hort 586:341–344
Famiani F, Proietti P, Farineli D, Tombesi A (2002) Oil quality in relation to olive ripening. Acta Hort 586:671–674
Fernández-Escobar R, Beltran G, Sánchez-Zamora MA, García-Novelo J, Aguilera MP et al (2006) Olive oil quality decreases with nitrogen over-fertilization. Hort Sci 41:215–219
Fernández-Escobar R, Marin L, Sánchez-Zamora MA, García-Novelo JM, Molina-Soria C, Parra MA (2009) Long-term effects of N fertilization on cropping and growth of olive tres and on N accumulation in soil profile. Eur J Agron 31:223–232
Ferreiro L, Aparicio R (1992) Influence of the altitude in the chemical composition of virgin olive oils. Grasas Aceites 43:149–156
Fiorino P, Alessandri S (1996) Agronomic techniques and characteristics of olive oil. In: World olive encyclopedia. International Olive Oil Council, Madrid, pp 195–217
García-González DL, Romero N, Aparicio R (2010a) Comparative study of virgin olive oil quality from single varieties cultivated in Chile and Spain. J Agric Food Chem 58:12899–12905
García-González DL, Tena N, Aparicio R (2010b) Quality characterization of the new virgin olive oil *var.* Sikitita by phenols and volatile compounds. J Agric Food Chem 58:8357–8364
García-González DL, Tena N, Aparicio R (2011) Describing chemical singularity of Spanish protected designations of origin of virgin olive oil. Grasas Aceites 63:26–34
Gomez del Campo M, Barranco D (2009) Situación del olivar en España y el seguro agrario, Ministerio de Agricultura. http://aplicaciones.mapya.es/documentos_pwe/seminarios/olivar_upm.pdf

Gómez-Caravaca AM, Cerretani L, Bendini A, Segura-Carretero A, Fernández-Gutiérrez A, Del Carlo M, Compagnone D, Cichelli A (2008) Effects of fly attack (*Bactrocera oleae*) on the phenolic profile and selected chemical parameters of olive oil. J Agric Food Chem 56:4577–4583

Gomez-del-Campo M (2010) Physiological and growth responses to irrigation of a newly established hedgerow olive orchard. Hort Sci 45:809–814

Gómez-González S, Ruiz Jiménez J, Luque de Castro MD (2011) Oil content and fatty acid profile of Spanish cultivars during olive fruit ripening. J Am Oil Chem Soc 88:1737–1745

Gomez-Rico A, Salvador MD, Moriana A, Perez D, Olmedilla N, Ribas F, Fregapane G (2007) Influence of different irrigation strategies in a traditional Cornicabra cv. olive orchard on virgin olive oil composition and quality. Food Chem 100:568–578

Grattan SR, Berenguer MJ, Connell JH, Polito VS, Vossen PM (2006) Olive oil production as influenced by different quantities of applied water. Agric Water Manage 85:133–140

Griggs WH, Hartmann MV, Bradley BT, Iwakiri BT, Whisler JE (1975) Olive pollination in California. California Agr Expt Sta Bul 869

Gucci R, Servili M, Selvaggini M, Baldioli M, Montedoro GF (2004) Oil quality of olive CV Leccino grown under irrigated or dry-farmed conditions. Acta Hort (ISHS) 664:297–302

Gucci R, Lodolini E, Rapoport HF (2007) Productivity of olive trees with different water status and crop load. J Hort Sci Biotechnol 82:648–656

Hermoso-Fernández M, González J, Uceda M, García-Ortiz A, Morales J et al (1998) Elaboración de Aceite de Oliva de Calidad II – Obtención por el sistema de dos fases. In: Informaciones Técnicas. Junta de Andalucía, Sevilla, pp 9–23

Idrissi A, Ouazzani N (2003) Apport des descripteurs morphologique à l'inventaire et à l'identification des variétés d'Olivier (*Olea europaea* L.). Plant Genet Resour Newsl 136:1–6

Inglese P, Barone E, Gullo G (1996) The effect of complementary irrigation on fruit growth and ripening pattern and oil characteristics of olive (*Olea europaea L.*) Cv. Carolea. J Hort Sci Botechnol 71:257–263

IOC (International Olive Council) (2011a) Country profiles. http://www.internationaloliveoil.org/estaticos/view/136-country-profiles

IOC (International Olive Council) (2011b) World market in figures. Olivae 115:26–29

Kalua CM, Allen MS, Bedgood DR, Bishop AG, Prenzler PD (2005) Discrimination of olive oils and fruits into cultivars and maturity stages based on phenolic and volatile compounds. J Agric Food Chem 53:8054–8062

Kalua CM, Allen MS, Bedgood DR, Bishop AG, Prenzler PD, Robards K (2007) Olive oil volatile compounds, flavour development and quality: a critical review. Food Chem 100:273–286

Koprivnjak O, Dminic I, Kosic U, Majetic V, Godena S et al (2010) Dynamics of oil quality parameters changes related to olive fruit fly attack. Eur J Lipid Sci Technol 112:1033–1040

Lavee S, Wodner M (1991) Factors affecting the nature of oil accumulation in fruit of olive. J Hort Sci 66:583–591

Lavee S, Wodner M (2004) The effect of yield, harvest time and fruit size on the oil content in fruits of irrigated olive trees (*Olea europaea*), cvs. Barnea and Manzanillo. Scientia Hortic-Amst 99:267–277

Lazzez A, Perri E, Caravita MA, Khlif M, Cossentini M (2008) Influence of olive maturity stage and geographical origin on some minor components in virgin olive oil of the Chemlali variety. J Agric Food Chem 113:796–807

Lombardo N, Marone E, Alessandrino M, Godino G, Madeo A et al (2008) Influence of growing season temperatures in the fatty acids (FAs) of triacilglycerols (TAGs) composition in Italian cultivars of *Olea europaea*. Adv Hort Sci 22:49–53

Luna G, Morales MT, Aparicio R (2006) Characterization of 39 varietal virgin olive oils by their volatile compositions. Food Chem 98:243–252

Mailer RJ (2005) Variation in oil quality and fatty acid composition in Australian olive oil. Aust J Exp Agric 45:115–119

Mailer RJ, Ayton J, Graham K (2010) The influence of growing region, cultivar and harvest timing on the diversity of Australian olive oil. J Am Oil Chem Soc 87:877–884

Mannina L, Fontanazza G, Patumi M, Ansanelli G, Segre A (2001) Italian and Argentine olive oils: a NMR and gas chromatographic study. Grasas Aceites 52:380–388
Mannina L, Dugo G, Salvo F, Locicero L, Ansanelli G et al (2003) Study of the cultivar composition relationship in Sicilian olive oils by GC, NMR and statistical methods. J Agric Food Chem 51:120–127
Morales-Sillero A, Jiménez R, Fernández JE, Troncoso A, Beltrán G (2007) Influence of fertigation in 'Manzanilla de Sevilla' olive oil quality. Hort Sci 42:1157–1162
Morelló JR, Moltilva MJ, Ramo T, Romero MP (2003) Effect of freeze injuries in olive fruit on virgin olive oil composition. Food Chem 81:547–553
Mousa YM, Gerasopoulos D (1996) Effect of altitude on fruit and oil quality characteristics of 'Mastoides' olives. J Sci Food Agric 71:345–350
Moutier NC, Pinatel A, Martre J-P, Roger (2004) Identification et caratérisation des varieties d'Olivier cultivées en France. Naturalia Publications, Turriers, 248 p. (20, rue des Grands-Augustins, 75006 Paris)
Mraicha F, Ksantini M, Zouch O, Ayadi M, Sayadi S, Bouaziz M (2010) Effect of olive fruit fly infestation on the quality of olive oil from Chemlali cultivar during ripening. Food Chem Toxicol 48:3235–3241
Ninfali P, Bacchiocca M, Biagiotti E, Esposto S, Servili M, Rosati A, Montedoro GF (2008) A 3-year study on quality, nutritional and organoleptic evaluation of organic and conventional extra-virgin olive oils. J Am Oil Chem Soc 85:151–158
Osman M, Metzidakis I, Gerasopoulos D, Kiritsakis A (1994) Qualitative changes in olive oil of fruits collected from trees grown at two altitudes. Riv Ital Sostanze Grasse 71:187–190
Ouazzani N (2005) Caractérisation et évaluation des performances agronomiques des génotypes locaux et variétés étrangères en collection dans les régions du Nord du Maroc. Rapport projet RESGEN CFC/IOOC/03
Padula G, Giordani E, Bellini E, Rosati A et al (2008) Field evaluation of new olive (*Olea europaea* L.) selections and effects of genotype and environment on productivity and fruit characteristics. Adv Hort Sci 22:87–94
Paliotti A, Famiani F, Proietti P, Boco M, Antognozzi E et al (1999) Effects training system on tree growth, yield and oil characteristics in different olive cultivars. Acta Hort 474:189–192
Pannelli G, Alfei B, Santinelli A (2001) Varietá di olivo nelle Marche. Publicazione dalla Agencia Servizi Settore Agroalimentare delle Marche, Ancona
Pastor M (1994) Plantaciones intensivas de olivar. Agricultura 746:738–744
Paz Aguilera M, Beltrán G, Ortega D, Fernandéz A, Jiménez A et al (2005) Characterization of virgin olive oil of Italian olive cultivars: 'Frantoio' and 'Leccino', grown in Andalusia. Food Chem 89:387–391
Pérez-López D, Ribas F, Moriana A, Olmedilla N, de Juan A (2007) The effect of irrigation schedules on the water relations and growth of a young olive (*Olea europaea L.*) orchard. Agr Water Manage 89:297–304
Pinelli P, Galarid C, Mulinacci N, Vincieri FF, Cimato A, Romani A (2003) Minor polar compound and fatty acid analysis in monocultivar virgin olive oils from Tuscany. Food Chem 80:331–336
Rallo L, Barranco D, Caballero JM, Del Rio C, Martin A et al (2005) Variedades de Olivo en España. Mundi-Prensa, Madrid
Ramos AF, Santos FL (2010) Yield and olive oil characteristics of a low-density orchard (cv. Cordovil) subjected to different irrigation regimes. Agric Water Manage 97:363–373
Ranalli A, Contento S (2010) Analytical assessment of destoned and organic-destoned extra-virgin olive oil. Eur Food Res Technol 230:965–971
Ranalli A, De Mattia G, Batumi M, Proietti P (1999) Quality of virgin olive oil as influenced by origin area. Grasas Aceites 50:249–259
Rapoport HF, Costagli G, Gucci R (2004) The effect of water deficit during early fruit development on olive fruit morphogenesis. J Am Soc Hort Sci 129:121–127
Ravetti L (2011) Medium density. Symposium at UC-Davis, California – April 4–5. http://www.olivecenter.ucdavis.edu/publications/publications#previous-course-powerpoints

Ravetti L, Robb S (2010) Continuous mechanical harvesting in modern Australian olive growing systems. Adv Hort Sci 24:71–77
Ripa V, De Rose F, Caravita MA, Parise MR, Perri E et al (2008) Qualitative evaluation of olive oils from new olive selections and effects of genotype and environment on oil quality. Adv Horti Sci 22:95–103
Rojas MA (2007) Curvas de producción del olivo: Experiencias en Chile. V Encuentro Nacional del Aceite de Oliva, Santiago. http://www.chileoliva.com/documentos/nuevo/Chileoliva%202007%20Marco%20%20Rojas
Romani A, Mulinacci N, Pinelli P, Vincieri FF, Cimato A (1999) Polyphenolic content of five Tuscan cultivars of *Olea europaea* L. J Agric Food Chem 47:964–967
Romero A, Tous J, Guerrero L (2005) Caracterización Sensorial del Aceite. In: Rallo L, Barranco D, Caballero JM, Del Río C, Martín A et al (eds) Variedades de olivo en España. Ediciones Mundi-Prensa, Madrid
Roselli L, De Gennaro B (2011) Olivo, confront economic tra intensive e superintensivo. L'Informatorio Agrario 12:63–67
Runcio A, Sorgona L, Mincione A, Santacaterina S, Poiana M (2008) Volatile compounds of virgin olive oil obtained from Italian cultivars grown in Calabria. Effect of processing methods, cultivar, stone removal, and antracnose attack. Food Chem 106:735–740
Servili M, Selvaggini R, Esposto S, Taticchi A, Montedoro GF, Morozzi G (2004) Health and sensory properties of virgin olive oil hydrophilic phenols: agronomic and technological aspect of production that affect their occurrence in the oil. J Chromatgr A 1054:113–127
Servili M, Esposto S, Lodolini E, Selvaggini R, Taticchi A et al (2007) Irrigation effects on quality, phenolic composition and selected volatiles of virgin olive oil cv Leccino. J Agric Food Chem 55:6609–6618
Skevin D, Rade D, Strucelj D, Mokrovcak Z, Nederal S et al (2003) The influence of variety and harvest time on the bitterness and phenolic compounds of olive oil. Eur J Lipid Sci Technol 105:536–541
Stefanoudaki E, Williams M, Chartzoulakis K, Harwood J (2009) Effect of irrigation on quality attributes of olive oil. J Agric Food Chem 57:7048–7055
Sweeney S (2005) National olive cultivar assessment – NOVA- Stage 2. Rural Industries Research and Development Corporation Publication no 05/155, project no SAR-47A. http://www.rirdc.gov.au
Tamendjari A, Angerosa F, Mettouchi S, Bellal MM (2009) The effect of fly attack (*Bactrocera oleae*) on the quality and phenolic content of Chemlali olive oil. Grasas Aceites 60:507–513
Tous J, Romero MA, Plana J, Guerrero L, Diaz I, Hermoso JF (1997) Chemical and sensory characteristics of "Arbequina" olive oil obtained in different growing areas of Spain. Grasas Aceites 48:415–424
Tous J, Romero MA, Díaz I (1999a) Fruit and oil characteristics of five Spanish olive cultivars. Acta Hort 474:639–642
Tous J, Romero A, Plana J (1999b) 'IRTA-i-18' a clone of the 'Arbequina' olive cultivar. Olivae 77(6):50–52
Tous J, Romero MA, Plana J, Baiges F (1999c) Planting density trial with 'Arbequina' olive cultivar in Catalonia (Spain). Acta Hort 474:177–180
Tous J, Romero MA, Plana J (2003) Plantaciones Superintensivas en Olivar. Comportamiento de 6 variedades. Agricultura 815:346–350
Tous J, Romero A, Plana J (2005a) Vigor. In: Rallo L, Barranco D, Caballero JM, Del Río C, Martín A et al (eds) Variedades de olivo en España. Ediciones Mundi-Prensa, Madrid
Tous J, Romero A, Díaz I (2005b) Composición del aceite (Banco de Germoplasma de Cataluña). In: Rallo L, Barranco D, Caballero JM, Del Río C, Martín A et al (eds) Variedades de olivo en España. Ediciones Mundi-Prensa, Madrid
Tura D, Gigliotti C, Pedó S, Failla O, Bassi D et al (2007) Influence of cultivar and site of cultivation on levels of lipophilic and hydrophilic antioxidants in virgin olive oils (*Olea europaea L.*) and correlations with oxidative stability. Scientia Hortic-Amst 112:108–119
Tura D, Failla O, Bassi D, Pedo S, Serraiocco A (2008) Cultivar influence on virgin olive (*Olea europea* L.) oil flavor based on aromatic compounds and sensorial profile. Scientia Hortic-Amst 118:139–148

Uceda M (2006) Olive oil quality decreases with nitrogen over-fertilization. Hort Sci 41:215–219
Uceda M, Beltrán G, Jiménez A (2005) Composición del aceite (Banco de Germoplasma Mundial de Córdoba). In: Rallo L, Barranco D, Caballero JM, Del Río C, Martín A et al (eds) Variedades de olivo en España. Ediciones Mundi-Prensa, Madrid
Varzakas TH, Zakynthinos G, Arapoglou D (2010) Fruit ripening in relationship to oil quality and some quality characteristics of the Greek olive cultivar koroneiki. Int J Food Sci 22:401–407
Vossen PM (2002) Super-high-density olive oil production. OLINT Mag Int 1(October):17–23
Vossen PM (2007) Olive oil: history, production, and characteristics of the world's classic oils – workshop Mediterranean fruits: ancient history and modern promise. Hort Sci 42:1093–1100
Vossen PM, Connell JH, Krueger WH, Klonsky KM, Livingston P (2007) Samples costs to establish a super-high-density olive orchard and produce oil in the Sacramento Valley. University of California, Cooperative Extension Division of Agriculture and Natural Resources, Oakland. http://coststudies.ucdavis.edu
Vossen PM, Elkins RB, Bianchi ML, Klonsky KM, Livingston P et al (2011) Sample costs to establish a medium-density olive orchard and produce bottled oil: North and Central Coasts. University of California Cooperative Extension Division of Agriculture and Natural Resources, Oakland. http://coststudies.ucdavis.edu
Wiesman Z, Itzhak D, Ben Dom N (2004) Optimization of saline water level for sustainable Barnea olive and oil production in desert conditions. Scientia Hort 100:257–266
Zarrouk W, Baccouri B, Taamalli W, Trigui A, Daouda D (2009) Oil fatty acid composition of eighteen Mediterranean olive varieties cultivated under the arid conditions of Boughrara (Southern Tunisia). Grasas Aceites 60:498–506
Zion B, Bechar A, Regev R, Shamir N, Weissblum A et al (2011) Mechanical harvesting of olives-an operations study. Isr J Plant Sci 59:71–84

Chapter 3
Technological Aspects

Luciano Di Giovacchino

Contents

3.1 The Olive Plant and Olive Oil: A Brief History

Today the olive plant (*Olea europea*, var. sativa) is distributed all over the countries of the Mediterranean basin, particularly in Spain and Italy in the central and western parts, mainly in Greece and Turkey in the eastern parts and Tunisia and Morocco in the southern parts. The present situation is the result of a slow but continuous development of the various civilisations in the coastal territories of the Mediterranean and more inland areas of the Middle East.

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties*,
DOI 10.1007/978-1-4614-7777-8_3, © Springer Science+Business Media New York 2013

Although fossil findings dating back to the tertiary period (1 million years ago) prove the existence of an ancestor of the olive tree in Italy a long time ago, and the olive stones or pits found in human settlements of the palaeolithic period (35,000–8,000 B.C.) confirm the presence of a plant similar to the olive tree in southern Europe (Schäfer-Schuchardt 1988), it seems certain that the olive tree as we know it today had its origin approximately 6,000–7,000 years ago in the region corresponding to ancient Persia and Mesopotamia (Boskou 1996). The olive plant later spread from these countries to nearby territories corresponding to present-day Syria and Palestine. Other sources (Schäfer-Schuchardt 1988) indicate that stone mortars and presses used to extract the oil from olives date back to 5000–4000 B.C. (these are now kept in the Haifa Olive Museum).

All ancient civilisations located around the Mediterranean basin and in the Middle East have left clear evidence that olive cultivation and oil production were activities that developed with the flourishing civilisations themselves.

At present, the most ancient known document in which the words *olive* and *olive oil* appeared for the first time is a tablet of clay discovered in the archaeological excavations of Ebla, near Aleppo (Syria), and date back to the third millennium B.C. (about 2500 B.C.) (Blazquez 1997). Another ancient document is a stone, found at Susa (Iran) and dating back to the second millennium B.C., where regulations regarding the commerce of agricultural products are reported, including ones for olive oil.

The archaeological findings kept in the Haifa Museum and those of the Knossos Palace in Crete are other examples confirming the production of olive oil in ancient times. The Cretan or Minoan civilisation, which experienced its major growth between 2000 and 1450 B.C., especially encouraged the production and trade of olives, thus stimulating oil export to other Mediterranean countries, notably Egypt.

Figure 3.1 shows the base plate of an early press that was used to extract oil. This press is among the archaeological finds of the Phestos Palace in Crete. Figure 3.2 shows vases (*pithoi*) used for olive oil storage that are kept in the Knossos Palace in Crete.

To extract olive oil, the olives were first ground in a mortar with stones and the paste obtained was placed on a stone base plate with grooves for drainage. It was then pressed by placing heavy rocks on top of them so as to cause the extrusion of oil globules. Virgin olive oil (VOO) and vegetation water were then separated by simple settling under the influence of gravity.

In ancient Egypt the olive plant was cultivated to obtain its oil for use in religious ceremonies and as an ointment. As mentioned previously, the Egyptians not only imported olive oil from Crete and Syria, but they also cultivated olive trees in the north, along the Nile valley, and in the south, in the region of the ancient town of Thebes. Evidence of olive cultivation is the press built in red granite and discovered close to the Temple of Pharaoh Ramses III (1150 B.C.) at Medinat Habu, near Luxor (Egypt) (Schäfer-Schuchardt 1988).

The Phoenicians also cultivated olive trees (first millennium B.C.), but they were also capable traders and navigators and probably introduced the olive tree to colonies in many countries of the Mediterranean basin, such as in Libya, in Tunisia (Carthage) and in Sicily, France and Spain.

Fig. 3.1 Archaeological find, in the Phestos Palace, Crete: base plate of a press (Reproduced with permission of Schäfer-Schuchardt 1988)

Fig. 3.2 Archaelogical find, in the Cnossos Palace, Crete: vases (pithoi) for olive oil storage (Reproduced with permission of Schäfer-Schuchardt 1988)

Many references to the olive tree and its oil are found in the Hebrew Scriptures and other holy texts, where the use of olive oil as a source of light for lamps used as offerings as well as reading is attested. Particularly important is the biblical reference, from 1000 B.C., concerning the return of the dove with a small olive branch to confirm to the patriarch Noah the end of the Deluge and that the olive trees cultivated near Mount

Ararat in Turkey were not still covered by water. Moreover, other references in the Hebrew Scriptures concern the kings of Israel, David and Solomon, who favoured olive cultivation to improve the country's economy (Schäfer-Schuchardt 1988).

In Greece, the olive tree and olive oil were present since the fourteenth and thirteenth centuries B.C., as demonstrated by the artefacts found in the excavations of Mycene, in the Argolis region. In the same period, references to olive trees are found in the Homeric poems, the *Iliad* and and *Odyssey*, such as the bed of Odysseus and the club of Cyclops built with the wood of olive, or the use of olive oil to anoint the body of Odysseus (Schäfer-Schuchardt 1988).

When Greek civilisation dominated and Athens became a very important city of the age, olive tree cultivation was improved, and the zenith of its development was reached in the eighth to seventh centuries B.C. The importance of olive trees in ancient Greece is confirmed by the mythology concerning the origin of the city of Athens, due to Athena, goddess of wisdom, who gave an olive tree as a gift to the city and won the dispute with Poseidon, god of the sea, who gave as a gift a salt spring. In other words, in Greek mythology, the olive tree was presented as a gift of the gods of Olympus to humans (Boskou 1996). In ancient Greece, olive oil was used not only to anoint the bodies of athletes but also as a prize for the winners of the Olympic games (Schäfer-Schuchardt 1997).

In Italy the olive tree was probably known since the rise of the Etruscan civilisation, but it is certain that it was introduced by Greek colonisers who founded several cities in the south of Italy (Magna Grecia), especially in Calabria and Sicily. The Romans later developed this cultivation, which in turn increased their commerce with all the conquered lands that made up the Roman Empire.

After the victory and the conquest of Carthage, the Romans increased the empire in North Africa, expanding their dominion into the territories of the West, corresponding to present-day Tunisia, Algeria and Morocco. In these countries, olive trees and oil were already known because the Phoenicians, as previously mentioned, founded colonies in these regions and imported olive oil through their maritime commerce. The Romans realised that the conquered territories were suitable for the production of olives and olive oil and therefore favoured the cultivation of olive trees in the south of Carthage, a region called Africa (Camps-Fabrer 1997), and to the south-west of Carthage, a region called Numidia (Camps-Fabrer 1997), where many ancient stone mills dating back to the second and third centuries A.D. were found.

In the territories corresponding to Algeria, the Romans left the traces of an ancient town and olive mills in the region called Caesarea, in particular the ruins are located near the city of Tipasa, on the coast of the Mediterranean Sea, where it is possible to admire some stone mills for crushing olives.

In ancient Mauritania, formerly called Tingitania and corresponding to present-day Morocco, the Romans favoured olive cultivation to produce olive oil and many traces of this activity remain, in particular in the ruins of the ancient city of Volubilis, near the city of Meknès, where it is possible to admire a wooden press with a beam (rebuilt) (Fig. 3.3) and some black stone mill.

Although the Romans, as did the Greeks, used olive oil mainly as an ointment, for pharmaceutical use, and for lighting (lampante oil), they began to use the oil also

Fig. 3.3 A wooden press with beam (rebuilt) present in the ruins of Volubilis (Morocco)

for food. In this regard, the Roman agronomist L. Junius Columella (late first century A.D.), in his book *De re Rustica*, indicated the varying quality of olive oils obtained from olive processing as follows: 'oleum acerbum', oil with an intense odour and obtained from green and unripe olives; 'oleum viride', oil obtained from red-yellow olives, handpicked from the tree; 'oleum maturum', oil obtained from ripe and black olives; 'oleum cibarium', the oil obtained from poor-quality olives and intended for lighting or as food for slaves.

The Romans also contributed to technological developments in olive processing by expediting the crushing operation with a millstone crusher, the trapetum (Fig. 3.4), and improving the separation system by the introduction of presses.

The screw press, first used by the Greeks (fourth century B.C.) (Schäfer-Schuchardt 1988) and later improved upon and disseminated by the Romans, represented a major advance in olive processing. This is especially true if one considers that even today, in some olive-growing countries of the Mediterranean basin, presses very similar to these are still used, as shown in Fig. 3.5.

With the fall of the Roman Empire and the barbarian (in Italy) and Arab invasions (in Spain), olive cultivation declined because of the destruction inevitably caused by war. Nevertheless, in the late Middle Ages, olive culture recovered either in Spain, under Arab rule, or in Italy, mainly with the efforts of religious communities. During a long period, for many centuries until the late nineteenth century, the innovations in olive processing were basically the following: the use of the cylindrical millstones as olive crushers (dating back to the Byzantine period); the use of animals (donkeys, horses, cows) to rotate the heavy cylindrical stone mill; the use of a press with a wooden or metallic screw (Fig. 3.6) to exert pressure on mats containing the olive paste, as shown in Fig. 3.6.

From the Middle Ages to the late 1800s, olive cultivation spread in the regions of central Italy and more to the south, in the Puglia region, where many old underground oil mills dug into the rock remain, as shown in Fig. 3.7.

Fig. 3.4 A trapetum, one of the first millstone crushers (Reproduced with permission of Schäfer-Schuchardt 1988)

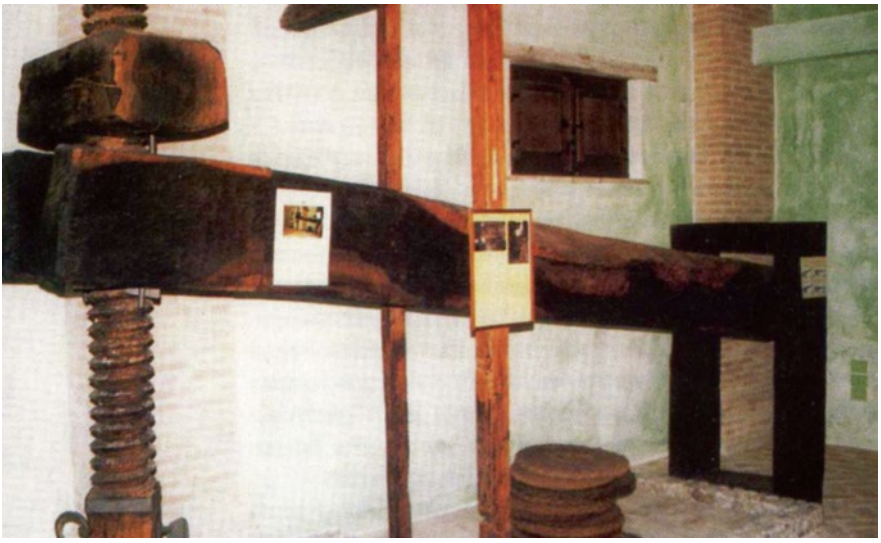

Fig. 3.5 A wooden press with beam (Reproduced with permission of MuTAC - Picciano, Italy)

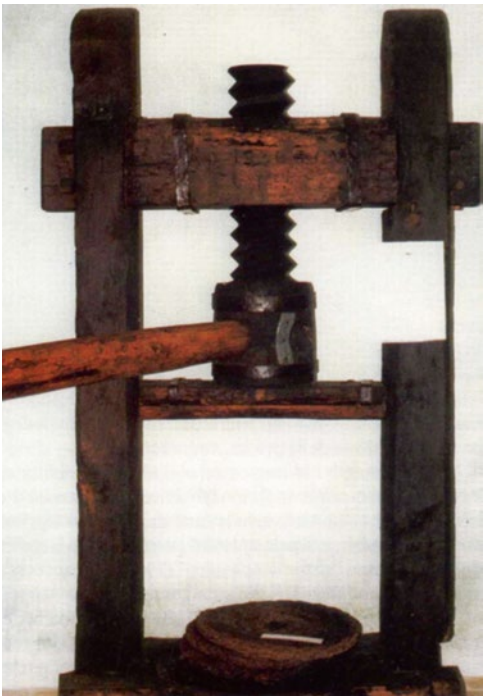

Fig. 3.6 A wooden press with screw (Reproduced with permission of MuTAC - Picciano, Italy)

Fig. 3.7 Old and underground oil mill (Puglia region) (Reproduced with permission of APROL – Associazione Produttori di Olive della Provincia di Lecce, Italy)

During the 1900s other possibilities presented themselves for mechanically extracting oil as a result of studies on percolation and centrifugation systems. These innovative systems materialised in 1951 with the Buendía patent for a percolation system, afterwards built in Italy under the name Sinolea, and towards the end of the 1960s with the introduction of the Centriolive plant, the first industrial decanter based on the continuous centrifugation of olive paste.

The pressing system underwent continuous improvement with the increasing application of electrically driven hydraulic pumps, the introduction of cage presses and column presses and, finally, with the arrival of the modern open monoblock superpresses that can reach pressures of 350–450 atm.

3.2 Olive Transport and Storage

After olive fruits are picked in olive groves, they need to be transported to oil mills as soon as possible. This operation is generally carried out using a tractor or a car having a tipping body possibly covered by a tarpaulin (or waterproof canvas), as is the general practice in Spain, Italy or other countries. Another good way to transport olives is through the use of small plastic cases (crates) with a capacity of 20–25 kg, as is often done in many countries, or using large crates (bins), with a capacity of 250–300 kg, as is sometimes done in Italy. To avoid the risk of reducing the quality of olives through squashing, the depth of the layers of olives and the time of transport must be limited. These conditions are generally respected using the means mentioned previously because the crates have special holes that allow air circulation and help to disperse heat from the fruit caused by their catabolic activity.

In contrast, the use of jute or plastic sacks to transport olives is not very rational since the drupes might be squashed, and then damaged, especially if they are very ripe. This method of transport could alter the oil quality by initiating biological processes caused by endogenous and exogenous microflora.

Olives must be processed in oil mills as soon as possible, especially if they are very ripe. If this is not possible, due to the reduced processing capacity of the mill, then the olives obviously must be stored. The duration of the storage should be as short as possible and must be carried out in such a way that the product quality is always protected. The method adopted to store olives can be different according to the modalities by which the oil mill works: (1) the oil mill offers to process olives for farmers and thus processes each batch for individual owners separately, as generally happens in Italy; (2) the oil mill is dedicated to its own land and can therefore process all the olives together, or the oil mill is a cooperative where all the olives are processed together without separation of the different fruits from individual farmers but on the basis of the quality of the olives, as often happens in Spain.

In the first case, the best way to store olives is by using large crates (bins) with holes, which has the advantages of ensuring the separation of the olives from various farmers, ensuring air circulation, avoiding thick layers of drupes, easily displacing the olives by electric forklift, and requiring a small surface (1.5–1.8 t/m^2), as shown in Fig. 3.8.

Fig. 3.8 Storage of olive using bins in an Italian olive oil mill

It is also a good idea to use small crates to store olives, but in this case it is not possible to employ forklifts, and so the fruits must be moved by hand, which increases expenses. Generally, small crates are used in small oil mills equipped with a pressure system, as happens in Italy.

In the second case, the oil mill is large and can process olives all together, and the best way to store olives and preserve their quality is to arrange thin layers (30–40 cm) of drupes and place them in an open but covered warehouse room, as often happens in Italy. Sometimes, the olives are stored outdoors, with or without a canopy, but this runs the risk of exposing the olives to harsh weather elements, particularly snow and frost, which are more frequent in the regions of central and northern Italy. Another method to store olives that makes it possible to process them all together is through the use of large hoppers where the drupes are stored following leaf removal and washing, as happens in Spain. This method, adopted by large oil mills having a capacity of greater than 200–300 t/day (and more), can have the disadvantage of exposing the drupes to squeezing when the layer is more than 40–50 cm thick because of the absence of leaves and the presence of water used for washing. In some cases, the hoppers at the large oil mills have a capacity of 30–40 t of olives, and therefore the drupes will be stored in very thick layers (2–3 m). Of course, if the olives are very ripe, the risk of squeezing is significant with implications for the quality of oil. Today, however, almost all the cooperatives process clean olives within 24–36 h of being harvested, avoiding the risk to reduce the quality of VOO.

Some studies have been carried out on the possible use of inert gases to store olives for long periods in controlled atmospheres (Castellano et al. 1993), but that solution has never been used because the current trend in the olive oil industry is to reduce the time of storage by increasing the capacity of mills through the use of larger centrifugal decanters, as is already done in Spain.

Table 3.1 Effects of added leaves to ripe olives on some qualitative characteristics of virgin olive oil obtained by centrifugation of olive paste prepared by a fixed hammer metallic crusher

		Added leaves (%)		
Determination	Control	1	2	3
Free fatty acids (%)	0.54	0.63	0.61	0.58
Peroxide value (meq/kg)	4.2	4.8	4.6	4.9
K_{232}	1.43	1.51	1.53	1.57
K_{270}	0.07	0.09	0.09	0.10
Organoleptic assessment (score)[a]	6.3	6.6	6.7	7.2
Total phenols (mg/kg)	69	66	68	68
Induction time (h)	8.0	8.2	8.2	8.7
Chlorophyll pigments (mg/kg)	3.4	5.5	6.6	8.4
(E)-2-Hexenal (mg/kg)	131	203	242	288
Green fruity (score)[b]	–	0.8	1.3	1.6

Data from Di Giovacchino et al. (1996)
[a]Score between 1 and 9
[b]Score between 1 and 5

3.3 Leaf Removal and Washing

Leaf-removal and washing operations are necessary to remove all foreign matter from the olives, whether vegetable or non-vegetable, that could be harmful to the machinery or contaminate the product. These operations are carried out by machinery equipped with a powerful aspirator, which removes leaves and sprigs, and a washing tank, with forced water circulation in which olives are washed. In addition, the machinery can be equipped with a special magnet to remove any iron objects that could be harmful to the metal crushers.

Leaf removal by fans and washing operations should always be done because clean olives enhance the quality of the oil. Many oil mills using a pressing system and crushing by millstones, however, do not perform these operations on olives picked from trees because they are not considered to present a significant risk to the protection of the machinery or to the quality of the oil. In some cases, the oil mill removes the leaves only, in particular when the olives are green and not ripe. When the olives are gathered from the ground, foreign matter must be removed by special equipment, such as vibrating screens.

If a mill uses a continuous centrifugation system (decanter) and the olives are ground with a metal crusher, leaf removal and olive washing operations should be performed to avoid negative consequences for olive oil sensory characteristics and damage to the rotating parts of the machinery. The presence of leaves will intensify the green-leaf taste organoleptic characteristic of oil that consumers find distasteful when it is excessive, in particular in regions or countries where olive trees are not cultivated. Table 3.1 shows the results obtained in a specific study (Di Giovacchino et al. 1996) indicating that very ripe

Fig. 3.9 Small machine for leaf removal and olive washing

olives supplemented with olive tree leaves increase the content of (E)-2-hexenal that (according to the panellists) intensifies the organoleptic perception of the green fruity odour of the oil and can improve its organoleptic assessment without varying the values of the total phenol content and other qualitative parameters.

The washing operation is useful for removing metal or mineral matter that can be harmful to the fast-moving mechanical parts of the crusher and decanter. In particular, the possible presence of sandy material (silica) with olives can cause dangerous abrasion on the body of the decanter, which rotates at a high speed and could become unbalanced. Moreover, the wash water helps to eliminate the residues of the plant protection products, or their metabolites, which can remain on the drupes, thereby reducing the risk that they will contaminate the oil. Figure 3.9 shows a small machine for leaf removal and washing olives in an Italian oil mill equipped with a centrifugal decanter.

3.4 Olive Processing

The object of processing olives is to obtain VOO as defined by the International Olive Council (IOC).

The mechanical extraction of oil from olives entails releasing it from the fruit tissues in such a way that the droplets can merge into larger drops until they form a

continuous liquid phase. Olive oil is mainly located inside the vacuoles of the mesocarp cells but is also somewhat scattered inside the colloidal system of the cytoplasm and, in small amounts, in the epicarp and endosperm.

Oil enclosed inside the vacuoles can be released by mechanical means, although oil dispersed inside the cytoplasm is quite difficult to extract and is generally lost with the pomace or vegetation water. Moreover, when olive paste is prepared by crushing and malaxation operations, an emulsion may sometimes form, which hinders the subsequent separation of the oil. This is in part due to the droplets of emulsified oil being surrounded by lipoproteins, which prevent them from coalescing.

Olive paste intended for mechanical oil extraction must therefore be prepared to obtain two results: the complete break-up of oil-containing mesocarp cells by the olive-crushing operation and the prevention or restriction of olive paste emulsion formation by malaxation (or mixing). These operations, common to all systems employed in processing olive fruits with oil mills, increase the amount of mechanically extractable oil and have two objectives: to preserve olive oil quality and to facilitate its release when separating the solid and liquid phases.

Until now, olive fruits for olive oil extraction were processed in mills by the following mechanical systems only: pressure, percolation, centrifugation.

3.5 Pressing Method

This extraction process, which has been the sole means of obtaining VOO for many centuries, is governed by various mechanical principles depending on the kind of pressing method.

Pressure is exerted on the olive paste and, under appropriate conditions, causes the separation of the liquid phases (VOO and vegetation water) from solids (pomace). A working diagram of the pressing system is shown in Fig. 3.10.

After leaf removal, the olive paste from which oil is extracted is prepared. To obtain high oil yields, it is necessary to release the greatest part of the oil content from the vacuoles of the olive mesocarp cells, and to that end, the olives are crushed.

Crushing is continually carried out by a stone mill when a pressing system is used. Modern stone mills have two or three (and sometimes even four or six) granite millstones that are cylindrical and have a diameter of 100–140 cm. In Spain these granite millstones have a conical or truncated-conical shape.

The crushing operation, carried out with granite millstones, has its pros and cons. The millstone makes it possible to establish the most appropriate crushing time (20–30 min) for each batch of olives and provides a partial mixing of the olive paste that helps the droplets of oil to merge into large drops. In addition, it is a very gentle operation, producing only a small increase in the olive paste temperature (3–5 °C), and its low rotation speed (12–15 rpm) avoids the formation of emulsions and, hence, increases the extraction yield. The millstone, however, has its disadvantages.

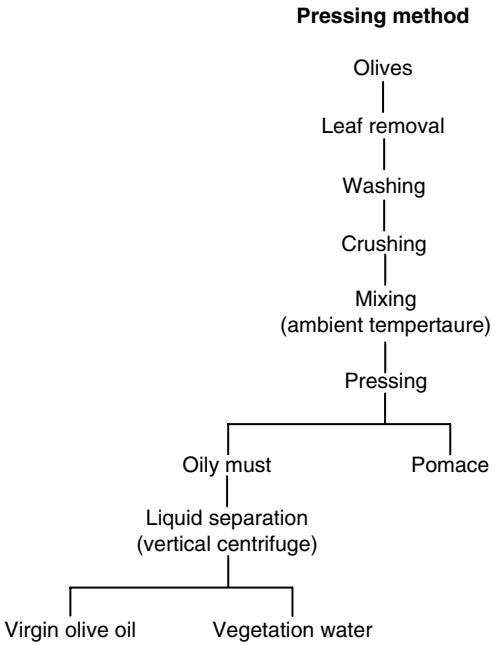

Fig. 3.10 Diagram of olive oil extraction by the pressing method?

It is very cumbersome and expensive, the whole process is slow and discontinuous and it has a low load capacity.

The olive paste obtained after crushing needs to be mixed to achieve maximum oil yields. The mixing operation consists of a slow and continuous stirring of the olive paste, which increases the percentage of 'free oil' by helping the droplets of oil to merge into large drops (Martinez Moreno et al. 1957a) and by breaking up the oil/water emulsion. During malaxation, the olive paste is heated by hot water circulating inside a heating jacket. The mixing time is limited to 20–30 min and the temperature of the paste should not exceed 22–25 °C. Modern mixers automatically spread the olive paste onto mats (filtering diaphragms of nylon fibre), which are then stacked for pressing.

To extract olive oil by the pressing method, pressure is applied to a large stack of mats spread with olive paste placed on a trolley with a central spike. Generally, 3-5 mats with olive paste are continually placed between two metallic discs. The perforations in the central spike have been a decisive factor in improving the separation of oily must (olive oil + vegetation water) from pomace as they allow the liquid phases to flow out from the middle of the stack. The large stack of mats and discs is placed under the press formed by an open monoblock scaffolding and a piston (35–40 cm diameter) that pushes the pile from the bottom (Fig. 3.11).

The oily must leaving the pile under the press is collected in a draining well (Fig. 3.11), and the speed of the flow depends on the olive characteristics and the

Fig. 3.11 Oily must (oil + vegetation water) is collected in a draining well

crushing and mixing operations. The oily must is then sent to a vertical centrifuge, rotating at 6,500 rpm, to separate the VOO from the aqueous phase (the vegetation water).

Many factors affect oil extraction yields during pressing, in particular the maximum level of pressure reached by the press. Super presses, with pistons 35 or 40 cm in diameter, reach a maximum pressure of between 350 and 400 kg/cm^2, corresponding to a specific pressure on the olive paste of 120–200 kg/cm^2. The high pressure reached and the huge force exerted on the olive paste allow extraction of a high percentage (82–86 %) of the oil present in the olives, as reported in some papers (De Felice et al. 1979; Di Giovacchino et al. 1994a).

Pressing systems have the following advantages (IOC 1990): (a) limited investment is needed; (b) simple and reliable machinery is used; (c) little electrical power is required and so energy consumption is low; (d) pomace is less wet; and (e) a small amount of vegetable water is produced and it contains little oil.

However, pressing systems also have disadvantages: (a) the machinery is cumbersome; (b) they require more labour; (c) the filtering mats could become contaminated; (d) the process is discontinuous; and (e) the working capacity is low.

With reference to olive oil quality, a pressing system can yield good results if the machinery and factory are clean, healthy olives are processed and the work is continuous, as reported in some papers (Di Giovacchino et al. 1994b; Di Giovacchino 1996; Boskou 1996).

At present, pressing systems are still viable in only small oil mills, which are common in Italy, where approximately 1,500–1,600 oil mills use such a system. In other countries, the number of oil mills equipped with a pressing system is rapidly decreasing, and in Spain such systems have almost disappeared entirely.

3.6 Percolation Method

Olive oil extraction from olives by the percolation method is based on the difference in the surface tension between oil and vegetation water. This difference allows a steel blade to be preferably coated with oil when it is plunged into olive paste. When the steel blade is withdrawn, olive oil drips off and separates from other phases, creating a flow of oily must. This is due to the fact that in the presence of olive paste solids, olive oil has an interfacial tension less than vegetation water in relation to the steel blade.

The first studies involving the percolation method date back to 1911, when the Acapulco method appeared. The industrial machinery was later modified (1929–1930) to become the Acapulco-Quintanilla method. The first machinery utilised olive paste without stones (kernels) and had some drawbacks which were later eliminated by Francisco Buendía, who built the Alfin prototype in 1951, which has been known as Sinolea since the 1950s (Fig. 3.12).

The Sinolea consists of a stainless steel semi-cylindrical grating with many small blades moving through slits in the grating. The movement of the blades is slow, and therefore, when they plunge into the olive paste, as it is continuously renewed, they are coated with oil. The oil drips off the blades when they are withdrawn.

The first Sinolea machinery had a loading capacity of 300–350 kg of olive paste and 5,120 small blades spread over a total surface of 1.18 m^2 (Martinez Moreno et al. 1965). A mechanical system, rotating at 7.5 rpm, moves the small blades and a scraper pushes the olive paste uniformly and continuously towards the grating plates. The Sinolea plant is able to extract up to 70–75 % of the oil contained in olives. It was used directly with a pressing method (Petruccioli 1965), but this combination had the drawbacks of the latter method – high labour costs and filtering diaphragms. Nowadays, a Sinolea plant is used in combination with a centrifugal decanter, thus reducing labour costs and obtaining higher oil yields. In this case, the machinery is fitted with fewer blades (4,608 over 1.06 m^2) because of the incorporation of a screw at the bottom to unload the partially de-oiled olive paste.

The theoretic equation (Martinez Moreno et al. 1968) to calculate the oily flow leaving the machinery is as follows:

$$Q = Q_0 \left(1 - e^{-bt}\right),$$

where Q is the amount of oil extracted in time t, and Q_0 is the amount of free oil that could be extracted in an infinite period of time.

Fig. 3.12 Sinolea machinery, used to extract olive oil by the percolation method?

This equation was later modified (Setti 1973; Petruccioli et al. 1973) for practical reasons:

$$\log \frac{Q_r}{Q_0} = H - h \log \frac{t}{10}$$

where Q_r is the residual oil in the olive paste at a specific time t, Q_0 is the olive oil contained in the initial paste, determined by Soxhlet apparatus, and h and H are kinetic constants of the process.

Percolation is the most natural process (Fig. 3.13) because it takes place at ambient temperatures, without the addition of diluting water and without employing mats, thereby avoiding any possibility of contamination due to the decomposition of organic vegetable matter on uncleaned mats. The characteristics of oil obtained by the percolation method in comparison with other extraction methods are shown in Table 3.8.

Oil yields obtained by the percolation method depend greatly on the rheological properties of olive paste and, particularly, on the percentages of water and hydrophilic solids. Figure 3.14 shows that increasing water content within olive paste (diminishing oil/water ratio) leads to a diminished oil yield (Di Giovacchino 1986), but increasing the hydrophilic solid content increases oil yield (Di Giovacchino and Mascolo 1988).

The percolation method is an automated process that requires a small workforce and uses a limited amount of energy but does not give high oil yields, especially when olives are difficult to process. Moreover, the produced pomace has a high water content (50–65 %) and a medium-high oil content (8–12 %). This last

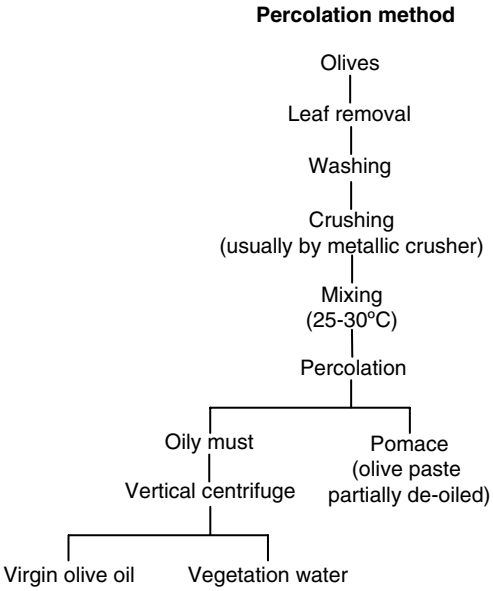

Fig. 3.13 Diagram of olive oil extraction by the percolation method?

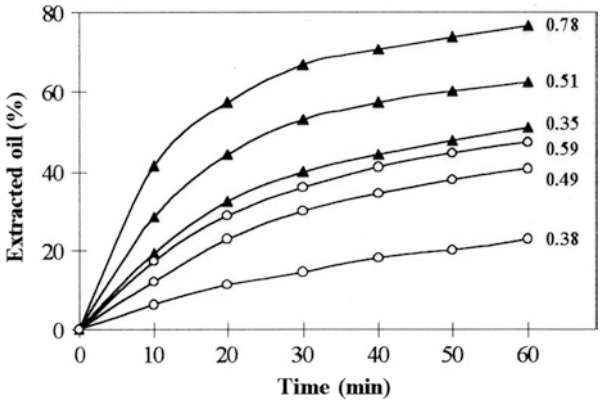

Fig. 3.14 Oil yields obtained at different times by percolation method, when olives with different oil/water ratios (0.38–0.59 and 0.36–0.78) are processed (*Source*: Di Giovacchino (1986), with permission of Rivista Italiana delle Sostanze Grasse)

drawback is overcome by combining the percolation method with centrifugation, thereby obtaining a semi-continuous process with all the advantages of automation. Less labour is needed, and because of the double extraction, the oil yields are similar to or higher than those obtained with the pressing method (Mascolo et al. 1980). In any case, at the present time the number of oil mills equipped with the percolation system is not very high and is decreasing as it is being replaced by the centrifugation system, using three- and two-phase decanters.

3.7 Centrifugation Method

The first centrifugal decanter was employed in an olive oil mill in the late 1960s. The machinery was the result of many years of research and represented the first step in olive mill automation with the consequent reduction of labour costs. The centrifugation method is a continuous or on-line process that is able to separate VOO from the other phases of the olive paste, either liquid (vegetation water) or solid (pomace), by means of centrifugal force. Stokes's modified law governs the speed at which a solid and a liquid are separated under centrifugal force:

$$V = \frac{D^2}{18}\frac{\left(d_2 - d_1\right)\omega^2 r}{\eta}$$

where V is the speed of separation, D is the diameter of the solid particles, d_1 is the specific weight of the liquid, d_2 is the specific weight of the solid, ω is the angular speed, r is the distance to the rotating arms, and η is the viscosity of the liquid.

Amirante and Catalano (1993) have formulated the relationship between the physical properties of olive paste and the decanter characteristics:

$$t_s = \frac{18\mu \ln\left(\frac{2(R_2)^2}{(R_1)^2 + (R_2)^2}\right)}{2(D_p)^2(\rho_s - \rho_l)K_c\omega^2}$$

where μ is the viscosity of the liquid phase, ρ_l is the specific weight of the liquid phase, ρ_s is the specific weight of the solid phase, D_p is the mean diameter of the solid particles, K_c is the correction factor depending on the concentration of the solid particles in the liquid, R_1 and R_2 are respectively the minimum and maximum radii of the ring of liquid being centrifuged, ω is the angular velocity of the centrifuge, and t_s is the particle sedimentation time.

This equation shows that the separation of the solid particles is more complete when the retention time is greater. The retention time is the ratio between the volume of the decanter and the volume of the olive paste plus the water added per minute. Figure 3.15 shows a basic diagram of VOO extraction by centrifugation systems.

The diagram in Fig. 3.15 is relative to a three-phase centrifugation system, a system which was widespread during the 1970s and 1980s and is still used at the present. However, it has been partially replaced by two-phase centrifugal decanters, especially in some countries like Spain. The difference between the two types of decanter is that it is possible to avoid, or reduce, the addition of water to the olive paste when the two-phase decanter is used to separate oil from other phases, liquid and solid, of the malaxed pastes. That interesting innovation led to some changes in olive oil quality as well as the work capacity of the decanter, as will be described below.

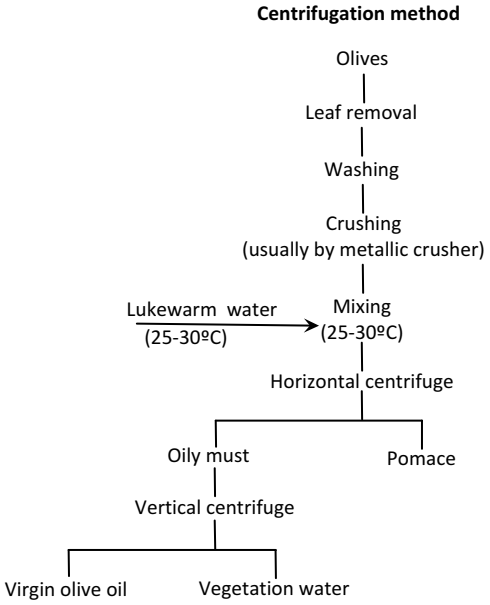

Fig. 3.15 Diagram of olive oil extraction by a centrifugation method with three-phase decanter

Leaf removal and olive washing are important and necessary operations when a centrifugation system is employed. When they are performed, it is possible to avoid damage to the mechanical parts rotating at high speeds. In addition, the sensory effect of leaves on VOO is also avoided.

3.7.1 Olive Crushing

When a centrifugal decanter is employed, olive crushing can be carried out by a range of different metallic crushers, including hammers, toothed discs, and cylindrical or roller crushers. Basically, these machines consist of a metallic body, of various shapes, which rotates at high speed and throws the olives against a fixed or mobile metal grating. The result is olive paste. The kind of metallic crusher used and the violence of the crushing action affect the size of the stone fragments, the extraction yields and the organoleptic properties of VOO.

A centrifugation system, with a hammer metal crusher and a grating with small holes (violent crushing), produces high extraction yields (Muñoz Aranda and Alba 1980), and the VOO contains high amounts of phenolic substances. Comparative studies between granite millstones and metal crushers pointed out that the latter produces VOOs with a higher content of total phenols (Angerosa and Di Giacinto 1995; Alloggio et al. 1996) that are more stable and with a more bitter taste (Angerosa and Di Giacinto 1995). Table 3.2 shows the effects of the different crushing methods on the characteristics of VOO.

Table 3.2 Effect of different crushing methods on some qualitative characteristics of virgin olive oils obtained by three-phase centrifugal decanter

	Olive cv Provenzale[a]		Olive cv Ogliarola[b]	
Determination	Millstone	Metallic crusher at discs	Millstone	Metallic crusher at hammer
Free fatty acids (%)	0.23	0.23	0.41	0.37
Peroxide value (meq/kg)	11.5	11.8	6.2	5.1
K_{232}	1.87	1.90	1.18	1.19
Total phenols (mg/kg)	133	246	186	435
Induction time (h)	7.8	10.6	9.4	12.7

[a]Angerosa and Di Giacinto (1995)
[b]Alloggio et al. (1996)

Metallic crushers, with fixed hammers or discs, are the most common machines used by farmers, and they have the main advantages that they are not very bulky or expensive, work continuously, and have a high hourly capacity. They have drawbacks, however: they can produce emulsions because of their rapid and violent operation, even though the olive paste temperature does not increase by more than 6–10 °C over the ambient temperature; they can increase the organoleptic characteristics of VOO, e.g. bitter and pungent taste; and the metallic parts rotating at high speed can suffer wear and tear.

For these reasons, some olive oil mills equipped with centrifugal decanters have replaced metallic crushers with millstones, both for historic image and for the organoleptic quality of oils. In these cases, oil extraction yields should be less than those obtained with metallic hammer crushers.

3.7.2 Olive Paste Mixing

As was previously mentioned, the mixing operation (also called malaxation) consists of a slow stirring of the olive paste with the aim of promoting contact between oil droplets to obtain larger drops, which then separate in a continuous oily phase. That operation is particularly important when the olives are crushed using violent metallic crushers, which can cause emulsions with negative effects on extraction yields. The mixing operation reduces or removes the aforementioned emulsions of olive paste by causing the droplets to coalesce, thereby increasing the free oil quantity and, therefore, oil extraction yields. Moreover, the mixing operation helps to increase the extractable oil by promoting the breaking up of unbroken cells containing oil for mechanical and enzymatic action.

Continuous centrifugal plants have two or three mixing units consisting of semi-cylindrical, large-capacity vats fitted with a horizontal shaft and an outer chamber for heated water circulation (Fig. 3.16).

The mixing of olive paste can be carried out for different times and at different temperatures, but in general, the mixing time varies between 30 and 60 min, and the

Fig. 3.16 Machine for olive paste mixing (capacity 500–600 kg)

Table 3.3 Effect of time and temperature of olive paste mixing on some characteristics of virgin olive oil obtained by two-phase centrifugal decanter

	Olive cv Leccino			Olive cv Cornicabra		
	Mixing time (minutes)[a]			Mixing temperature (°C)[b]		
Determination	30	60	90	20	28	40
Free fatty acids (%)	0.31	0.28	0.30	0.21	0.21	0.25
Peroxide value (meq/kg)	4.5	4.1	4.0	5.9	6.2	6.8
K_{232}	1.49	1.50	1.51	1.60	1.67	1.70
Total phenols (mg/kg)	285	306	265	450	649	788
Induction time (h)	14.6	14.6	13.6	14.7	21.8	24.7

[a]Di Giovacchino et al. (2002a)
[b]Inarejos-Garcia et al. (2009)

temperature varies between 25 °C and 32 °C. The effects of time and temperature in olive paste mixing are shown in Table 3.3 (Di Giovacchino et al. 2002a; Inarejos-Garcia et al. 2009).

The data show that the adopted time and temperature used to mix olive paste do not influence the commercial characteristics of VOO. In particular, only a mixing temperature of 40 °C determines a small, insignificant increase in the peroxide value and K_{232} of oil, whereas the same temperature value causes a significant increase in total phenol content and induction time of oil, as confirmed by Stefanoudaki et al. (2011). In contrast, an increase in the mixing time causes a small, insignificant reduction in the total phenol content and the correlated values of induction time, but only when the operation lasts 90 min.

During olive paste mixing, the oil is protected from oxidation by two important phenomena: (1) the large quantity of phenolic substances present in the paste and their reaction with oxygen, whose quantity is reduced; (2) the consumption of the

Fig. 3.17 Modern mixers placed in parallel, closed and equipped with a window

oxygen, due to cellular respiration, and the consequent formation of CO_2. As demonstrated in some papers (Parenti et al. 2005, 2006), the mixing operation of olive paste carried out in a closed mixer causes a strong reduction in the oxygen contained in the olive paste and in the atmosphere, because of cellular respiration, and the formation of the corresponding quantity of carbon dioxide, the percentage of which can reach 25 % of the gas present in the headspace.

In the past, mixers (two or three) were placed in series, either horizontally or vertically, whereas currently mixers are independent because they are placed in parallel. Moreover, modern mixers (Fig. 3.17) are tightly closed as a result of laws on industrial safety and the HACCP (Hazard Analysis Critical Control Point) regulations for olive oil mills.

Even though the mixing operation does not promote oil oxidation, some constructors have suggested, and built, mixer machines that make it possible to modify the gaseous atmosphere by replacing air with an inert gas like nitrogen. The purpose is to avoid, or reduce, any decrease in the content of phenolic substances in the oil due to the mixing operation when it is carried out for a long time. The results obtained in some studies, carried out using an industrial scale machine (Servili et al. 2008) or small laboratory machines (Romano et al. 2010; Ridolfi et al. 2010), indicated that the use of nitrogen to modify and control the atmosphere during the mixing operation helped to obtain oils with a higher content of total phenols and *o*-diphenols, compared to the content of the same substances in oils obtained under normal conditions.

3.7.3 Olive Paste Centrifugation (Three- and Two-Phase Decanters)

The separation of oil from other liquid and solid phases of olive paste is produced by a horizontal centrifuge (Fig. 3.18). The centrifuge, rotating at high speed (in general at 3,500–3,600 rpm), accentuates the difference between the specific weights of the non-miscible liquids (oil and water) and the solid matter (pomace). In this way, olive oil and vegetation water are separated by nozzles from olive pomace, which is pushed to one

Fig. 3.18 Centrifugal decanter in medium-size to large Italian oil mill (Reproduced with permission of OL.MA.- Braccagni, Italy)

end of the centrifuge. Centrifugation, therefore, permits extraction of olive oil in a continuous operation, whereas the pressing and percolation methods are discontinuous.

The decanters have a theoretical hourly load capacity of olive paste depending largely on their size. Decanters, in relation to the length, diameter and angular speed of the rotating body, yield the best quantitative results when the feed flow of the olive paste is 70–90 % of the theoretical load capacity, depending on the olive characteristics. If the flow of the olive paste is too high, oil yields drop because of the increasing oil content in the pomace and vegetation water.

When a three-phase centrifugal decanter is used, the mixed olive paste is pumped through to the decanter by the addition of lukewarm water to raise the fluidity of the mixture and to promote the separation of the liquid and solid phases by centrifugation. The quantity of water added to the olive paste affects the extraction yield and the phenol content. Generally, oil extraction yields decrease when the amount of water added to dilute the paste is very low or very high; the best results correspond to olive paste/water (w/w) ratios of between 1:0.4 and 1:0.7.

Phenols are important constituents of VOO (Tsimidou et al. 1992), and their content is also affected by the quantity of water added to the olive paste because phenolic substances are more soluble in water than in oil. An increase in the amount of diluting water diminishes the phenol content and, hence, the stability of VOO during storage. Table 3.4 shows the results obtained in olive processing using pressure and three-phase centrifugation systems (Di Giovacchino et al. 1994a).

Moreover, the different extraction systems affect the composition of the volatile substances present in the headspace of oil, as shown in Table 3.5.

Table 3.4 Characteristics of oil, pomace and vegetable water obtained in olive processing by pressing and 3-phase centrifugation systems

	Olive processing system	
Determinations	Pressing	3-phase centrifugation
Characteristics of oil		
Free fatty acids (%)	0.35	0.29
Peroxide value (meq/kg)	4.8	5.7
K_{232}	1.91	1.96
Total phenols (mg/L)	155	114
Induction time (h)	12.0	9.5
Characteristics of pomace		
Oil content (% of fresh matter)	7.7	4.0
Moisture (%)	27.9	51.2
Characteristics of vegetation water		
Oil content (g/L)	6.7	12.5
Dry matter (%)	16.4	9.0

Data from Di Giovacchino et al. (1994a)

Table 3.5 Average content of some volatile substances of oils obtained in olive processing by pressing and 3-phase centrifugation systems (good-quality olives)

	Olive processing system	
Determinations	Pressing	3-phase centrifugation
n-Octane	24.3	7.1
Ethyl acetate	13.5	6.8
Hexanal	41.3	36.1
Isobutyl alcohol	20.1	4.0
Isoamyl alcohol	78.6	10.9
(E)-2-Hexenal	425.5	435.4
Hexanol	107.3	35.6
(E)-2-Hexenol	97.5	39.9
Acetic acid	6.0	1.7

Data from Di Giovacchino and Serraiocco (1995)

The data, as reported in the original paper (Di Giovacchino and Serraiocco 1995), indicate that the oils extracted by pressure and centrifugation systems have the same content of hexanal and (E)-2-hexenal, because it depends on the olive cultivar and its ripening degree, whereas oil obtained by a pressure system has a higher content of *n*-octane, isobutyl and isoamyl alcohols, with an undesirable aroma (Aparicio et al. 1996). These substances are especially present in oils obtained by a pressure system because of the prolonged use of filtering diaphragms, or mats; the vegetable organic matter remains on the mats for many days and can therefore ferment and decompose. Use of a centrifugation system instead makes it easy to remove the olive paste from the machinery by short and quick washing with lukewarm water.

One of the classic drawbacks of the continuous three-phase centrifugation method is the production of a considerable volume of vegetation water that varies between 60

and 100 L per 100 kg olives. To reduce the amount of wastewater, it has been suggested that the vegetation water could be recycled, partially replacing the total need for lukewarm mains water (Amirante et al. 1993). This process has the following advantages: (1) increased oil extraction yields; (2) increase (20–25 %) in phenolic content of oil; and (3) reduction (30–40 %) in the volume of vegetation water.

The continuous centrifugal method (either three or two phases) is the most common in all of the olive-producing countries. This method is now used in 96–98 % of Spanish olive oil mills, 80 % or more of Greek olive oil mills, 60–70 % of Italian olive oil mills, and a similar percentage in other Mediterranean countries, such as Tunisia, Turkey, Syria and Morocco. The success of this method is due to the facts that the machinery does not require much space, all operations are automated, operating costs have been drastically reduced because of automation, and pomace contains low amounts of olive oil (3–5 %). The continuous centrifugal method, however, has drawbacks: the machinery requires large outlays because it is built with stainless steel, the consumption of thermal energy is a limiting factor, the pomace is wetter than that produced by the pressing method, and the aforementioned high volume of vegetation water has a medium-high content of oil, which varies between 5 and 15 g/L (when three-phase decanters are used).

In the late 1980s, to avoid some of the drawbacks of the three-phase decanter, the industries in the sector launched a new decanter, the so-called two-phase decanter, which allowed the olive paste to be centrifuged without adding water in order to separate the oil and the pomace only, avoiding the production of vegetation water. The results obtained in olive processing by the two types of decanter indicated that the oil yields were similar, whereas the pomace was wetter (moisture of approximately 65–72 %) when the two-phase decanter was used (Hermoso Fernandez et al. 1995; Amirante et al. 1993; Di Giovacchino 1994; Di Giovacchino et al. 1994c; Koutsaftakis and Stefanoudaki 1996; Stefanoudaki et al. 2011). With reference to the quality of oil, Table 3.6 shows the results obtained in olive processing by two- and three-phase decanters (Di Giovacchino et al. 2002b).

The data indicate that the two types of decanter do not influence the values of parameters correlated with the qualitative characteristics of oils, such as the percentage of free fatty acids, peroxide value, values of K_{232} and K_{270} and the score of the organoleptic assessment because they largely depend on the qualitative characteristics of olive fruits and their ripeness degree. The total phenol content and the value of the oil induction time can differ simply because of the addition of water to the olive paste when a three-phase centrifugal decanter is used. The dilution of olive paste by lukewarm water allows a reduction in the concentration of phenols of the liquid and solid phases and, therefore, of the oil as well.

Thanks to its characteristics and to the large water savings, this technology promises to expand throughout the Mediterranean basin, a geographical area with limited water supply. At present, the two-phase decanter is now used in 95 % of Spanish olive oil mills because it dramatically reduces the production of vegetation water. This fact is particularly important because in Spain, the disposal of this liquid by-product of oil mills is a big environmental problem. Moreover, the use of the two-phase centrifugal decanter, especially in large oil mills, allows for the adoption

Table 3.6 Average values of some parameters determined in oils obtained by two- or three-phase centrifugal decanter

	Centrifugal decanter	
Determinations	Two-phases	Three-phases
Free fatty acids (%)	0.34	0.32
Peroxide value (meq/kg)	4.3	4.7
K_{232}	1.56	1.50
K_{270}	0.11	0.10
Organoleptic assessment (score)[a]	7.2	7.2
Total phenols (mg/L)	292	197
Induction time (h)	14.2	11.0
Chlorophyll pigments (mg/kg)	6.9	7.5
Hexanal (mg/kg)	32.5	32.8
(E)-2-Hexenal (mg/kg)	275.0	253.0
Hexanol (mg/kg)	19.0	17.1
(E)-2-Hexenol (mg/kg)	26.5	29.1

Data from Di Giovacchino et al. (2002b)
[a]Score between 1 and 9

Table 3.7 Advantages and disadvantages of the two types of decanters

Type of decanter	Advantage	Disadvantage
Two-phases	Oil has higher phenol content	Pomace is very wet
	Oil is more stable	Lower working capacity
	Lower water consumption	Oil can be more bitter
	Lower energy consumption	Oil can be more pungent
	No production of vegetation water	
Three-phases	Higher working capacity	Oil has lower phenol content
	Pomace has normal moisture content	Oil is less stable
		Production of vegetable water
		More consumption of water
		More consumption of energy

of a particular programme for olive processing that is based on the recovery of a small quantity of oil and a large quantity of stones from the wet pomace, as described in the following sections.

At the end of this section, the pros and cons of the use of the two types of decanter are given (Table 3.7).

3.8 New Trends in Olive Processing and Olive Oil Quality

The quality of VOO is defined by certain analytical parameters, as established by European law and the documents of the IOC. These institutions establish the limit values of the qualitative parameters for classifying VOOs in various categories.

The various mechanical methods used to extract oil from olives affect VOO quality and the content of some chemical compounds like phenolic substances, pigments

Table 3.8 Average results of qualitative characteristics of oils obtained in olive processing by different mechanical systems

	Olive processing system		
Determination	Pressing	Percolation	3-phase centrifugation
Free fatty acids (%)	0.23	0.23	0.22
Peroxide value (meq/kg)	4.0	4.6	4.9
K_{232}	1.93	2.03	2.01
K_{270}	0.12	0.12	0.13
Organoleptic assessment (score)	6.9	7.0	7.0
Total phenols (mg/L)	158	157	121
Induction time (h)	11.7	11.2	8.9
Chlorophyll pigments (mg/kg)	5.0	8.9	9.1

Data from Di Giovacchino et al. (1994a)

and volatile substances. Table 3.8, based on the results reported by Di Giovacchino et al. (1994a), shows the data obtained in olive processing by various systems in industrial oil mills.

The results indicate that all systems yielded extra VOOs when good-quality olives were processed. However, the centrifugation system produced oils with a lower content of total phenols and a lower value for induction time because of the addition of lukewarm water to the olive paste. Moreover, olive processing systems can affect the content of some volatile substances that characterise the aroma of VOOs, as shown in Table 3.5. At present, the general and most important aim of olive growers and olive oil producers is to obtain a good, or very good, VOO (extra virgin) by adopting the best conditions during all phases of the process, from cultivation to extraction, including storage and bottling. When the olives are sound and of appropriate ripeness, the quality of the oil must be very good, of course, if the adopted system for processing olives helps to avoid any deterioration of the oil due to pollution from dirty machinery on which some vegetable residues can remain for a long time. It is possible to avoid that drawback when the oil mill is equipped with a centrifugal decanter and other machinery built with stainless steel, which is very easy to clean using water to remove residues. Regarding this problem, in a pressing system, in particular, it may be difficult to clean the mats on which some vegetable and oily residues inevitably remain and which are broken down further because of the activity of micro-organisms. The same problem may be encountered in a percolation system if the machinery is rarely cleaned.

Today, the chances of obtaining an oil of good quality have been improved by the introduction of the two-phase centrifugal decanter, which helps to avoid the use of water added to the olive paste. This produces an oil that has a higher content of phenolic substances and, thus, with higher nutritional properties, is more bitter and pungent and more stable during storage, as shown in Table 3.6.

In regions where there is high production of olives, another important aim is to extract VOO at the lowest possible cost. This goal is reached through the use of large centrifugal decanters with a theoretical capacity of 10–15 t of olives/h that in general operate in two phases in large oil mills having a theoretical capacity to

Table 3.9 Qualitative characteristics of oils obtained by double centrifugation of olive paste of good and poor quality

	Olives cv Coratina (good quality)		Olives cv Cima (poor quality)	
Determinations	First extract	Second extract	First extract	Second extract
Free fatty acids (%)	0.56	0.80	2.1	2.6
Peroxide value (meq/kg)	7.6	10.4	10.9	11.1
Total phenols (mg/L)	308	416	156	174
Erythrodiol+Uvaol (%)	3.9	9.1	2.3	7.0
Waxes (mg/kg)	45	87	154	726
Aliphatic alcohols (mg/kg)	103	342	253	1,407
Total triterpene alcohols (mg/kg)	1,128	1,321	1,709	1,669

Data from Di Giovacchino et al. (2002c)

process 1,000–1,500 t of olives/24 h. In Spain, many oil mills have the aforementioned capacity, whereas a small number of oil mills in Italy or other countries of the Mediterranean basin have a theoretical capacity of 500–800 t of olives/24 h.

When a two-phase centrifugal decanter is used, the wet pomace obtained (partially de-oiled olive paste) contains all the oil not extracted. Thus, it seems useful to recover part of that oil by a second centrifugation of the pomace in another decanter that can operate in two or three phases (Di Giovacchino and Costantini 1991; Alba Mendoza et al. 1996). From the second extraction, the quantity of the recovered oil is very small, varying between 0.3 and 0.6 kg/100 kg of olives, and the oil may have different qualitative characteristics, as shown in Table 3.9, (Di Giovacchino et al. 2002c).

The data show that the oils of the second extraction were very green because of the high content of the chlorophyll pigments and had an irregular composition because of the percentage of erythrodiol and uvaol and the content of waxes. European law establishes that a VOO must have a percentage of erythrodiol + uvaol no higher than 4.5 % and a waxes content no higher than 250 mg/kg. If the preceding values are higher than the established limits, the oil must be classified as pomace oil.

Double extraction of olive oil is a common practice in Spain (Alba Mendoza et al. 1996), although it is also done in other producing countries, e.g. in a small number of the large oil mills of Italy.

Moreover, the wet pomace obtained by two-phase centrifugal decanters is not used in the industry for solvent extraction of oil because of the high percentage of moisture (65–72 %), which gives rise to high costs because of its drying by heating. For that reason, many oil mills, operating with two-phase centrifugation, in order to generate income from the solid by-product, have introduced stages in olive processing that use a specific machine able to separate the fragments of stone from the fibre of the pomace. The stones of the pomace are useful as fuel because of their high heating power and other interesting characteristics, as will be described subsequently in Sect. 3.11.

3.9 Use of Co-adjuvants for Olive Oil Extraction

The rheological characteristics of olive pastes influence oil extraction yields with every mechanical method employed during processing. Oil yields generally vary between 80 % and 85 % of oil contained in olives as determined by the Soxhlet extractor. If the olive paste is difficult to work (a so-called hard paste), the oil yield can diminish to 70–75 %. The reason for this is the high quantity of oil trapped in the colloidal tissues of cytoplasm or emulsified with vegetation water, the so-called non-free olive oil.

It is possible to improve oil yields using certain co-adjuvants that are able to increase the quantity of free oil of olive paste. In the past, the first co-adjuvants utilised were lyophilised enzymes with pectolytic and cellulosolytic activity (De Soroa y Pineda 1952; Martinez Moreno et al. 1957b; Montedoro and Petruccioli 1973; Montedoro and Petruccioli 1978). After the 1980s, liquid enzymatic products having more specific pectolytic activity were employed (Alba Mendoza et al. 1987; Siniscalco and Montedoro 1988), and their influence on oil yield is shown in Table 3.10.

Data derived from Hermoso Fernández et al. (1991) indicate that the use of enzymes helps to improve oil yield, which rises, on average, from 20.0 kg/100 kg olives to 21.1 kg/100 kg olives and, in consequence, increases the overall extracted oil from 81.2 % to 85.8 % of the maximum oil contained in olive fruits and determined by the Soxhlet extractor.

With reference to the quality of olive oil extracted from olive paste containing added enzymes, the results reported in several papers (Alba Mendoza et al. 1987; Di Giovacchino 1991, 1993) indicated that the use of co-adjuvants did not influence the basic quality characteristics of oils, such as the percentage of free fatty acids, the peroxide value and other parameters correlated with the commercial quality of VOO. However, in some cases a small increase (not statistically significant) in the total phenolic content was found (Di Giovacchino 1993). Of course, the quantitative contents of fatty acids, sterols, aliphatic and triterpenic alcohols, triterpenic dialcohols and others were not obviously influenced by the use of enzymatic products.

In general, the use of enzymes, when olive pastes are difficult to process, gives good results either in terms of increasing the oil yield or improving the efficiency of the centrifugal decanter. However, some international institutions have worried about the image of VOO, which has always been considered a natural product obtained only by mechanical and physical means. The use of a biochemical product could change that image, and for that reason the European Community wanted to

Table 3.10 Average results obtained in olive processing by three-phase centrifugation of olive pastes with enzymes added

	Olive paste – control		Olive paste – with enzymes	
Oil content of olives (kg/100 kg olives)	Industrial yield (kg/100 kg olives)	Theoretical yield (%)	Industrial yield (kg/100 kg olives)	Theoretical yield (%)
24.6	20.0	81.2	21.1	85.8

Data from Hermoso Fernández et al. (1991)

Table 3.11 Average results obtained in olive processing by three-phase centrifugation of olive paste added with different percentages of mineral talc added

Added talc (%)	Oil yield (%)	Oil lost in by-products (kg/100 kg olives)
–	78.3	4.1
2	80.5	3.7
4	84.4	3.0
6	83.1	3.2

Data from Di Giovacchino (1988)

revise the definition of VOO, and with Regulation 1513 of 2001, it was established that the use of any substance with chemical or biochemical activity was forbidden during olive processing in oil mills.

Another co-adjuvant used to increase oil yields is the micronised mineral talc, mainly used, and still used, in Spain (Martinez Suarez et al. 1975; Muñoz Aranda et al. 1979). The results of experiments carried out using talc to process difficult olives (or hard pastes) by centrifugation methods indicate that higher yields are obtained when the water content of olive paste is above 50 % and the talc added at 2–4 %, as shown in Table 3.11, which reports data from Di Giovacchino (1988).

Talc has lipophilic properties and its positive action is due to the reduction in the emulsion oil/water and to the consequent increase in free oil. This positive effect is more evident in the significant reduction in olive oil lost with the vegetation water. Moreover, the talc, because of its high specific weight (2.8 g/cm^3), flows into the pomace and compacts it, thereby helping to better separate the solid phase from the liquid phases inside the decanter. This effect improves the efficiency of the decanter and increases the feed flow of olive paste. This is the most important reason for the common use of talc in large-capacity Spanish oil mills.

Of course, the use of talc does not affect the sensory or other qualitative characteristics or the chemical composition of the saponifiable and unsaponifiable fractions of VOO (Di Giovacchino 1988).

3.10 Storage of Virgin Olive Oil

VOO, obtained by various extraction systems, is cloudy because it contains a small quantity of vegetable water and, in some cases, small vegetable fragments. The oil needs to be cleaned by centrifugation in a vertical centrifuge rotating at high speed (6,500–7,000 rpm). In this way, the oil is separated from most impurities but remains cloudy because of the unavoidable emulsion between the oil and a very small quantity of water, dispersed as microdroplets. In that condition, VOO should be properly stored in large containers while it awaits bottling and marketing.

The best and most common system to store VOOs is to utilise large metallic tanks made of stainless steel and kept under cover inside buildings. In some cases, large underground vats, with walls coated with inert material, i.e. stainless steel, are also still used to store VOO.

The storage premises must be free of all aromas pleasant or unpleasant. Moreover, during storage the oil should not come into contact with unsuitable materials, which may cause spoilage, such as plastic or reactive metallic surfaces.

During storage, the quality of VOO may deteriorate because it can undergo both biological and chemical processes due to the following reactions:

– Hydrolysis of triglycerides caused by enzymes;
– Chemical oxidation of fatty acids, promoted by the presence of oxygen and free radicals.

Alteration of oil due to hydrolysis is possible when enzymes and water are present, as commonly happens with cloudy oils that are freshly obtained in oil mills. Under these conditions, the aqueous phase, consisting of a small quantity (approximately 0.5 %) of vegetation water, contains enzymes and, in particular, lipase, which is able to hydrolyse triglycerides to release free fatty acids and, as a consequence, increases the acidity of the oil. That reaction is promoted by storage temperatures higher than 18–20 °C.

Moreover, the small droplets of emulsified water that is present in the oil slowly settle on the bottom of the container, forming a layer of sediment containing sugars, proteins and enzymes. It can ferment under certain temperature conditions and then produces some substances (e.g. short-chain fatty acids) that give a typical defect. If VOO is left in contact with the aqueous layer for a long time, it may acquire the organoleptic defects of muddy sediment or putrid. In order to avoid this type of oil spoilage, VOO should be separated from the sediment as quickly as possible, either by pouring or by filtering it through hydrophilic materials. In general, the oil stored in a container is poured into another container after 20–30 days. If there is a need to bottle the oil, separation of the aqueous emulsion is carried out by a suitable filtration using a filter-press equipped with cellulose paperboard.

Another important process, particularly dangerous for VOO quality, is chemical oxidation, also called autoxidation. The oxidative deterioration of VOO can be delayed by employing suitable methods, but oxidation cannot be avoided. The autoxidation of the oil depends on the following factors: the composition of the fatty acids and the content of phenols, the material of the container, exposure to light, the storage temperature, and any contact with air (oxygen).

All oils and fats deteriorate by oxidative processes, but the process is relatively slow for VOO because of its fatty acid composition and phenolic content. Polyunsaturated fatty acids are oxidised faster than monounsaturated ones. In general, VOO contains a high percentage (60–80 %) of oleic acid (monounsaturated), a medium content (12–18 %) of palmitic and stearic acids (saturated) and a low percentage of polyunsaturated fatty acids, like linoleic acid (5–13 %) and linolenic acid (less than 1 %). This explains the higher stability of VOO in comparison with many other vegetable oils, such as seed oils, which can have a percentage of polyunsaturated fatty acids higher than 30–40 %.

Moreover, VOO is the only vegetable oil that contains significant natural antioxidants like phenolic substances (in particular the secoiridoid compounds).

With reference to the material of VOO containers, they must not transfer foreign substances to the oil, as may happen when the container is made of plastic or reactive metals.

Table 3.12 Variation in peroxide value and K_{232} of oils stored at 12–20 °C, in closed bottles, in the dark and under different conditioner gases

Determinations	Storage time (months)	Conditioner gas: air		Conditioner gas: nitrogen	
		Volume of oil (98 %)	Volume of oil (60 %)	Volume of oil (98 %)	Volume of oil (60 %)
Peroxide value	0	9.2	9.2	9.2	9.2
(meq/kg)	10	8.4	17.4	8.2	10.9
	24	18.7	36.2	15.0	15.0
	0	1.75	1.75	1.75	1.75
K_{232}	10	1.72	2.50	1.72	1.75
	24	1.74	2.55	1.70	1.73

Data from Di Giovacchino et al. (2002c)

Another risk factor for VOO stability is light (natural or artificial), which may stimulate some photosensitisers able to generate singlet oxygen ($^1O_2$), which can rapidly cause polyunsaturated fatty acid oxidation. VOO, in comparison to other vegetable oils, may suffer that type of oxidation because it contains significant quantities of pigments (like chlorophyll and pheophytin), photosensitisers that are able to activate singlet oxygen. To avoid that risk, VOO should be stored in opaque containers or in the dark.

Temperature is also a risk factor for VOO stability when stored in either large or small containers. It is advisable to store oil at temperatures varying only between 13 °C and 18 °C, avoiding lower or higher values. A temperature lower than 7–8 °C makes the oil solid and hinders other operations, like filtration or bottling. A temperature higher than 20–22 °C, on the other hand, is dangerous because it can increase the risk of oil auto-oxidation. The speed of the oxidation of fatty acids in the oil, in fact, depends on the storage temperature, which must be controlled between the aforementioned values (13–18 °C).

The most dangerous risk factor for oxidation of VOO is contact with air. During storage, in the presence of oxygen, the oil can be oxidised because of the activity of the lipoxidase enzymes or by a chain reaction due to free radicals. Enzymatic oxidation is possible only if water is present in the oil; thus, it is possible to avoid this risk by suitable filtration. More difficult is avoiding the risk of oxidation due to the strong reactivity of free radicals that form for many reasons, as explained in Chap. 13.

To avoid, or reduce, the speed of oil oxidation, it is recommended that oil be stored in a fully filled container (the volume of air must be no more than 3–5 % of the total volume), well closed, in the dark and at a temperature between 13 °C and 18 °C, as stated earlier. The risk of oxidation is greater when the oil occupies only a small part of the container, thus favouring continued solubilisation of oxygen in the oil and, therefore, reactions with fatty acid radicals and the formation of hydroperoxides. In these cases, there is a need to control the atmosphere in contact with oil by replacing air by an inert gas, like nitrogen, as indicated in Table 3.12.

Data derived from Di Giovacchino et al. (2002d) indicate that the peroxide value and the value of K_{232} of extra VOO stored in bottles not completely full (with the oil occupying only 60 % of the total volume) and conditioned with air increased, after

10 months, up to the limit values established for these oils and exceeded them after 24 months of storage, forming an oil known as lampante. By contrast, when the headspace was filled with nitrogen, the quality parameters remained within the limits established for the extra virgin category.

To conclude this section, it would be useful to summarise sound practices for storing VOO:

- VOO must be stored as soon as it is produced in the mill.
- The storage container must be made of inert material, like stainless steel or glass for small containers, to avoid transfer of polluting substances to the oil.
- Oil must be protected from light and stored at controlled temperatures (13–18 °C).
- Oil must be stored in a completely full container (with air accounting for no more than 3–4 % of the total volume).
- If the container is not completely filled with oil, the air should be replaced with an inert gas, like nitrogen.

3.11 By-Products of Olive Processing

When olives are mechanically processed into oil, pomace and vegetation water by-products are obtained.

Pomace, a solid by-product, is a source of income for oil mills since it is sold to industrial factories where the residual oil (olive-pomace oil) is extracted by solvent (hexane). The characteristics of olive pomace (Table 3.13) indicate that the pomace obtained by pressing is richer in oil content, while pomace obtained by centrifugation is wetter.

Olive pomace should be delivered directly to the pomace factory (on the same day it is produced) for drying in order to avoid any increases in acidity in the olive-pomace oil. Drying reduces the activity of enzymes. The dried pomace is then extracted by hexane to yield raw olive-pomace oil, which must be refined to become edible. The edible product sold as olive-pomace oil is refined olive-pomace oil blended with a small quantity of VOO (approximately 5 %).

After oil extraction, the solid by-product, de-oiled pomace, is used mainly as a low-grade fuel, with a heating power variable between 3,200 and 3,600 Kcal/kg. Another alternative is the careful separation of the stone fragments from the de-oiled pulp fraction. The de-oiled pulp could then be used in animal foodstuffs while the stone

Table 3.13 Characteristics of olive pomace obtained in olive processing by different systems

	Extraction system		
Determination	Pressing	Three-phase centrifugation	Two-phase centrifugation
Quantity (kg/100 kg olives)	25–35	45–55	80–85
Moisture (%)	22–35	45–55	65–75
Oil (%)	6–8	3.5–5.0	3.0–4.0
Stones (%)	30–45	20–28	12–18

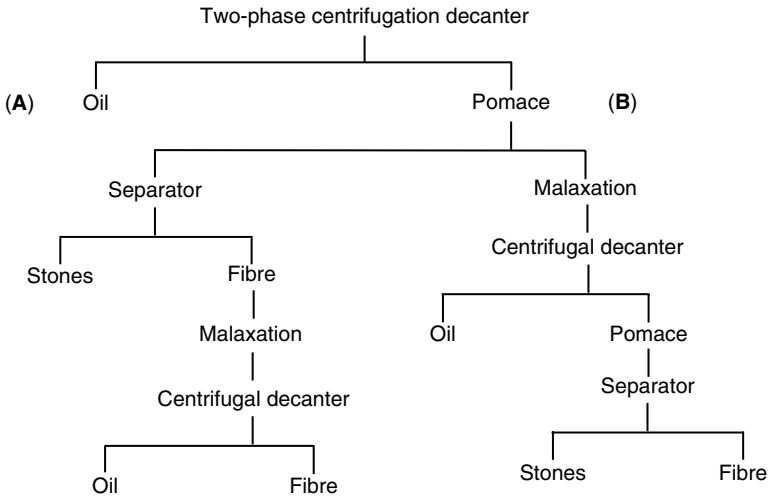

Fig. 3.19 Diagram of olive-pomace processing to separate stone fragments and to obtain second extraction oil

fragments could be used as a fuel since they have a higher heating power than the whole dried pomace, or they could be used in the manufacture of furfural or active carbon.

At present, Italian factories prefer pomace obtained from pressing methods to extract the residual oil by solvent and, therefore, pomace obtained in oil mills equipped with a centrifugal decanter (especially those of two phases) has a low value because of its high water content and low oil content. For that reason, many oil mills have adopted a suitable procedure to process olive pomace using a machine capable of separating the fragments of stones from the fibre, as shown in the scheme (Fig. 3.19).

The diagram shows that the recovery of stones can be carried out in two ways:

(A) The olive pomace obtained from a centrifugal decanter is sent to a suitable machine (separator) that can separate the stones from the fibre. The fibre is malaxed (at a temperature of 40–45 °C) and still centrifuged to extract a small quantity of oil (about 0.3–0.5 kg/100 kg olives), with the characteristics in Table 3.9.

(B) The olive pomace is malaxed (at a temperature of 40–45 °C) and sent to another centrifugal decanter to obtain a small quantity of oil (0.4–0.6 kg/100 kg olives), very green in colour and with the characteristics described in Table 3.9, and the pomace, which is then sent to the separator machine to obtain stone fragments and fibre.

The stone fragments are used as fuel because of their high calorie power (4,000–4,200 kcal/kg); moreover, their combustion produces a very low quantity of ash and smoke with a negligible quantity of gas containing sulphur and nitrogen. The wet fibre can be mixed with other vegetable residues to prepare a compost useful as a fertiliser in agriculture.

Table 3.14 Characteristics of olive mill wastewater obtained in olive processing by different systems

Determination	Extraction system		
	Pressing	Three-phase centrifugation	Two-phase centrifugation
Quantity (L/100 kg olives)	40–50	50–80	10–20[a]
pH	4.5–5.7	4.5–6.0	4.5–5.0
Dry matter (%)	8–20	4–15	3
Oil content (%)	0.2–0.8	0.6–2.0	0.1
Phenols	2–10	2–8	0.5–1.6
C.O.D. (g O_2/kg)	60–200	50–170	10
Nitrogen (%)	0.10–0.15	0.05–0.10	–
Phosphorous (%)	0.05–0.10	0.02–0.06	–
Potassium (%)	0.2–0.4	0.1–0.2	–

Data from Di Giovacchino and Preziuso (2006)
[a]Water used to wash olive oil in vertical centrifuge

The vegetation water is a liquid by-product obtained from mechanical olive processing in olive oil mills. The volume and characteristics of this by-product depend on the extraction method employed, as shown in Table 3.14.

The data in Table 3.14 (from Di Giovacchino and Preziuso 2006) show that vegetation water is mainly a wastewater due to its high content of organic matter, but it also contains mineral elements, e.g. potassium, that are potentially of interest for further utilisation. Because of the high pollutant properties of oil mill waste, many countries prohibit its discharge into water courses or urban sewage networks. In some olive-producing countries around the Mediterranean, the disposal of vegetation water is possible by industrial purification or by controlled spreading on agricultural land. In particular, Spanish oil mills have partially solved the problem by changing olive-processing technology through the adoption of a two-phase centrifugation system, thereby avoiding the production of vegetation water. In any case, in Spain, the water used to wash olives and to wash oil (in the vertical centrifuge) must be collected in a suitably protected evaporation pond. In Greece, vegetation water from an oil mill can be evaporated naturally in a protected lagoon or it can be treated to reduce its pollutant power and, therefore, may be spread on soil. A similar law regulates the disposal of vegetation water in Portugal. In Italy, in contrast, the law permits but regulates the controlled spread of vegetation water on agricultural land.

The different solutions to the problem are due to the fact that the purification of vegetation water by industrial plants is a difficult operation due to the water's high content of organic matter. Many industrial methods have been proposed to purify vegetation water, but only two have been used: (1) concentration by solvent evaporation and (2) concentration by membrane filtration (reverse osmosis and ultra-filtration).

The concentration of vegetation water by evaporation of water (an expensive process requiring energy and fuel) does not solve the problem because it produces a concentrate (similar to jam) that is not usable. Also, disposing of the concentrate is difficult. The concentration of vegetation water by membrane filtration is an expensive process that has some drawbacks, such as the frequent occlusion of membranes.

Table 3.15 Average results obtained in olive tree cultivation for soils treated with olive vegetation wastewater

Determinations	Control	Quantity of oil mill wastewater		
		5 L/m^2	10 L/m^2	30 L/m^2
Olive production (kg/tree)	4.3	4.6	5.4	5.2
Moisture of olive fruits (%)	51.8	52.0	52.0	52.6
Oil of olive fruits (%)	17.3	17.2	16.6	17.4
Oil characteristics				
Free fatty acids (%)	0.50	0.50	0.50	0.54
Peroxide value (meq/kg)	8.1	6.3	7.9	7.3
Organoleptic assessment (score)	7.1	7.0	7.1	7.1
Soil characteristics				
pH	7.15	7.20	7.32	7.27
Organic matter (%)	1.97	2.02	2.04	2.08
Organic nitrogen (%)	0.12	0.12	0.13	0.13
Reducing substances (mg/100 g)	0.24	0.38	0.39	0.48

Data from Di Giovacchino and Preziuso (2006)

A few Italian olive oil mills were equipped in the past with industrial plants for vegetation water purification. Today in Italy, almost all vegetation water is used in controlled spreading on agricultural land.

A more rational method to dispose of and utilise vegetation water is its controlled spreading on agricultural land, as is permitted by Italian law (50 or 80 m^3/ha, depending on the extraction system used, pressing or centrifugation). The aim of this practice is to give back to the land the residue of organic substances produced from the land itself, which can have positive effects on vegetation and crops, as reported in several papers (Lombardo et al. 1990; Bonari et al. 1993; Di Giovacchino et al. 2001; Di Giovacchino 2005). The best way to utilise vegetation water from oil mills is to spread it on soil cultivated with olive trees, as a natural supply of organic and mineral substances to the land from which they came. Table 3.15 shows results obtained for olive trees cultivated on soil treated with vegetation water.

Data from Di Giovacchino and Preziuso (2006) demonstrated that the olive crop and its characteristics, the quality of VOO and the characteristics of the soil did not change when olive vegetation water was spread in large quantities on the soil and regular fertilisers were not used or only partially used. This is due to the important quantity of organic matter, nitrogen, phosphorous and potassium supplied to the cultivated soil, in particular when large quantities of vegetation water were applied. The simple and complex organic and mineral substances are slowly utilised and transformed by soil micro-organisms as well as being absorbed by the roots. Results reported in other papers confirm that the spreading of oil mill vegetation water on cultivated soil helps to improve the chemical (Levi Minzi et al. 1992) and microbiological (Picci and Pera 1993) characteristics of the land. Finally, the recycling of oil mill wastewater to the soil fits in with the principle of sustainable organic farming and reduces the consumption of fuel and other energy sources, thereby avoiding the production of CO_2 as well.

3.12 Sound Control Practices

This chapter has pointed out that olive oil extraction is a complex process that requires considerable attention from the people involved in it. Each person should master the tasks to be carried out in addition to the appropriate time frame for doing so. A list of good control practices (Aparicio et al. 1994; IOC 2006) follows.

1. Olive Reception
    1.1. Olives must be cleaned of impurities using a leaf-removal and washing machine.
    1.2. Olives should be processed within 24–48 h after harvesting, but if that is impossible, then they should be stored under controlled conditions.
2. Olive Processing
    2.1. Crushing time should be as short as possible to prevent negative effects on aroma, oxidative processes and emulsion formation.
    2.2. Malaxation should be controlled with regard to two aspects: temperature and time.
    2.3. Malaxation time is generally 60 min; but this can be higher (90 min) or lower (30 min) depending on the rheological properties of the olive paste.
    2.4. The temperature of the olive paste should be maintained between 28°C and 30 °C to obtain good olive oil. Temperatures higher than 35–40 °C have a negative effect on oil quality.
    2.5. Mixers with two, three or more independent chambers are recommended.
    2.6. Extraction aids, like natural micronised talc, can be used to prevent an excess of colloids in the paste if the rheological characteristics of the olive paste requires it.
3. Separation of Phases by Centrifugation
    3.1. The total time of the process should be as short as possible.
    3.2. Water should be added at a temperature between 25 °C and 30 °C and must be potable and of high quality. However, newer systems do not add water for the centrifugation process.
    3.3. The olive oil temperature should not be above 30 °C.
4. Olive Oil Storage
    4.1. Olive oil should be stored in stainless steel tanks or in large underground vats.
    4.2. The temperature should be kept in the neighbourhood of 13–18 °C.
    4.3. The maximum capacity of every tank should be no more than 10 % of factory production, and the olive oils should be classified in different levels and hence stored in different tanks.

References

Alba Mendoza J, Ruiz Gomez A, Prieto Gonzalez C, Gutierrez RF (1987) Eficacia de la formulación enzimática "Rohament O" en la tecnología del aceite de oliva. Composición y valoración organoléptica de los aceites obtenidos. Grasas Aceites 38:271–277

Alba Mendoza J, Hidalgo Casado F, Ruiz Gomez A, Martinez Roman F, Moyano Perez J et al (1996) Caracteristicas de los aceites de oliva de primera y segunda centrifugacion. Grasas Aceites 47:163–181

Alloggio V, Caponio F, De Leonardis T (1996) Influenza delle tecniche di preparazione della pasta di olive sulla qualità dell'olio. Nota I. Profilo quali-quantitativo delle sostanze fenoliche, mediante HPLC, in olio d'oliva vergine della cv Ogliarola Salentina. Riv Ital Sost Grasse 73:355–360

Amirante P, Catalano P (1993) Analisi teorica e sperimentale dell'estrazione dell'olio d'oliva per centrifugazione. Riv Ital Sost Grasse 70:329–335

Amirante P, Di Renzo GC, Di Giovacchino L, Bianchi B, Catalano P (1993) Evoluzione tecnologica degli impianti di estrazione dell'olio di oliva. Olivae 48:43–53

Angerosa F, Di Giacinto L (1995) Caratteristiche di qualità dell'olio di oliva vergine in relazione ai metodi di frangitura. Nota II. Riv Ital Sost Grasse 72:1–4

Aparicio R, Calvente JJ, Alonso MV, Morales MT (1994) Good control practices underlined by an on-line fuzzy control database. Grasas Aceites 45:75–81

Aparicio R, Morales MT, Alonso MV (1996) Relationship between volatile compounds and sensory attributes of olive oils by the sensory wheel. J Am Oil Chem Soc 71:1253–1264

APROL (2002) Oleum Divinae Gratiae. Mario Congedo Editore, Galatina (I)

APROL Lecce, Associazione Produttori di Olive della Provincia di Lecce (I), Italy

Blazquez JM (1997) Origine e diffusione della coltivazione. In: Enciclopedia Mondiale dell'olivo, 1st edn. IOC, Madrid, pp 19–21

Bonari E, Macchia M, Angelini LG, Ceccarini L (1993) The waste waters from olive oil extraction: their influence on the germinative characteristics of some cultivated and weed species. Agricoltura Mediterranea 123:273–280

Boskou D (1996) Olive oil. Chemistry and technology. AOCS Press, Champaign

Camps-Fabrer H (1997) La cultura dell'olivo nell'Africa del nord. In: Enciclopedia mondiale dell'olivo, 1st edn. IOOC, Madrid, pp 30–33

Castellano LM, Garcia JM, Morilla A, Perdiguero S, Gutierrez F (1993) Quality of picual olive fruits stored under controlled atmospheres. J Agric Food Chem 41:537–539

De Felice M, Gomes T, Catalano M (1979) Estrazione dell'olio dalle olive con sistemi continui di centrifugazione delle paste. Risultati di ricerche triennali. Riv Ital Sost Grasse 56:361–369

De Soroa y Pineda JM (1952) Technique d'extraction de l'huile d'olive sans pression et sans solvent. Oléagineux 12:621–623

Di Giovacchino L (1986) Variazione delle costanti cinetiche di estrazione dell'olio per percolamento in dipendenza della temperatura e del rapporto olio/acqua delle paste di olive. Riv Ital Sost Grasse 63:119–123

Di Giovacchino L (1988) L'impiego del talco micronizzato nell'estrazione dell'olio dalle olive con il sistema continuo. Riv Ital Sost Grasse 65:623–638

Di Giovacchino L (1991) Situation en Italie de la voie enzymatique pour la production d'huile d'olive: résultats des essais et perspectives. Rev Fr Corps Gras 3–4:85–94

Di Giovacchino L (1993) L'impiego dei preparati enzimatici nella estrazione dell'olio dalle olive con i sistemi continui di centrifugazione. Nota I. Risultati di esperienze pluriennali. Riv Ital Sost Grasse 70:279–287

Di Giovacchino L (1994) Risulati conseguiti nella estrazione dell'olio dalle olive con un nuovo decanter a 2 fasi. Olivae 50:42–44

Di Giovacchino L (1996) Influenza dei sistemi di estrazione sulla qualità dell'olio di oliva. Olivae 63:52–63

Di Giovacchino L (2005) Characteristics of olive oil mill wastewater and prospects for its rational re-use. Olivae 104:55–63

Di Giovacchino L, Costantini N (1991) L'estrazione dell'olio dalle olive mediante doppia lavorazione. Nota II. Risultati ottenuti con la doppia centrifugazione. Riv Ital Sost Grasse 68:519–527
Di Giovacchino L, Mascolo A (1988) Incidenza delle tecniche operative nell'estrazione dell'olio con il sistema continuo. Nota II. Riv Ital Sost Grasse 65:283–289
Di Giovacchino L, Preziuso S (2006) Utilization of olive mill by-products. In: Proceedings of Olivebioteq 2006, pp 379–389. Marsala-Mazara del Vallo (I), November 5–10, 2006
Di Giovacchino L, Serraiocco A (1995) Influenza dei sistemi di lavorazione delle olive sulla composizione dello spazio di testa degli li. Riv Ital Sost Grasse 72:443–450
Di Giovacchino L, Solinas M, Miccoli M (1994a) Aspetti qualitativi e quantitativi delle produzioni olearie ottenute dalla lavorazione delle olive con i differenti sistemi di estrazione. Riv Ital Sost Grasse 71:587–594
Di Giovacchino L, Solinas M, Miccoli M (1994b) Effect of extraction systems on the quality of virgin olive oil. J Am Oil Chem Soc 71:1189–1194
Di Giovacchino L, Costantini N, Di Febo M (1994c) La centrifugazione delle paste di olive senza acqua di dilizione. Riv Ital Sost Grasse 71:555–559
Di Giovacchino L, Basti C, Costantini N, Ferrante ML, Surricchio G (2001) Effects of olive vegetable water spreading on soil cultivated with maize and grapevine. Agricoltura Mediterranea 131:33–41
Di Giovacchino L, Costantini N, Ferrante ML, Serraiocco A (2002a) Influence of malaxation time of olive paste on oil extraction yields and chemical and organoleptic characteristics of virgin olive oil obtained by a centrifugal decanter at water saving. Grasas Aceites 53:179–186
Di Giovacchino L, Sestili S, Di Vincenzo D (2002b) Influence of olive processing on virgin olive oil quality. Eur J Lipid Sci Technol 104:587–601
Di Giovacchino L, Mucciarella MR, Costantini N, Surricchio G (2002c) Double oil extraction from olive paste and olive pomace by centrifugal decanter at 2-phases, type integral. Riv Ital Sost Grasse 79:351–355
Di Giovacchino L, Mucciarella MR, Costantini N, Ferrante ML, Surricchio G (2002d) Use of nitrogen to improve stability of virgin olive oil during storage. J Am Oil Chem Soc 79:339–344
Di Giovachino L, Angerosa F, Di Giacinto L (1996) Effect of mixing leaves with olives on the organoleptic quality of oil obtained by centrifugation. J Am Oil Chem Soc 73:371–374
Hermoso Fernández M, Uceda Ojeda M, García-Ortiz A, Morales Bernardino J, Fríaz Ruiz L et al (1991) Elaboracion de aceite de oliva de calidad. Apuntes no 5/1991, Consejeria de Agricultura y Pesca, Junta de Andalucia
Hermoso Fernandez M, Gonzalez Delgado J, Uceda Ojeda M, Garcia-Ortiz A, Morales Bernardino J et al (1995) Elaboracion de aceite de oliva de calidad. II. Obtencion por el sistema de dos fases. Apuntes no 11/94, Junta de Andalucía, Sevilla
Inarejos-Garcia AM, Gomez-Rico A, Desamparados Salvador M, Fregapane G (2009) Influence of malaxation conditions on virgin olive oil yield, overall quality and composition. Eur Food Res Technol 228:671–677
International Olive Council (IOC) (2006) Quality management guide for the olive oil industry: olive oil mills. COI/T.33/Doc, no 2–4, Madrid
International Olive Council IOC (1990) Collection: Technical Handbooks. Olive Oil Quality Improvement. International Olive Oil Council, Madrid
Koutsaftakis A, Stefanoudaki E (1996) Diminuzione dell'umidità e dell'olio contenuto nella sansa prodotta con decanter a due fasi. Riv Ital Sost Grasse 73:23–27
Levi Minzi R, Saviozzi A, Riffaldi R, Falzo L (1992) L'épandage au champ des margines: effets sue les propriétées du sol. Olivae 40:20–25
Lombardo N, Briccoli Bati C, Marsilio V, Di Giovacchino L, Solinas M (1990) First observations on the disposal of olive oil mills vegetation waters on cultivated soil. Acta Horticulturae 286:493–496
Martinez Moreno JM, Gomez Herrera JM, Janer del Valle C (1957a) Estudios fisico-quimicos sobre las pastas de aceitunas molidas. IV. Las goats de aceite. Grasas Aceites 8:112–118
Martinez Moreno JM, Gomez Herrera C, Janer del Valle C (1957b) Estudios fisico-quimicos sobre las pastas de aceitunas molidas.V. Afinidades entre las fases sòlidas y liquidas. Grasas Aceites 8:155–161

Martinez Moreno JM, Martinez Suarez JM, Gomez Herrera C, Janer del Valle C, Pereda Marin J et al (1965) Estudios fisico-quimicos sobre las pastas de aceitunas molidas. XXIV. Mecanismo de funcionamiento del extractor Alfin. Grasas Aceites 16:223–229

Martinez Moreno JM, Pereda Martin J, Flores Luque V, Gomez Herrera C, Borrero ME (1968) Estudios fisico-quimicos sobre las pastas de aceitunas molidas. XXVIII. Ensayos con el prototipo industrial del nuevo sistema Alfin. Grasas Aceites 19:45–48

Martinez Suarez JM, Munoz Aranda E, Alba Mendoza J, Lanzon RA (1975) Informe sobre utilizaciòn del analizador de rendimientos Abencor. Grasas Aceites 28:379–385

Mascolo A, Cucurachi A, Di Giovacchino L, Solinas M, Seghetti L (1980) Confronto tra i rendimenti in olio conseguibili con i sistemi della pressione e della centrifugazione nella lavorazione delle olive. Ann Ist Sper Elaiotecnica VIII:165–174

Montedoro G, Petruccioli G (1973) Aggiornamenti sui trattamenti con additivi enzimatici nell'estrazione dell'olio con mezzi meccanici. Riv Ital Sost Grasse 50:331–343

Montedoro G, Petruccioli G (1978) Trattamenti biochimici effettuati sulle paste integrali e snocciolate di oliva al fine di facilitarne l'estrazione meccanica dell'olio. Riv Ital Sost Grasse 55:150–159

Muñoz AE, Alba MJ (1980) Informe de la Almazara Experimental del Istituto de la Grasa correspondiente a la campaña 1979-80. Grasas Aceites 31:423–428

Muñoz Aranda E, Alba Mendoza J, Lanzon Rey A, Aparicio R (1979) Informe de la Almazara Experimental del Instituto de la Grasa correspondiente a la Campaña 1978-79. Grasas Aceites 30:251–261

MuTAC. Museo delle Tradizioni ed Arti Contadine, Picciano (I)

OL.MA. Oleificio del Madonnino – Cooperative oil mill at Madonnino di Braccagni (I)

Parenti A, Spugnoli P, Masella P, Calamai L (2005) Carbon dioxide emission from olive oil pastes during the transformation process: technological spin offs. Eur Food Res Technol 222:521–526

Parenti A, Spugnoli P, Masella P, Calamai L, Pantani OL (2006) Improving olive oil quality using CO_2 evolved from olive pastes during processing. Eur J Lipid Sci Technol 108:904–912

Petruccioli G (1965) Prove tecnologiche sul complesso "Sinolea-Pressa" e sulla centrifuga-chiarificatrice Westfalia. Industrie Agrarie III:347–353

Petruccioli G, Peri C, Montedoro G, Setti D (1973) L'estrazione dell'olio d'oliva per percolamento. Riv Ital Sost Grasse 50:315–321

Picci G, Pera A (1993) Relazione su un triennio di ricerche microbiologiche sullo spargimento delle acque di vegetazione (AAVV) dei frantoi oleari su terreno agrario. Genio Rurale 5:72–77

Ridolfi M, Scamosci M, Patumi M (2010) Influenza del tempo di gramolazione in atmosfera a ridotto contenuto di ossigeno sulle caratteristiche qualitative ed aromatiche dell'olio di oliva. In: Proceedings of the national seminar on olive and olive oil, Portici (I), 1–2 Oct 2009. Italus Hortus 17:51–54

RISG 63 (1986) Rivista Italiana delle Sostanze Grasse, Milano (I)

Romano R, Giordano A, Formato A, Sacchi R, Spagna Musso S (2010) Effetti del processo di estrazione in atmosfera inerte (N2) sulla qualità dell'olio vergine d'oliva. In: Proceedings of the national seminar on olive and olive oil, Portici (I), 1–2 Oct 2009. Italus Hortus 17:22–26

Schäfer-Schuchardt H (1988) L'oliva. La grande storia di un piccolo frutto, Favia-Bari (I)

Schäfer-Schuchardt H (1997) Espansione culturale ed artistica. In: Enciclopedia mondiale dell'olivo. International Olive Council, Madrid, pp 21–26

Servili M, Taticchi A, Esposto S, Urbani S, Selvaggini R et al (2008) Influence of the decrease in oxygen during malaxation of olive paste on the composition of volatile and phenolic compounds in virgin olive oil. J Agric Food Chem 56:10048–10055

Setti D (1973) Ottimizzazione del processo di estrazione dell'olio d'oliva per percolamento. Riv Ital Sost Grasse 50:322–324

Siniscalco V, Montedoro GF (1988) Estrazione meccanica dell'olio di oliva mediante l'impiego di coadiuvanti tecnologici. Nota I: drenanti ed enzimi. Riv Ital Sost Grasse 65:675–680

Stefanoudaki E, Koutsaftakis A, Harwood JL (2011) Influence of malaxation conditions on characteristic qualities of olive oil. Food Chem 127:1481–1486

Tsimidou M, Papadopoulos G, Boskou D (1992) Determination of phenolic compounds in virgin olive oil by reverse-phase HPLC with emphasis on UV detection. Food Chem 44:53–60

Chapter 4
Lipid Metabolism in Olive: Biosynthesis of Triacylglycerols and Aroma Components

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Contents

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties*,
DOI 10.1007/978-1-4614-7777-8_4, © Springer Science+Business Media New York 2013

4.1 Introduction

The olive fruit, classified as a drupe from a botanical perspective, accumulates lipids in the form of different molecular species of triacylglycerols (TAGs). Hence, to understand the biosynthesis of olive oil, one must consider the source of carbon skeletons, de novo fatty acid synthesis, and their assembly into complex lipids. Indeed, the relative importance of both biosynthetic pathways in olive (fatty acid synthesis and lipid assembly) has been studied in olive tissue cultures by applying top-down (metabolic) control analysis (TDCA) (Ramli et al. 2002a, b). Results showed that fatty acid formation exerted a somewhat higher control than lipid assembly, with flux control coefficients of 0.57 and 0.43, respectively.

4.2 Source of Carbon for Oil Synthesis

In contrast to most seeds, developing fruits contain active chloroplasts, which enables them to fix CO_2 under photosynthetic conditions. Therefore, developing fruits possess two sources of reduced carbon. First, as a sink organ, they import photosynthate from the leaves, in the same way as seeds do. Second, owing to their photosynthetic capability, developing fruits can fix CO_2.

4.2.1 Export of Photoassimilates from Leaves

In higher plants, CO_2 is photosynthetically fixed in the leaf chloroplasts to yield triose phosphate, which is exported to the cytosol to form sucrose. Sucrose is then exported via the phloem to the rest of the plant to supply reduced carbon and metabolic energy necessary for the growth and development of nonphotosynthetic sink organs, such as developing seeds. This is the universal pathway of photosynthetic CO_2 fixation and distribution of photoassimilates, but a number of plant families, such as the *Apiaceae*, also possess the capability of forming sugar alcohols (mainly mannitol and sorbitol) from triose phosphates in the cytosol of the leaf cell, and these can be translocated, together with sucrose, to the rest of the plant. Other plant species, such as legumes, can synthesize oligosaccharides (commonly raffinose and stachyose) from sucrose, and these carbohydrates are exported to the rest of the plant. The metabolic capability of synthesizing either sugar alcohols or oligosaccharides is characteristic of certain plant genera or families, and usually the presence of one pathway excludes the other (Zimmermann and Ziegler 1975). The olive tree, belonging to the family *Oleaceae*, is exceptional, among other things, for its ability to form both mannitol and oligosaccharides of the raffinose family. Thus, sucrose, mannitol, raffinose, and stachyose have been detected in exudates from olive leaves (Flora and Madore 1993), indicating that they are all translocated through the phloem from the leaves to the sink tissues,

including developing fruits. The catabolic pathways involved in the utilization of mannitol and stachyose in nonphotosynthetic tissues have been described in a number of plant species (Stoop et al. 1996; Kandler and Hopf 1982).

Very little is known about these pathways in olives. In vivo experiments carried out with tissue slices from developing olives fed with ^{14}C-labeled sucrose or mannitol resulted in very poor incorporation of the label into glycerolipids in comparison with that obtained with either ^{14}C-acetate or ^{14}C-pyruvate (del Cuvillo 1994). These results might reflect a slow rate of uptake of the carbohydrate precursors rather than a low activity of the biochemical pathways responsible for their utilization. Interestingly, high α-galactosidase (the enzyme that catalyzes the hydrolysis of the galactosyl moiety of stachyose and raffinose) activity has been detected in cell-free extracts from the pulp of developing olives (Fernández-Bolaños et al. 1995), indicating that they are able of degrading stachyose imported from the leaves to form sucrose. Furthermore, cell suspension cultures were able to use sucrose, glucose, galactose, fructose, and mannitol as the sole carbon and energy sources, confirming the role of this polyol as an important photoassimilate in olive (Oliveira et al. 2002). More recently, mannitol transport mechanisms operating in olive heterotrophic-suspension-cultured cells have been investigated in detail (Conde et al. 2007).

4.2.2 Fruit Photosynthesis in Developing Olives

Olives remain green for a considerable period and retain active chloroplasts even when they change color as they approach maturity. While chlorophyll is localized mostly in the exocarp, the mesocarp contains significant amounts of phosphoenolpyruvate carboxylase (Sánchez 1994), the CO_2 fixation enzyme of the CAM and C4 photosynthetic pathways. Because photosynthesis in fruits displays certain characteristics, different from those observed in either C3 or C4/CAM plants, it has been suggested this synthesis should be considered as a new type, and the term *fruit photosynthesis* has been proposed (Blanke and Lenz 1989). According to this concept, fruits are considered strong sink organs. During the developmental period, and due to both mitochondrial respiration of photoassimilates imported from the leaves and the impermeability of the fruit cuticle, CO_2 accumulates in the cell-free space of the fruit in high concentrations. Then, after CO_2/HCO_3^- equilibration, phosphoenolpyruvate carboxylase, present in the cytosol, catalyzes the fixation of bicarbonate into oxaloacetate, which is converted into malate by malate dehydrogenase. In due course, malate can be decarboxylated to yield pyruvate (which is the precursor of fatty acid synthesis) and CO_2. The latter is photosynthetically fixed into triose phosphate in the fruit chloroplasts. Fruit photosynthesis, therefore, should play an important role in refixing the CO_2 produced by mitochondrial respiration of photoassimilates imported from leaves (Sánchez and Harwood 2002).

Indeed, developing olives have been observed to accumulate levels of CO_2 of up to 2 % in the intercellular space, whereas alkaline extraction of the pulp tissue yielded amounts of CO_2/HCO_3^- in the range of 50–60 μmol/g of fresh tissue. Such

olives were also capable of photosynthetic $^{14}CO_2$ fixation (Sánchez 1994). When fruits were exposed to $^{14}CO_2$ in the light, the label from the precursor was actively incorporated into glycerolipids, carbohydrates, and organic acids, whereas in the dark total incorporation was severely reduced and most of the label was associated with organic acids (Sánchez and Salas 1997).

4.2.3 Contribution of Fruit Photosynthesis to Oil Biogenesis

Because photosynthesis in fruits, measured by CO_2 exchange (infrared gas analysis), rarely reaches the compensation point, it has been suggested that the contribution to the carbon economy of the organ is rather modest (Blanke and Lenz 1989). However, gas exchange measurements give information about the overall balance of CO_2 exchange of the plant organ, which is the algebraic sum of photosynthesis, respiration, and photorespiration, and provides no information about the extent of CO_2 refixation carried out in the fruit through the phosphoenolpyruvate carboxylase pathway described earlier. Therefore, a field experiment was designed to assess the relative contribution of fruit photosynthesis to olive oil biogenesis (Sánchez 1995). Complete defoliation of certain branches was carried out some 12 weeks after anthesis (WAA), at a time when lignification of the fruit endocarp takes place. Fruits grew on these branches under autotrophic conditions because they were deprived of a supply of photoassimilates from adjacent leaves. In parallel, selected fruits growing on other branches not submitted to defoliation were enclosed in dark cages to develop under heterotrophic conditions, i.e., in the absence of fruit photosynthesis. Fruits were harvested at the cherry stage of ripening, the beginning of the ripening period (28 WAA), when the accumulation of storage TAGs reached its plateau and the color of the fruits changed from green to purple. Clear differences were observed in the size and oil content of fruits submitted to the two growing regimes (Sánchez and Harwood 2002). Olives developed in the dark (heterotrophic) reached a size close to that of the controls but were etiolated, and their oil content was significantly reduced. On the other hand, fruits growing on defoliated branches (autotrophic) were significantly smaller than the controls, but their oil content, expressed as a percentage of fresh weight, was higher than that of the controls. These results show that both fruit photosynthesis and import of photoassimilates contribute to the biosynthesis of TAGs in olives. Indeed the oil content per fruit was equally reduced in heterotrophic and autotrophic olives as compared to the controls, i.e., one-third in both cases (Sánchez 1995). These results strongly suggest that fruit photosynthesis contributes to the carbon economy of developing olives and hence to olive oil biogenesis. Indeed, Proietti et al. (1999) reported that during daylight, for a large part of the fruit-growing period, CO_2 intake by an olive fruit permitted the reassimilation of a large part (40–80 %) of the CO_2 produced by the dark respiration rate.

4.3 Fatty Acid Biosynthesis

The crucial precursor for de novo fatty acid biosynthesis is acetyl-CoA. Biosynthesis of the latter can be achieved in a number of ways, of which three main pathways have been identified (Nikolau et al. 2000). First, pyruvate is formed from carbohydrates via glycolysis, and then the oxidative decarboxylation of pyruvate inside the plastid by the pyruvate dehydrogenase complex produces CO_2 and acetyl-CoA. Another route for the synthesis of plastidial pyruvate (and thence acetyl-CoA) is through the decarboxylation of imported malate by the plastidial NADP-dependent malic enzyme. In the third pathway, acetyl-CoA is formed from pyruvate in the mitochondria and is hydrolyzed to acetate, which leaves the mitochondria to reach the plastid where it is activated to acetyl-CoA by the acetyl-CoA synthetase (Weselake et al. 2009). The relative contribution of these pathways to fatty acid biosynthesis has not been studied so far in olive. However, labeling experiments carried out using tissue slices from developing olives showed that both acetate and pyruvate were efficiently incorporated into acyl lipids (Salas et al. 2000), suggesting that both pathways could be operative in the olive pulp.

De novo fatty acid biosynthesis is a process that occurs in plant plastids and needs the combined activity of two multienzyme complexes, acetyl-CoA carboxylase and fatty acid synthase.

4.3.1 Acetyl-CoA Carboxylase

Acetyl-CoA carboxylase (ACCase, EC 6.4.1.2) catalyzes the formation of malonyl-CoA from acetyl-CoA, the first committed reaction in the biosynthesis of fatty acids, and is considered to be an important regulatory enzyme for fatty acid synthesis in many tissues (Alban et al. 2000; Harwood 1996; Sasaki and Nagano 2004). This reaction, which uses bicarbonate as substrate, involves the participation of biotin and is driven by the hydrolysis of ATP (Harwood 1988). The reaction takes place in two steps: biotin is carboxylated by biotin carboxylase and carbon dioxide is transferred to acetyl-CoA by carboxyl transferase (Fig. 4.1). Therefore, the enzyme is a complex consisting of at least three separated proteins or domains on a multifunctional protein: the previously cited biotin carboxylase and carboxyl transferase, and the biotin

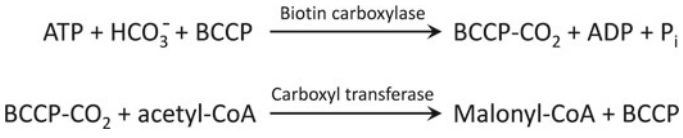

Fig. 4.1 Acetyl-CoA carboxylase reaction

carboxyl carrier protein (BCCP), which contains biotin linked to a lysine residue. In higher plants, there are two isoenzymes located in the cytosol and the plastid. In dicotyledons, such as olive, the cytosolic isoform consists of a single multifunctional polypeptide, and all three domains are part of a single polypeptide. This is called the eukaryotic form because it is a type of complex present in animals and yeast. In contrast, the plastidial isoform is a multienzyme complex, consisting of four separate proteins: biotin carboxylase, BCCP, and carboxyl transferase, which is a heterodimer (α- and β-subunits). Since this multienzyme complex is similar to that in bacteria or cyanobacteria, it is called a prokaryotic form (Harwood 1996). On the other hand, in monocotyledons, both are multifunctional enzymes. Three of the genes for the plastidial prokaryotic form of ACCase (biotin carboxylase, BCCP, and α-subunit of carboxyl transferase) are nuclear-encoded. The fourth gene coding for the β-subunit of carboxyl transferase is plastid-located (Harwood 2005).

As shown in Fig. 4.1, in the first step, a carboxybiotin intermediate is formed at the expense of one ATP. Biotin is carboxylated by biotin carboxylase that binds bicarbonate to nitrogen in the biotin ring of BCCP. The flexible biotin arm of BCCP carries the activated CO_2 from the biotin carboxylase active site to the carboxyl transferase site. The carboxyl transferase transfers the activated CO_2 from biotin to acetyl-CoA, yielding malonyl-CoA (Knowles 1989; Sánchez and Harwood 2002). Biochemical evidence indicates that plastid ACCase is responsible for providing most, if not all, the malonyl-CoA required for de novo biosynthesis of fatty acids (Sasaki and Nagano 2004).

4.3.2 Fatty Acid Synthase

Fatty acids are synthesized from malonyl-ACP (acyl carrier protein), which is formed from the malonyl-CoA generated by ACCase, in a reaction catalyzed by malonyl-CoA:ACP transacylase (MCAT, EC.2.3.1.39). Fatty acids are subsequently produced by an easily dissociable multisubunit complex consisting of monofunctional enzymes and referred to as fatty acid synthase (FAS). As shown in Fig. 4.2, the enzymatic complex includes six enzymes as well as the ACP, which binds the intermediate acyl chains (Harwood 1996): β-ketoacyl-ACP synthases I, II, and III (KAS I, EC.2.3.1.41; KASII, EC.2.3.1.179; and KASIII EC.2.3.1.180 respectively), β-ketoacyl-ACP reductase (KAR, EC.1.1.1.100), β-hydroxyacyl-ACP dehydrase (DH, EC.4.2.1.-) and enoyl-ACP reductase (ENR, EC.1.3.1.9).

The cycle of elongation starts with the condensation of malonyl-ACP with acetyl-CoA by the action of KAS III to produce acetoacetyl-ACP. This compound is subsequently reduced by KAR, an enzyme usually dependent on NADPH, to yield the β-hydroxyacyl derivative (Harwood 1988). The latter compound is then dehydrated by DH and finally reduced by ENR, resulting in a four-carbon (butyryl) acyl-ACP derivative, which can be submitted to a further chain elongation cycle (Fig. 4.2). The next condensations are catalyzed by KAS I, which, unlike KAS III, uses acyl-ACPs as the primer substrates instead of acetyl-CoA. This enzyme is involved in most of the condensations necessary to produce the acyl-chains, so that

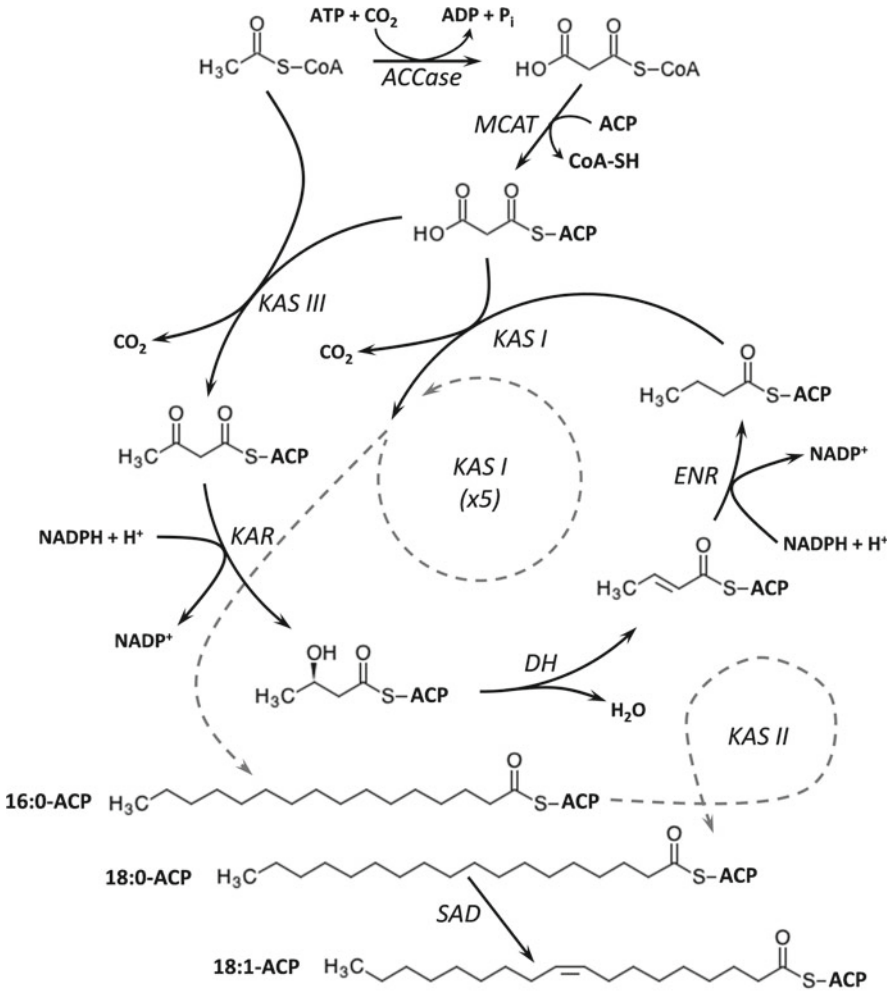

Fig. 4.2 De novo fatty acid biosynthesis in plants. Abbreviations: *ACP* acyl carrier protein, *ACCase* acetyl-CoA carboxylase, *MCAT* malonyl-CoA:ACP transacylase, *KAS* ß-ketoacyl-ACP synthase, *KAR* ß-ketoacyl-ACP reductase, *DH* ß-hydroxyacyl-ACP dehydrase, *ENR* enoyl-ACP reductase, *SAD* stearoyl-ACP desaturase (*Source*: Authors)

the final product of the cycles in which it takes part is palmitoyl-ACP (Stumpf 1987). Finally, this compound can be elongated to stearoyl-ACP in a further cycle involving the participation of the last condensing enzyme, KAS II (Fig. 4.2). This step is especially relevant because it will determine the C16/C18 ratio of the resulting pool of fatty acids.

Although the individual enzymes of the olive fatty acid synthase complex have not been separated and characterized so far, its overall activity has been studied in soluble fractions from the pulp of developing olives using radiolabeled malonyl-CoA as the precursor (Sánchez and Harwood 1992). As in other species, total activity

was found to be stimulated by the addition of ACP and inhibited by the antifungal antibiotic cerulenin, which specifically inhibits KAS I and to a lesser extent KAS II. The activity showed a strong dependence on the presence of reduced pyridine nucleotides (NADH and, in particular, NADPH) and thiol reagents (2-mercaptoethanol). Peculiar to this enzyme system were the high proportions of medium-chain fatty acids, ranging from C8 to C14, found among the products synthesized in vitro (Sánchez and Harwood 1992; Sánchez et al. 1992; Salas et al. 2000).

Though some of the enzymes catalyzing the partial reactions of fatty acid synthesis have been purified from plant species and genes coding for them have been isolated and sequenced, in olive only the enoyl-ACP reductase gene has been cloned and its temporal and transient expression during flower and fruit development studied (Hatzopoulos et al. 2002; Poghosyan et al. 2005).

The process of de novo fatty acid synthesis finishes with the release of the acyl moieties from the ACP derivatives by the action of plastid thioesterases or their transfer into complex lipids by acyltransferases inside the plastid. Most species, including oil fruits, have two types of acyl-ACP thioesterases showing different substrate specificities for C16 and C18 acyl-ACPs: the FATA type preferentially hydrolyzes oleoyl-ACP, whereas the FATB type has the highest activity with saturated acyl-ACPs (Jones et al. 1995; Harwood 1996).

4.3.3 Fatty Acid Modification

Although the final products of FAS are mainly C16 or C18 saturated acyl-ACPs, olive oil and most vegetable oils are enriched in unsaturated fatty acids, like oleic (C18:1), linoleic (C18:2), or α-linolenic (C18:3) acids. The formation of oleic acid, the most abundant constituent of olive oil, takes place by desaturation of stearoyl-ACP, a reaction catalyzed by stearoyl-ACP Δ9-desaturase (SAD, EC 1.14.99.6), which is also present in the plastid stroma and is responsible for the formation of oleoyl-ACP (Fig. 4.2). This is, in general, a very active enzyme, thus explaining why, except for a few cases such as cocoa, stearate does not accumulate, but oleate is the main product of fatty acid synthesis in plastids. The enzyme has been isolated from several plant species, purified to homogeneity and eventually crystallized (Shanklin and Cahoon 1998). The gene coding for this protein has been cloned and sequenced from various plant species, including olive (Baldoni et al. 1996; Angiolillo et al. 1997; Haralampidis et al. 1998).

The formation of linoleic acid, the most abundant polyunsaturated fatty acid in vegetable oils, mainly occurs outside the plastid. Thus, it is produced in the endoplasmic reticulum (ER) by a reaction catalyzed by an oleate desaturase (Δ12 fatty acid desaturase, EC 1.3.1.35). In this case, the substrate oleoyl moiety is esterified in a membrane phospholipid, generally phosphatidylcholine (Harwood 1996; Sperling and Heinz 2001). The reaction has been extensively studied in oil seeds, and the genes encoding for the enzyme of several plant species have been cloned

and sequenced (Harwood 1996). Two different genes encoding microsomal Δ12 oleate desaturase, FAD2-1 and FAD2-2, with a 73 % identity, have been cloned and sequenced from olive (Hatzopoulos et al. 2002; Banilas et al. 2005; Hernández et al. 2005). Expression analysis of FAD2 genes suggests a differential physiological role for the two olive genes, with FAD2-1 involved in the desaturation of storage lipids in the young seed and FAD2-2 being mostly responsible for reserve lipid desaturation in the mature seed and the mesocarp (Hernández et al. 2005, 2009). On the other hand, attempts to detect oleate desaturase activity in membrane preparations from developing olives have been unsuccessful so far.

Further desaturation producing α-linolenic acid may occur by the action of a microsomal linoleate desaturase (Δ15 fatty acid desaturase, EC 1.14.99.-). Olive oil is characterized by low amounts (0.6–0.8 %) of α-linolenic acid, relative to the other major unsaturated fatty acids, i.e., oleic acid (55–85 %) and linoleic acid (7–19 %). The olive gene encoding for linoleate desaturase activity in the ER, FAD3, has been sequenced and its expression patterns in different seed tissues and mesocarps studied during olive fruit development, showing that its contribution, as expected from the olive oil fatty acid composition, to olive oil biosynthesis and modification is minimal (Hatzopoulos et al. 2002; Banilas et al. 2007).

Very long-chain fatty acids, which are found in small amounts in olive oil [arachidic (C20:0), behenic (C22:0) and lignoceric acids (C24:0)], are released from the ER as acyl-CoAs after sequential addition of two-carbon units at the carboxyl end of preexisting long-chain acyl-CoAs. This reaction is catalyzed by the fatty acid elongase (FAE) complexes (Cassagne et al. 1994), which are membrane-bound multienzyme complexes located in the ER. Cycles of fatty acid elongation in the ER are analogous to intraplastidial de novo fatty acid biosynthesis and involve four enzymatic reactions catalyzed by 3-ketoacyl-CoA synthase (FAE1), 3-ketoacyl-CoA reductase, 3-hydroxyacyl-CoA dehydrase, and enoyl-CoA reductase (Lessire et al. 1998; Barrett and Harwood 1998; Domergue et al. 2000).

4.4 Complex Lipid Synthesis: Formation of Triacylglycerols

The assembly of complex lipids from glycerol-3-phosphate and the fatty acids formed initially in the plastids proceeds in the ER initially by the Kornberg–Pricer acylation reactions and completed by the Kennedy pathway, which were named after their discoverers (Fig. 4.3). This simple pathway must be modified by additional reactions such as the phospholipid:diacylglycerol acyltransferase (PDAT) reaction as well as “acyl editing” (Bates et al. 2009). Nevertheless, it is very important to bear in mind that the additional ancillary reactions have not been evaluated in olive in vivo. Moreover, when PDAT and diacylglycerol: diacylglycerol acyltransferase were assayed in vitro only small (less than 10 % of total), activities were detected (Ramli et al. 2009). The general topic of triacylglycerol synthesis and its regulation in oil crops has been summarized recently (Weselake et al. 2009).

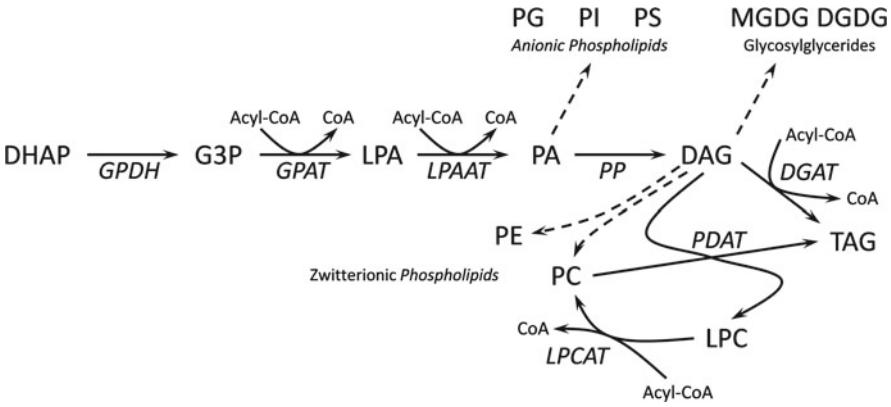

Fig. 4.3 Assembly of fatty acids onto glycerol backbone. Abbreviations: *GPDH* glycerol 3-phosphate dehydrogenase, *GPAT* glycerol 3-phosphate acyltransferase, *LPAAT* lysophosphatidate acyltransferase, *PP* phosphatidate phosphohydrolase, *DGAT* diacylglycerol acyltransferase, *PDAT* phospholipid; diacylglycerol acyltransferase, *LPCAT* lysophosphatidylcholine acyltransferase, *DHAP* dihydroxyacetone phosphate, *G3P* glycerol 3-phosphate, *LPA* lysophosphatidate, *PA* phosphatidate, *DAG* diacylglycerol, *TAG* triacylglycerol, *PG* phosphatidylglycerol, *PS* phosphatidylserine, *PI* phosphatidylinositol, *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *MGDG* monogalactosyldiacylglycerol, *DGDG* digalactosyldiacylglycerol, *lysoPC* lysophosphatidylcholine (*Source*: Authors)

The Kennedy pathway consists of a series of four reactions, three of them being acylations of the glycerol backbone catalyzed by acyl-CoA-dependent acyltransferases, which yield TAGs as end products. In the first step, a glycerol 3-phosphate acyltransferase (GPAT; EC 2.3.1.15) catalyzes the acylation in the *sn*-1 position of the glycerol 3-phosphate to form lysophosphatidic acid (LPA). Secondly, a lysophosphatidate acyltransferase (LPAAT; EC 2.3.1.51) transfers a fatty acid to the *sn*-2 position, generating phosphatidate (PA). This is a branch point for the synthesis of anionic phospholipids (phosphatidylglycerol, PG; phosphatidylinositol, PI; phosphatidylserine, PS). The third enzyme of this pathway is the phosphatidate phosphatase (PP; EC 3.1.3.4), which hydrolyzes phosphatidic acid to yield diacylglycerol (DAG), a substrate for zwitterionic phospholipid (phosphatidylcholine, PC; phosphatidylethanolamine, PE) and glycosylglyceride (monogalactosyldiacylglycerol, MGDG; digalactosyldiacylglycerol, DGDG; sulphoquinovosyldiacylglycerol, SQDG) synthesis. Finally, in the only step specific to TAG synthesis, diacylglycerol is acylated at the *sn*-3 position by a diacylglycerol acyltransferase (DGAT; 2.3.1.20) (Harwood 1996). As stated earlier, other pathways have been described for TAG formation (Fig. 4.3), one of them, the transfer of acyl groups from phospholipids to diacylglycerols, an acyl-CoA-independent reaction catalyzed by the enzyme PDAT, must be mentioned because it has been recently established in vivo that PDAT and DGAT have overlapping functions that are essential for normal pollen and seed development of Arabidopsis (Zhang et al. 2009).

In olives, the whole pathway of TAG synthesis has been studied in microsomal fractions, both from the pulp tissue of developing olives using radiolabeled acyl-CoAs

as substrates (Sánchez et al. 1992) and from tissue cultures using radiolabeled glycerol 3-phosphate (Rutter et al. 1997). The incorporation of acyl-CoAs into TAGs by microsomes from developing olives seemed to involve their initial incorporation into PC; further incorporation into TAGs was stimulated at pH 8 and by addition of Mg^{2+} and glycerol 3-phosphate. Oleoyl-CoA was found to be a better substrate than palmitoyl-CoA, which agrees with analytical data for the fatty acid composition of olive oils (Sánchez et al. 1992). Further studies with olive callus cultures using radiolabeled acyl-CoAs as substrates have shown that lysophosphatidylcholine acyltransferase (LPCAT) is important for the entry of unsaturated fatty acids (oleate and linoleate) into olive callus lipid metabolism, and PDAT may also be involved in TAG biosynthesis (Hernández et al. 2008).

The specificities of the three acyltransferases involved in the *Kennedy* pathway dictate the molecular species of the resulting TAGs and, hence, the quality and physicochemical properties of a particular vegetable oil. Several studies of the selectivity of the enzymes involved in TAG synthesis have been carried out with olive cell cultures or deduced from studies with other plant species (Sánchez and Harwood 2002; Williams et al. 1993; Rutter et al. 1997). Although not all the specificities of the acyltransferases involved in the Kennedy pathway have been examined in olive fruits, the results from stereospecific analysis of olive oil TAG can indicate the selectivity of these enzymes for different fatty acids (Santinelli et al. 1992; Sacchi et al. 1992; Gallina-Toschi et al. 1993; Damiani et al. 1994, 1997; Gabrielli-Favretto et al. 1999; Vichi et al. 2007). These analyses have shown that (1) unsaturated fatty acids are preferentially esterified at the *sn*-2 position, which is consistent with previous findings indicating that LPAAT has a strong selectivity for oleoyl-CoA in most plants (Sánchez and Harwood 2002); (2) the ratio of the saturated fatty acids 16:0, 18:0, and 20:0 between positions *sn*-1 and *sn*-3 decrease as the chain length becomes longer, indicating the preference of GPAT for palmitoyl-CoA among saturated fatty acids, although it can also accept oleoyl-CoA, especially if this is present in high proportions in the pool of acyl-CoAs available for the enzyme; (3) the *sn*-1 position of TAG is enriched in linoleate, which suggests that linoleoyl-CoA is also an effective substrate for olive GPAT; and (4) it seems likely that fatty acids esterified at the *sn*-3 position of glycerol reflect the composition of the acyl-CoA pool, suggesting that DGAT is an enzyme with a broad selectivity and specificity (Harwood and Page 1994).

Although the enzymatic properties of DGAT enzymes from olive [in plants there are generally two DGATs; see Weselake et al. (2009)] have not been studied in detail, in vivo labeling experiments using ^{14}C-acetate and tissue slices from developing olive pulp have shown that TAG formation is strongly reduced at temperatures above 40 °C (Sánchez et al. 1990). Similarly, the formation of TAGs from [^{14}C]-glycerol-3-phosphate by microsomal fractions from developing olives was reduced when the incubation temperature was increased from 30 °Cto 40 °C, with a commensurate rise in the relative labeling of DAG (Rutter et al. 1997). These results were indicative of the limiting activity of olive DGAT at higher temperatures, which suggests that this reaction might exert significant flux control in the biogenesis of olive oil under climatic conditions during the ripening season and in typical regions

of olive growing. In fact, the conclusion that DGAT activity could be important for lipid accumulation in olive has been confirmed by metabolic control analysis using callus cultures (Ramli et al. 2002a). Furthermore, additional experiments applying metabolic control analysis to the Kennedy pathway for triacylglycerol formation in olive tissue cultures using 2-bromooctanoate as a specific inhibitor of DGAT showed that this enzyme had a flux control coefficient in the Kennedy pathway (under the experimental conditions) of 0.74 (Ramli et al. 2005). This indicates that most of the control is at the final step (DGAT) of the pathway and is in contrast to other oil crops, such as oil palm (Ramli et al. 2005). Two major unrelated gene families have been shown to encode DGAT in plants, DGAT1 (type-1) and DGAT2 (type-2), both of which are ER-localized. DGAT1 and DGAT2 genes have been cloned from several plant species, including olive (Giannoulia et al. 2000; Hatzopoulos et al. 2002; Banilas et al. 2011). Comparative transcriptional analysis revealed that DGAT1 and DGAT2 are developmentally regulated and share an overall overlapping but distinct transcription pattern: DGAT1 appears to contribute for most of the TAG deposition in seeds, whereas in the mesocarp, both DGAT1 and DGAT2 share an overlapping expression pattern.

4.5 Storage of Triacylglycerols in Olive Fruits

In oil seeds, the storage and packaging of the TAGs synthesized in the ER involves the formation of oil bodies through a well-known process in which oleosin proteins participate (Murphy 2001). However, in fruits like olive the majority of the oil accumulates in the fleshy pericarp, which, unlike seeds, does not possess proper oil bodies. Ultrastructural studies have shown that, in olive, TAGs tend to fuse to produce oil droplets that develop with fruit maturation to a size of approximately 30 μm (Rangel et al. 1997). This process probably takes place because oleosins (as has been demonstrated by immunoblotting and transcriptional analysis) are absent from olive pulp (Ross et al. 1993; Giannoulia et al. 2007), so there is no constraint on oil coalescence, and this also explains why olive oil can be easily extracted from olive pulp by mild mechanical methods. Moreover, any other role that oleosins may play, for example by acting as an anchor for lipase activity, is superfluous in olive pulp since the latter's TAGs are not reused by the plant to feed the olive embryo, and the function of the fruit is merely to aid dispersal of seeds, often by providing an attractive nutritious meal for an animal (Murphy 2001).

4.6 Biosynthesis of Virgin Olive Oil aroma

Virgin olive oil (VOO) differs from other vegetable oils in that it is prepared from fresh fruits and is retailed and consumed without any refining because it is a natural product that possesses a pleasing taste and aroma. Olive oil aroma is strongly related

to its minor volatile components, which were extensively characterized in previous studies using dynamic headspace sampling (Morales et al. 1994). These studies revealed that olive oil contains volatile aldehydes, alcohols, ketones, hydrocarbons, and esters, among which aldehydes, alcohols, and esters of alcohols of five and six carbon atoms typically synthesized through the lipoxygenase (LOX) pathway are especially abundant (accounting for 60–80 % of the total volatile fraction). The impact of these compounds for the aroma of olive oil and other food products has also been investigated using techniques of sniffing, indicating that they are responsible for the fresh green fruity notes valued by consumers (Morales et al. 1995; Angerosa 2002). During the process of olive oil processing the LOX pathway is triggered by olive crushing and proceeds during the malaxation step. The volatile compounds formed during these operations are incorporated into the oil, giving rise to the aroma. Thus, the aroma of a given oil is determined by the relative activity of the enzymes involved in the pathway, which can vary depending on many factors like the variety and degree of maturation of olives used or the conditions used during the extraction process.

4.7 Lipoxygenase Pathway

The LOX pathway consists of a cascade of oxidative reactions that give rise to a variety of metabolites with different functions from polyunsaturated fatty acids (either linoleic or linolenic acids as the initial substrates) (Feussner and Wasternack 2002). These compounds have received the generic name oxylipins. Among its different parts the branch of the LOX pathway directly related to aroma biosynthesis (Fig. 4.4) involves the oxygenation of PUFAs by the action of the enzyme LOX, which introduces a hydroperoxide function in the carbons adjacent to the 1,4-pentadiene group of the fatty acid substrate (Grechkin 1998; Brash 1999). The resulting hydroperoxide is quickly cleaved to two carbonyl moieties through a reaction catalyzed by the hydroperoxide lyase (Matsui 1998). This enzyme produces C6 aldehydes and C12 oxoacids from 13-hydroperoxides and two C9 moieties from 9-hydroperoxides. This step is very important from the point of view of the aroma notes arising from the food product because, unlike C6 carbonyl compounds that give rise to green fruity notes, C9 carbonyls are responsible for cucumber- and melon-like notes (Matsui et al. 1991, 2000). Moreover, depending on the initial substrate, the aldehydes produced by this enzyme will be saturated (hydroperoxides from linoleic acid) or unsaturated (hydroperoxides from α-linolenic acid). The breakage of hydroperoxides can also occur in absence of HPL by homolytic lysis catalyzed by LOX. Unlike the main branch of the pathway, this reaction yields C5 alcohols and enals and C13 aldehydes (Salch et al. 1995). These C-5 volatile compounds are also components of the olive oil aroma producing important positive attributes like fruity and sweet notes (Morales et al. 1995). Furthermore, C6 alcohols can be produced by the reduction of aldehydes generated by HPL through a reaction catalyzed by alcohol dehydrogenases (Yamashita et al. 1976; Olías et al. 1993). Finally, alcohols can be esterified to acyl moieties by the enzyme

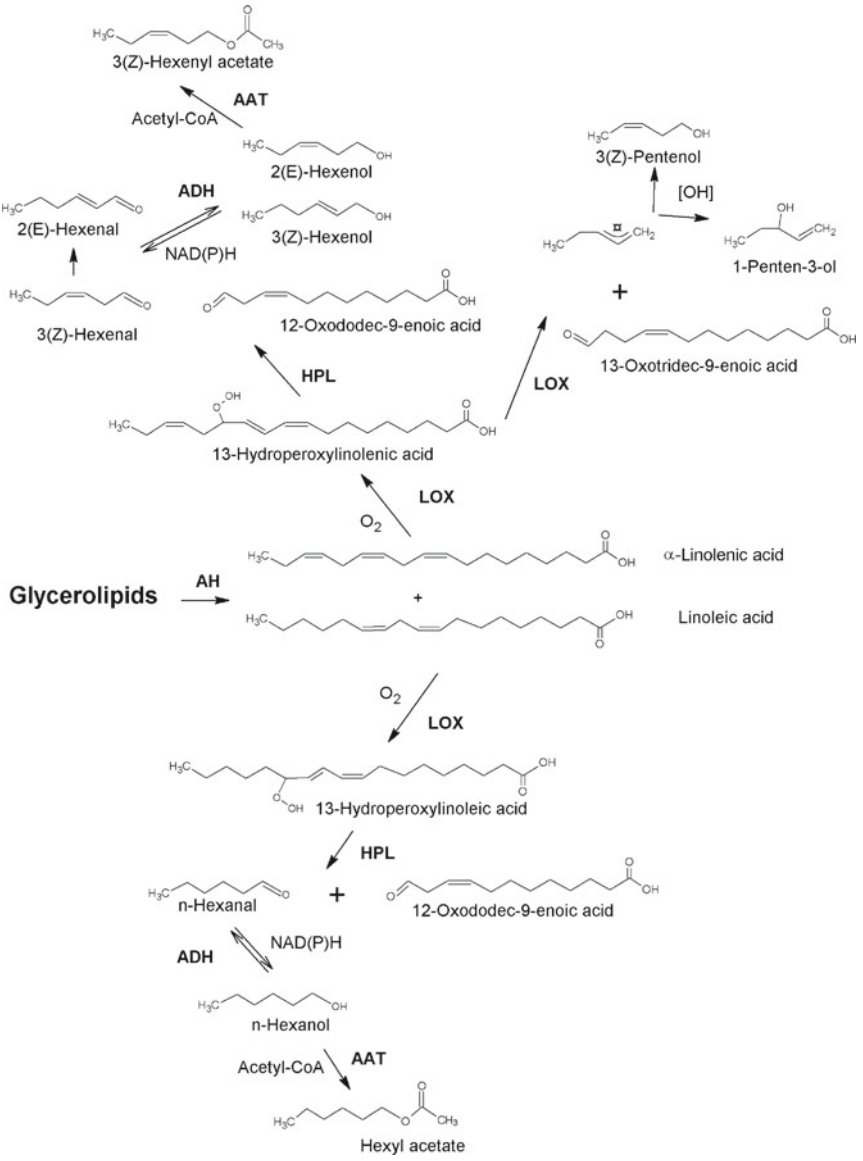

Fig. 4.4 Scheme of reactions that take place in synthesis of volatile compounds present in virgin olive oil through lipoxygenase pathway. Abbreviations: *AH* acyl hydrolase, *LOX* lipoxygenase, *HPL* hydroperoxide lyase, *ADH* alcohol dehydrogenase, *AAT* alcohol acyltransferase (*Source*: Authors)

alcohol acyltransferase to produce esters responsible for fruity notes appreciated by consumers (Pérez et al. 1993). The final aroma of a given oil will be a function of the activity and specificity of all these enzymes and will be determined by biochemical and molecular factors inherent to the fruits, environmental factors affecting olive fruit development, and operation parameters during processing like the temperature and pH of the olive pulp paste.

4.8 Release of Fatty Acid Substrates for Lox Pathway

Although several LOX forms acting on fatty acids esterified to glycerolipids have been reported (Pérez-Gilabert et al. 1998; Feussner et al. 2001), there is extensive experimental evidence indicating that the synthesis of volatile compounds in plants takes place from unesterified PUFAs and is dependent on their previous hydrolysis from complex lipids by the action of acyl hydrolases (Hatanaka and Harada 1973). Thus, studies on homogenates from different plant tissues showed that a rapid hydrolysis of membrane lipids and catabolism of the released fatty acid moieties take place simultaneously with volatile formation (Hatanaka et al. 1976; Galliard et al. 1976). In the case of olive pulp no direct evidence yet exists on how free fatty acids are released from disrupted tissues (to give rise eventually to volatile compounds). Following an examination of typical VOO volatile composition, one can conclude that the majority of components (up to 80 %) originate from the degradation of α-linolenic acid. In this regard, some studies indicated that the synthesis of aroma in olive pulp is largely dependent on the levels of α-linolenic acid, and not so much on those of the major polyunsaturated fatty acid linoleic acid, suggesting that there may be a limiting role for the hydrolysis of the latter fatty acid in this process (Sánchez-Ortiz et al. 2007). The most abundant lipids in developing olive fruits are, by far, the TAGs of the oil. However, olive TAGs contain only very low amounts of α-linolenic acid, which is not degraded to a significant extent during olive crushing, indicating that these lipids are not the main source of PUFAs involved in VOO aroma biosynthesis, and this would exclude a significant role of TAG lipases in the process. This point was confirmed by the different acyl composition of the unesterified fatty acid fraction compared to the oil TAGs (Sánchez-Ortiz et al. 2007). In this regard, systematic analysis of the acyl composition of the different polar lipids species from olive pulp indicated that those containing the highest proportion of α-linolenic acid are galactolipids, which in olives (as in other green tissues) contained 70–85 % α-linolenic acid in their acyl moieties (Vioque and De la Maza 1973). Thus, these lipids are promising candidates for being the source of substrates for the LOX pathway during olive oil elaboration, mediating their hydrolysis by the action of specific enzymes. In this regard, recent studies reported that the synthesis of jasmonic acid (JA) (which is also formed via LOX activity) in Arabidopsis leaves was dependent on the expression of the *DLG* gene, encoding for a chloroplast-targeted galactolipase (Hyun et al. 2008). Hence, both the basal levels of JA in this plant and the burst in the synthesis of this hormone after wounding are strongly dependent on this enzyme. Since JA and aldehyde biosynthesis share the initial steps within the LOX pathway, it would be expected that galactolipases of the DGL type are involved in the regulation of both branches of the pathway. As of today no galactolipases have been described in olive, so this represents an interesting field for future research.

4.9 Olive Lipoxygenase

Lipoxygenase (LOX) is a dioxygenase that catalyzes the oxidation of polyunsaturated fatty acids such as linoleic and α-linolenic acid, which contain a 1(Z), 4(Z)-pentadiene sequence, yielding a fatty acid hydroperoxide. The oxygen molecule can be introduced

into any of the double bonds in the pentadiene sequence to form a hydroperoxide group, while the double bond is changed to an E-configuration and migrates one carbon toward the other double bond to become conjugated (Siedow 1991).

LOX also catalyzes the anaerobic cleavage of n-6(S)-hydroperoxylinolenic acid to yield a C13-oxoacid (13-oxo-12,9-tridecadienoic acid) and a C5 alcohol (Z-2-pentenol or 1-penten-3-ol) (Salch et al. 1995) (Fig. 4.4), which are present in appreciable amounts in the aroma of VOO (Morales et al. 1995; Angerosa et al. 2002).

Plant LOXs have been classified into two gene subfamilies (type 1 and type 2) in relation to their primary structures (Feussner and Wasternack 2002). Type 1 LOXs are soluble proteins with a cytosolic location. These LOX forms can display different product specificities and show a preference to produce either 13- or 9-hydroperoxides. Type 2 LOXs are chloroplast-targeted proteins that are found in the particulate fraction of plant tissues and yield almost exclusively 13-hydroperoxides. Several forms of LOXs are often expressed simultaneously in a given plant tissue, which probably means that there is a specialization for each isoform. These enzymes are involved in the regulation of plant growth and the activation of responses induced by stress (Feussner and Wasternack 2002). Thus, in general, the production of volatile aldehydes is related to wound-induced gene expression and interaction of plants with insects, parasites, or pathogens (Porta and Rocha-Sosa 2002).

4.9.1 Olive Pulp Lipoxygenase

LOX activity has been characterized in the developing tissues of olive pulp. This activity is bound to the particulate fraction of olive tissues, mainly to high-density membranes, typically isolated from plastids or chloroplast or from low-density microsomal fractions (Salas et al. 1999) (Table 4.1). Despite the fact that a high proportion of olive LOX is located in high-density membranes, there was no more activity associated with fractions containing higher levels of chlorophyll. Thus, activity was regularly distributed along the whole pulp tissue, so that the green epicarp of the fruit had approximately the same LOX activity level as the nongreen mesocarp (Table 4.1). The level of activity varied during fruit development, ranging from 20 nkat/g fresh weight at the beginning of the period of oil accumulation to 2.5 nkat/g fresh weight in mature olives. Olive LOX displayed an acidic optimal pH value (pH 5.5). With regard to its substrate specificity this activity showed a clear preference for the oxidation of α-linolenic acid in its unsterified form, which was oxidized at a rate double that of linoleic acid and approximately 100-fold higher than linoleic acid esterified to phosphatidylcholine (Table 4.2). Furthermore, analysis of the hydroperoxide products resulting from the enzyme reaction revealed that the LOX activity in olive pulp yielded mainly 13-hydroperoxides from both α-linolenic and linoleic acids and only minor amounts of 9-hydroperoxides (Table 4.3).

Table 4.1 Distribution of lipoxygenase activity among different tissues and membrane fractions from olive fruit pulp

	Epicarp				Mesocarp			
	LOX		Chlorophyll		LOX		Chlorophyll	
Fraction	(nkat)	(%)	(mg)	(%)	(nkat)	(%)	(mg)	(%)
3K	1.61 ± 0.01	12.4	0.06	24	1.14 ± 0.04	8.9	0.02	8
15K	2.4 ± 0.2	18.7	0.06	24	2.1 ± 0.3	16.4	0.02	8
40K	0.91 ± 0.05	7	0.05	20	0.50 ± 0.2	3.9	0.01	4
150K	1.8 ± 0.3	14	0.02	8	2.4 ± 0.4	18.7	0.01	4
Total	6.7	52.1	0.19	76	6.1	47.9	0.06	24

Data from Salas et al. (1999). Activity is given as the mean and the standard deviation of three determinations

Table 4.2 Substrate specificity of lipoxygenase from crude fractions extracted from olive fruit pulp

	LOX	
Substrate	(nkat/mg prot)	%
Linoleic acid	1.67 ± 0.06	100
α-linolenic acid	0.77 ± 0.04	46
Dilinoleyl-phosphatidyl choline	0.01 ± 0.00	1

Data from Salas et al. (1999). Activity is given as the mean and the standard deviation of three determinations

Table 4.3 Product specificity of lipoxygenase from crude fractions extracted from olive fruit pulp

	Hydroperoxide isomer			
Substrate	13-ZE(Z)	13-EE(Z)	9-EZ(Z)	9-EE(Z)
Linoleic acid	67	11	17	7
α-linolenic acid	73	15	12	nd

Data from Salas et al. (1999) and expressed as percentage of total isomers
nd not detected

Developing olive pulp tissues are a complex biological system in which it is expected that a variety of LOX forms could be expressed at a given time. Nevertheless, based on results obtained from olive tissues, it can be concluded that LOX forms predominating during the processes of olive oil biosynthesis and fruit maturation are of type 2, displaying an acidic optimum pH, membrane location, and preference for the production of 13-hydroperoxides. The levels of LOX activity in olive pulp are easily sufficient to produce the amount of volatile compounds found in olive oil, and its substrate and product specificities are the most straightforward for explaining the efficient production of 13-ZEZ-hydroperoxy linolenic acid, the main precursor of the most abundant volatile compounds found in VOO: E(2)-hexenal and Z(3)-hexenal. Moreover, characterization of this activity revealed that hydrolysis of fatty acid precursors is really necessary because activity with esterified fatty acids was almost negligible. Results also pointed to high levels of LOX

activity bound to plastids, which are organs typically containing linolenate-enriched galactolipids. This was further evidence to support the hypothesis of a role for a galactolipid hydrolase within this pathway (or acyl hydrolases that typically have high activity with galactolipids) (Burns et al. 1979).

4.9.2 *Functional Characterization of Lipoxygenase Genes from Olive Pulp*

Characterization of LOX from olive pulp was completed in recent years with the cloning and characterization of LOX genes expressed in the fruit during the period of maximal olive oil biosynthesis. Plant tissues usually express different forms of LOX with distinct functions and regulation. In the case of olive pulp three genes coding for LOX, called OeLOX1, Oep1LOX2, and Oep2LOX2, have been reported (Lorenzi et al. 2006; Padilla et al. 2009). They coded for proteins of around 900 amino acids, with molecular weights of approximately 100 KDa (Table 4.4). The OeLOX1 was a soluble protein displaying high homology with other LOXs of type 1 from hazelnut, tobacco, and almond. The other two proteins contained an N-terminal chloroplast transit peptide and displayed sequences typical for type 2 LOXs like those from tobacco and tea leaves. The number of copies of homologous genes in the olive genome was also investigated by restriction plus transference studies and showed the presence of a single copy of OeLOX1, three copies of Oep1LOX2, and one copy of Oep2LOX2. The functional characterization of these LOXs was completed by their overexpression in *E. coli* followed by purification by affinity chromatography. The purified enzymes were kinetically characterized using both linoleic and α-linolenic fatty acids. The kinetic parameters of these enzymes are shown in Table 4.5. The type 1 LOX displayed similar parameters for both substrates, with higher affinity (lower K_m value) for linoleic acid. With regard to the membrane-bound LOX forms, both displayed their higher V_{max} values for α-linolenic acid, with activity values that were about tenfold higher than those found for linoleic acid. Moreover, these enzymes displayed important differences in their affinity for substrates. Thus, Oep1LOX2 showed higher affinity and catalytic efficiencies

Table 4.4 General characteristics of gene coding from lipoxygenases and hydroperoxide lyase cloned from olive fruit pulp tissues

		Gene size		Protein size		
Gene	Gene bank accession	bp	aa	KDa	PI	Copies
Oe LOX1[a]	EU678670	2,592	864	98.4	6.0	1
Oep1LOX2[b]	EU513352	2,898	913	103.4	7.2	3
Oep2LOX2[b]	EU513353	2,821	901	101.7	5.9	1
OePHPL[c]	EUS13350	1,641	491	55.0	7.4	1

[a]Lorenzi et al. (2006)
[b]Padilla et al. (2009)
[c]Padilla et al. (2010)

Table 4.5 Kinetic parameters from different lipoxygenase enzymes cloned from olive pulp fruits and expressed in *E. coli*

					Optimum values	
		K_m	V_{max}			Temperature[a]
		(μM)	(nmol/min^{-1})	K_m/V_{max}	pH	(°C)
OeLOX1[b]	α-linolenic acid	174.94	105.0	0.28	6.0	nd
	Linoleic acid	343.66	103.2	0.14		
Oep1LOX2[c]	α-linolenic acid	28.0	210.5	7.52	6.75	45
	Linoleic acid	14.6	23.1	1.58		
Oep2LOX2[c]	α-linolenic acid	138.9	166.6	1.20	6.25	35
	Linoleic acid	129.8	10.3	0.08		

nd not detected
[a]On basis of initial activity
[b]Lorenzi et al. (2006)
[c]Padilla et al. (2009)

than Oep2LOX2, which displayed K_m values one order of magnitude higher. The product specificity of these enzymes was also characterized by analyzing the hydroperoxide isomers produced by each isomer. OeLOX1 produced 9- and 13-hydroperoxides in a 2:1 ratio, whereas both type 2 LOXs produced 13-hydroperoxides as the main product, displaying profiles similar to ones found in crude fractions from olive mesocarp and LOX purified from these tissues. When considering the localization and specificity it seemed clear that type 2 LOXs are those that could take part in the process of volatile biosynthesis in olive pulp. The contribution of these enzymes to VOO aroma biosynthesis was further investigated by studying their temporal expression in different tissues. Thus, Oep2LOX1 was expressed at low levels in olive pulp but had high levels of expression in leaf tissues. In contrast, the other form, Oep2LOX2, was expressed in olive pulp at much higher levels, displaying homology with other LOX forms involved in volatile biosynthesis for plant tissues such as tomato or potato leaf. Moreover, this was expressed at the maximum level at a time when VOO is extracted from fruits.

Another interesting aspect of olive LOX research has been the level of activity or expression of these enzymes in different olive cultivars and their contribution to the differences found among them. Thus, Padilla et al. (2009) demonstrated different expression profiles for type 2 LOXs in the cultivars *Arbequina* and *Picual*. Furthermore, Ridolfi et al. (2002) reported a correlation between the level of LOX activity and the final amount of volatile compounds of the oil from three different Italian and Greek cultivars.

4.10 Olive Hydroperoxide Lyase

Hydroperoxide lyase (HPL) catalyzes the cleavage of fatty acid hydroperoxides at the bond situated between the carbon atom carrying the hydroperoxide group and the adjacent E-double bond, thus yielding a volatile aldehyde of 6 or 9 carbon atoms

Table 4.6 Distribution of hydroperoxide lyase activity among different tissues and membrane fractions from olive fruit pulp

	Epicarp				Mesocarp			
	HPL		Chlorophyll		HPL		Chlorophyll	
Fraction	(nkat)	(%)	(mg)	(%)	(nkat)	(%)	(mg)	(%)
3K	13 ± 1	14.7	0.06	24	9.9 ± 0.2	11.2	0.02	8
15K	16.6 ± 0.8	18.7	0.06	24	13.6 ± 0.1	15.3	0.02	8
40K	9.0 ± 0.2	10.2	0.05	20	8.2 ± 0.2	9.3	0.01	4
150K	8.8 ± 0.1	9.9	0.02	8	9.5 ± 0.4	10.7	0.01	4
Total	47.7	53.5	0.19	76	41.2	46.5	0.06	24

Data from Salas and Sánchez (1999a)
Activity is given as the mean and the standard deviation of three determinations

and an oxoacid of 9 or 12 carbon atoms, depending on the substrate used (Fig. 4.4). This enzyme was first isolated from watermelon seedlings (Vick and Zimmerman 1976) and since then has been described in a number of plant species and tissues, and it is considered a ubiquitous enzyme within the plant kingdom. The gene coding for this enzyme was first cloned and sequenced from pepper by Matsui et al. (1996). The later molecular characterization of HPLs revealed that these enzymes belong to a novel family of cytochrome P450 proteins that was given the name CYP74. Enzymes within this family do not require molecular oxygen or reducing equivalents and are closely connected to the synthesis of oxylipins (Noordermeer et al. 2001). Furthermore, all were targeted to plastids by transit peptides in the N-terminal side of their sequences, as in the case of type 2 LOXs.

The most common plant HPL enzymes display strict specificity toward 13-hydroperoxides and, thus, produce C6-aldehydes (Vick and Zimmerman 1976; Matsui et al. 1991). However, there are other types of HPLs that are less common, showing specificity toward 9-hydroperoxides (Kim and Grosh 1981) or even HPLs unspecific for both positional isomers (Noordermeer et al. 1999). The aldehydes produced by these enzymes are involved in plant resistance to pests and could also be used to attract insect predators. In the case of olive, the C6-aldehydes produced by the LOX pathway have been demonstrated to be repellent to the olive fruit fly, one of the most important pests of this crop (Scarpati et al. 1993).

4.10.1 Hydroperoxide Lyase in Olive Fruit Pulp

Olive pulp HPL is bound to the particulate fraction of the tissues, displaying a distribution similar to that found for crude olive LOX (Salas and Sánchez 1999a). Thus, this enzyme is found in a higher proportion in high-density membranes typically from chloroplasts (Table 4.6). However, the distribution of activity did not correlate with the content of chlorophyll and was found equally in both pericarp and mesocarp, indicating that this enzyme is probably present in both green chloroplasts

Table 4.7 Kinetic parameters from olive hydroperoxide lyase. HPL I and HPL II correspond to enzymes purified from olive pulp. OepHPL corresponds to recombinant enzyme expressed in *E. coli*

	Hydroperoxide substrate	K_m (μM)	V_{max} (nkat/mg prot)	K_m/V_{max}	Optimum pH
HPL I[a]	13-ZEZ-HP-18:3	9.6	58.0	–	
	13-ZE-HP-18:2	17.1	23.1	–	6
HPL II[a]	13-ZEZ-HP-18:3	9.7	34.1	–	
	13-ZE-HP-18:2	78.3	32.1	–	6
			(nmol/min^{-1})		
OepHPL[b]	13-ZEZ-HP-18:3	55.7	29.6	0.53	8–9
	13-ZE-HP-18:2	47.7	11.6	0.25	

[a]Salas and Sánchez (1999a)
[b]Padilla et al. (2010)

and nongreen plastids. This result showed that the LOX and HPL have a similar location in organelles typically rich in the PUFAs that are the substrates of the pathway.

HPL from olive pulp was solubilized from membranes using Triton X-100 and purified by ion exchange and hydroxyl apatite chromatography (Salas and Sánchez 1999a). Two peaks of activity were obtained from the second column (HPL I and HPL II), corresponding to enzymes with identical properties, which probably indicated that they are indeed the same enzyme that has been modified after translation (Table 4.7). Olive pulp HPL displayed strict specificity toward 13-ZEZ-hydroperoxides from both linoleic and α-linolenic acid and displayed no activity toward 9-hydroperoxides or 13-EE-hydroperoxides. This substrate specificity explains the fact that no C9-aldehydes are present in olive oil aroma, although olive LOX was able to produce a small amount of 9-hydroperoxides. These enzymes were also kinetically characterized and had K_m values on the micromolar order. They also displayed higher affinity and, in the case of HPL I, higher V_{max} for the 13-ZEZ-hydroperoxylinolenic acid, which was again in good agreement with the volatile composition of VOO, due to this compound being the precursor of its major products, 3(Z)-hexenal and E(2)-hexenal. HPL from olive pulp displayed an acidic optimum pH and showed thermal lability in the conditions in which it was assayed, with stability around 15 °C. The activity of this enzyme in olive pulp tissues was approximately 20 nkat/g fresh weight (13-ZEZ-hydroperoxylinolenic acid substrate), which was higher than LOX activity and enough to account for the volatile aldehydes produced by olive pulp during olive oil processing.

4.10.2 Functional Characterization of Hydroperoxide Lyase from Olive Pulp

One gene of HPL has been amplified from cDNA from olive pulp tissues (OepHPL). This gene had a size of 1,641 bp, coding for a protein of 491 amino acids (Table 4.4) with a PI of 7.4 (Padilla et al. 2010). The OepHPL gene displayed a high degree of

homology with other HPLs cloned from plants, clustering in the group of the 13-HPLs from dicotyledon plants. Furthermore, the OepHPL sequence displayed an N-terminal extension that probably was a chloroplast transit peptide, which reinforced data suggesting a plastidial localization of this enzyme. Further studies on the genomic organization of the gene pointed to the existence of a single copy of it in the olive genome. The HPL from olive pulp was heterologously expressed in *E. coli* and the resulting protein purified by affinity chromatography. The kinetic parameters of the purified enzyme were similar to those reported for HPL I from olive pulp, with K_m values on the micromolar order and the highest V_{max} for 13-ZEZ-hydroperoxide linolenic acid (Table 4.7). Some differences, however, were found with regard to the optimum pH and temperature stability. Thus the optimum pH was found to be acidic for the enzyme purified from pulp tissues and alkaline in the case of the recombinant enzyme. The divergence with regard to temperature stability was also high. Thus, the recombinant OepHPL was found to give maximal activity at 45 °C, under the assay conditions that contrasted with the 15 °C reported for HPL purified from olive tissues. These differences could be due to methodological aspects such as the solubilization necessary for purification of the enzyme from pulp tissues.

Interesting results were also obtained in studies of the expression of the HPL gene in different tissues of the olive tree (Padilla et al. 2010). This gene displayed high levels of expression in leaf and mesocarp tissues, with barely detectable levels of the transcript in the seed kernel. The higher levels of expression in the pulp of fruit tissues were similar to those of LOX, with a peak around 28 weeks after flowering. This period was coincident with the onset of fruit maturation and is well known to be a time in which the oils produced display higher amounts of volatile compounds and an especially intense aroma. Moreover, the levels of expression of this enzyme were studied in two olive varieties: *Picual* and *Arbequina*. This study demonstrated that different cultivars also displayed different levels of expression, although there was no correlation between the amount of HPL transcripts expressed and the final concentration of aldehydes in the oil, which could mean that the biosynthetic capacity of this pathway may be affected by other factors such as the final content of phenols in the fruit.

4.11 Modification of Aldehydes: Isomerization, Reduction, and Further Esterification of Alcohols

The sequential action of LOX and HPL on α-linolenic acid results in the production of 3(Z)-enals of either six or nine carbon atoms, depending on the specificity of the former enzymes. In the aroma of olive oil, however, the most prominent volatile is a 2(E)-enal, 2(E)-hexenal, which accounts for more than 50 % of the total amount of volatile compounds detected by headspace analysis (Morales et al. 1995). This prevalence of 2(E)-enals over 3(Z)-enals is also common in the aroma composition of other species, indicating the presence of isomerase enzymes or chemical isomerization factors

Table 4.8 Kinetic parameters from different alcohol dehydrogenases purified from olive pulp

	NAD-ADH		NADP-ADH I		NADP-ADH II	
	Km	Vmax	Km	Vmax	Km	Vmax
Propyl-aldehyde	–	–	–	–	4.4	190
Hexanal	2.1	320	1.9	280	0.04	740
E-2-hexenal	–	–	–	–	0.012	520
Nonanal	–	–	–	–	0.03	560
NADPH	–	–	–	–	0.006	660

Data from Salas and Sánchez (1998)

(Phillips et al. 1979; Hatanaka et al. 1989; Noordermeer et al. 1999). These catalytic activities have been described in cucumber fruit, soybean seeds, and tea leaves, although no data about them have been reported in olives to date. The high degree of conversion of 3(Z) to 2(E)-enals that is observed in VOO indicated the existence of a very active isomerization mechanism during the processing of this oil.

Reduction of the aldehydes produced by the LOX pathway to alcohols is catalyzed by alcohol dehydrogenases (ADHs) through a reversible reaction dependent on reducing equivalents in the form of pyridine nucleotides (Fig. 4.4). These enzymes are widely distributed in plants, where they are involved in the resistance to anoxia (Sachs et al. 1980) and the synthesis of cinnamic alcohol (Davies et al. 1973). Olive fruit ADHs have been characterized in soluble fractions prepared from pulp acetone powders (Salas and Sánchez 1998). Crude olive-soluble fractions displayed ADH activity dependent on both NAD and NADP. In these preparations the NADP-ADH activity was found to be 20-fold higher than the NAD-dependent one. Purification of these activities by means of ion exchange and affinity chromatography revealed that these activities were due to three enzymes, NAD-ADH, NADP-ADH I, and NADP-ADH II. These enzymes were characterized by their kinetic parameters and substrate specificity, which revealed a very different range of affinities and specificities of these enzymes for the reduction of aliphatic aldehydes. Thus, NAD-ADH displayed preferences for short- and medium-chain saturated aldehydes, with a K_m value for hexanal on the millimolar order. The enzyme NADP-ADH I displayed preference for long-chain aldehydes and low affinity for hexanal, whereas the NADP-ADH II displayed both high activities and affinities for the C6-aldehydes produced by the combined action of LOX and HPL from olive fruit (Table 4.8). Thus, it was concluded that the latter enzyme was the one contributing, to a large extent, to the synthesis of the C6-alcohols present in this oil. The NADP-ADH activity was assayed in olives harvested at different stages of development, and it was found to reach a peak at about 25 weeks after flowering, just before the beginning of the ripening process, and declined after this point. This observation fitted well with the analytical data reported in previous studies.

Together with the volatile aldehydes and alcohols described previously, volatile esters of alcohols with acetate or butyrate are important constituents of the aroma of many fruits. These compounds are formed by esterification of alcohols and carboxylic acids in the form of acyl-CoA derivatives in a reaction catalyzed by alcohol

acyltransferase (Pérez et al. 1993). Studies on olive tissue slices reported synthesis of hexyl acetate upon incubation with hexanol and acetic acid (Olías et al. 1993). This activity was later characterized in crude extracts prepared from acetone powders obtained from developing olives using hexanol and acetyl-CoA as the substrates (Salas 2004). Low activity values were found, together with low affinities for both acetyl-CoA (2 mM) and hexanol (20 mM). These low levels of enzyme activity might be the reason why volatile esters, which are important constituents of the aroma of many fruits, are minor constituents of the aroma of olive oil. The substrate specificity of the olive enzyme has been studied using alcohols from one to six carbons with acetyl-CoA as the second substrate. n-Hexanol and 3(Z)-hexenol were the best substrates among those alcohols tested, which would agree with the presence of low amounts of hexyl acetate and 3(Z)-hexenyl acetate, as detected in the headspace analysis of certain olive oil samples (Salas 2004).

4.12 Improvement of Aroma of Olive Oil: Effect of Temperature on Volatile Production

Research on the molecular and biochemical mechanisms involved in the synthesis of olive oil volatile compounds could be useful for future improvements to the quality of VOOs by impacting on the production process. In this regard, previous research using green leaves from potato plants demonstrated that it was possible to alter the aroma of crushed leaves by altering the activities of different enzymes in the LOX pathway (Salas et al. 2005). Thus, leaves depleted in the expression of the LOX-H1 gene displayed very low amounts of volatiles and, in consequence, hardly any aroma, which indicated that this form of LOX controlled the total amount of volatile compounds. Furthermore, leaves with HPL activity reduced by means of antisense RNA technology displayed reduced levels of C6 aldehydes, but this was compensated by an increase in the C5-alcohols produced by the lysis of hydroperoxides mediated by LOX. These modifications in volatile composition induced important changes in the nature of the aroma of leaves that were evaluated to be sweeter and fruitier. These results were reproduced in Arabidopsis plants (Salas et al. 2006), where the leaves from a HPL-KO plant displayed similar alterations in the aroma resulting from its leaves. All these results show that it is possible to alter the aroma of plant food products by manipulating the LOX/HPL system.

VOO is considered a natural product extracted by physical means from fresh fruits. Therefore, any change made to its processing conditions in order to improve the aroma should concern only physical parameters. In this regard, several studies have focused on the influence of malaxation temperature for the aroma of the final oil and its impact on the LOX/HPL system from olive pulp. Olive oil elaboration consists of three main steps involving fruit grinding, malaxation of the resulting paste, and phase separation by centrifugation. It is well known that the conditions used in the malaxation step have an impact on the aroma of the final oil. Thus, high malaxation temperatures (40–45 °C) give higher oil yields at the expense of

lower amounts of volatile compounds and poorer organoleptic scores (Stefanoudaki et al. 2011). The causes of this effect were first investigated by studying the impact of temperature on the enzymes LOX and HPL from olive pulp. The first approach involved in vitro determination of both the whole system and of individual enzymes (Salas and Sánchez 1999b). These studies pointed to the thermal lability of HPL as the main cause of the decrease in the amount of volatile compounds at high malaxation temperatures. However, later studies on thermal inactivation on recombinant Oep1LOX2, Oep2LOX2, and OepHPL enzymes pointed to the former enzymes as those displaying higher lability, with important inactivation rates at temperatures higher than 30 °C, whereas OepHPL was fairly stable at temperatures around 45 °C (Padilla 2009). These data indicated that the changes in the aroma could be produced by a decrease in the ratio of LOX/HPL activities during the elaboration of the oil. The divergence between these two studies could be caused by the different methodologies used in them. Nevertheless, further studies on the stability of LOX and HPL in crude enzymatic preparations confirmed the pattern of thermal inactivation of these activities in olive pulp, which fitted well with the existence of two LOX forms showing different thermostability and a HPL that displayed higher stability than the LOXs (Luaces et al. 2007). These results fitted well with studies on the inactivation of the recombinant enzymes and were completed with a study on the variation in the volatile composition of oils obtained at different malaxation temperatures. Thus, malaxation at higher temperatures induced a significant decrease in the total amount of volatile compounds as well as important decreases in the ratio between C5/C6 volatiles. This was compatible with inactivation of LOX and prevalence of HPL at high temperatures. Thus, lower levels of LOX give rise to reduced amounts of total volatile compounds and lower rates of homolytic cleavage of hydroperoxides, which yield C5 volatile compounds (in agreement with Salas et al. 2005). The decrease in total volatile compounds and the lower content of C5 alcohols and enals resulted in a less intense aroma and a loss of sweet and fruity notes that are conferred by the latter compounds.

4.13 Conclusions and Perspectives

The main enzymes responsible for the biosynthesis of olive oil aroma, which are LOX and HPL, have been characterized at both the biochemical and molecular levels. The kinetic parameters and specificity of these enzymes broadly explain the volatile composition of VOO, and their profiles for thermal inactivation are able to explain the changes that take place in the aroma of virgin oils when different temperatures of malaxation are applied. The biochemical or molecular characterization of other steps involved in aroma biosynthesis is still pending and will be the subject of interesting research in the future. This research should indicate the initial steps of lipid hydrolysis, modification of enals by isomerization, aldehyde reduction, and esterification of alcohols.

Such studies should also provide a biochemical basis for the selection of olive varieties and lines with better potential to yield oils with improved aroma. Thus, it would be possible to screen lines of olives with levels of transcripts adequate for individual genes that could be backcrossed to yield oils with improved aromas. In this regard, results in model systems showed that higher levels of LOX activity in combination with low HPL cleaving rates would yield oils with a more intense aroma, in which sweet and fruity notes would be enhanced (Salas et al. 2005). These approaches will always be limited by the difficulties inherent in breeding olive trees, such as the long times of growth and development of this species. In this regard, modern techniques of tissue culture transformation and regeneration have opened the possibility of easier genetic modification of the olive tree, which would allow the modification of levels of activity of the LOX pathway enzymes to alter oils in shorter periods of time. Thus, at present there are some studies on this direction. However, a large-scale planting of transgenic olive trees has yet to take place due to consumer concerns about GMO organisms and the recognition of olive oil as a healthy traditional food.

Other fields of interest could be the interaction of the LOX pathway enzymes with minor components of olive fruit like the phenols. Such compounds, which can vary considerably in different olive cultivars, can alter the rates of lipid peroxidation. Therefore, the phenolics have a strong influence on the amount of volatile compounds in the resulting oil. Results in this area have been achieved for olives of *Arbequina* and *Picual*, where the higher contents of volatile compounds of the latter cannot be predicted simply by the levels of enzyme activity (Padilla et al. 2010).

References

Alban C, Job D, Douce R (2000) Biotin metabolism in plants. Ann Rev Plant Physiol Plant Mol Biol 51:17–47

Angerosa F (2002) Influence of volatile compounds on virgin olive oil quality evaluated by analytical approaches and sensor panels. Eur J Lipid Sci Technol 104:639–660

Angiolillo A, Baldoni L, Albert AG (1997) The olive gene for stearoyl-ACP desaturase. Abstract to the third international symposium on olive growing, 1997, Chania, Olea 24, p 163

Baldoni L, Georgi LL, Abbott AG (1996) Plant gene register PGR 96–052: nucleotide sequence of a cDNA clone from *Olea europaea* encoding a stearoyl-acyl carrier protein desaturase (accession no. U58141). Plant Physiol 111:1353–1354

Banilas G, Moressis A, Nikoloudakis N, Hatzopoulos P (2005) Spatial and temporal expressions of two distinct oleate desaturases from olive (*Olea europaea* L.). Plant Sci 168:547–555

Banilas G, Nikiforiadis A, Makariti I, Moressis A, Hatzopoulos P (2007) Discrete roles of a microsomal linoleate desaturase gene in olive identified by spatiotemporal transcriptional analysis. Tree Physiol 27:481–490

Banilas G, Karampelias M, Makariti I, Kourti A, Hatzopoulos P (2011) The olive DGAT2 gene is developmentally regulated and shares overlapping but distinct expression patterns with DGAT1. J Exp Bot 62:521–532

Barrett PB, Harwood JL (1998) Characterization of fatty acid elongase enzymes from germinating pea seeds. Phytochemistry 48:1295–1304

Bates PD, Durret TP, Ohlrogge JB, Pollard M (2009) Analysis of the fluxes through multiple pathways of triacylglycerol synthesis in developing soybean embryos. Plant Physiol 150:55–72

Blanke MM, Lenz F (1989) Fruit photosynthesis. Plant Cell Environ 12:31–46
Brash AL (1999) Lipoxygenases: occurrence, functions, catalysis, and acquisition of substrate. J Biol Chem 274:23679–23682
Burns DD, Galliard T, Harwood JL (1979) Purification of acyl Lydrolase enzymes from the leaves of *Phaseolus multiflorus*. Phytochemistry 18:1793–1797
Cassagne C, Lessire R, Bessoule JJ, Moreou P, Créach A et al (1994) Biosynthesis of very long chain fatty acids in higher plants. Prog Lipid Res 33:55–69
Conde C, Silva P, Agasse A, Lemoine R, Delrot S et al (2007) Utilization and transport of mannitol in *Olea europaea* and implications for salt stress tolerance. Plant Cell Physiol 48:42–53
Damiani P, Santinelli F, Simonetti MS, Castellini M, Rosi M (1994) Comparison between two procedures for stereospecific analysis of triacylglycerols from vegetable oils. I: olive oil. J Am Oil Chem Soc 71:1157–1163
Damiani P, Cossignani L, Simonetti MS, Campisi B, Favretto L et al (1997) Stereospecific analysis of the triacylglycerol fraction and linear discriminant analysis in a climatic differentiation of Umbrian extra-virgin olive oils. J Chromatogr A 758:109–116
Davies DD, Patila KD, Ugochukwua EN, Towers GHN (1973) Aliphatic alcohol dehydrogenase from potato tubers. Phytochemistry 12:523–530
del Cuvillo MT (1994) Biosynthesis of glycerolipids in developing olive pulp. Role of fruit photosynthesis, Ph.D. thesis, University of Seville, Seville
Domergue F, Chevalier S, Créach A, Cassagne C, Lessire R (2000) Purification of the acyl-CoA elongase complex from developing rapeseed and characterization of the 3-ketoacyl-CoA synthase and the 3-hydroxyacyl-CoA dehydratase. Lipids 35:487–494
Fernández-Bolaños J, Rodríguez R, Guillén R, Jiménez A (1995) Activity of cell wall-associated enzymes in ripening olive fruit. Physiologia Plantarum 93:651–658
Feussner I, Wasternack C (2002) The lipoxygenase pathway. Ann Rev Plant Biol 53:275–297
Feussner I, Kühn H, Wasternack C (2001) Lipoxygenase-dependent degradation of storage lipids. Trends Plant Sci 6:268–273
Flora LL, Madore MA (1993) Stachyose and mannitol transport in olive (*Olea europaea* L.). Planta 189:484–490
Gabrielli-Favretto L, Campisi B, Favretto L, Simonetti MS, Cossignani L et al (1999) Cross-validation in linear discriminant analysis of triacylglycerol structural data from Istrian olive oils. J AOAC Int 82:1489–1494
Galliard T, Matthew JA, Fishwick MJ, Wright AJ (1976) The enzymic degradation of lipids resulting from physical disruption of cucumber (*Cucumis sativus*) fruit. Phytochemistry 15:1647–1650
Gallina-Toschi T, Christie WW, Conte LS (1993) Capillary gas chromatography combined with high performance liquid chromatography for the structural analysis of olive oil triacylglycerols. J High Res Chromatogr 16:725–730
Giannoulia K, Haralampidis K, Poghosyan Z, Murphy DJ, Hatzopoulos P (2000) Differential expression of diacylglycerol acyltransferase (DGAT) genes in olive tissues. Biochem Soc Trans 28:695–697
Giannoulia K, Banilas G, Hatzopoulos P (2007) Oleosin gene expression in olive. J Plant Physiol 164:104–107
Grechkin A (1998) Recent developments in biochemistry of the plant lipoxygenase pathway. Prog Lipid Res 1998:317–352
Haralampidis K, Milioni D, Sánchez J, Baltrusch M, Heinz E et al (1998) Temporal and transient expression of stearoyl-ACP carrier protein desaturase gene during olive fruit development. J Exp Bot 49:1661–1669
Harwood JL (1988) Fatty acid metabolism. Ann Rev Plant Physiol Plant Mol Biol 39:101–138
Harwood JL (1996) Recent advances in the biosynthesis of plant fatty acids. Biochem Biophys Acta 1301:7–56
Harwood JL (2005) Fatty acid biosynthesis. In: Murphy DJ (ed) Plant lipids: biology, utilisation and manipulation. Blackwell, Oxford, pp 27–66
Harwood JL, Page RA (1994) Biochemistry of oil synthesis. In: Murphy DJ (ed) Designer oil crops. VCH, Weinheim, pp 165–194

Hatanaka A, Harada T (1973) Formation of cis-3-hexenal, trans-2-hexenal and cis-3-hexenol in macerated *Thea sinensis* leaves. Phytochemistry 12:2341–2346

Hatanaka A, Kajiwara T, Sekiya J (1976) Seasonal variations in trans-2-hexenal and linolenic acid in homogenates of *Thea sinensis* leaves. Phytochemistry 15:889–1891

Hatanaka A, Kajiwara T, Matsui K, Matsunaga T (1989) Non-enzymatic isomerization of 12-hydroxy-3(Z)-dodecenal to the 2(E)-isomer after enzymatic cleavage of 13-hydroperoxylinoleil alcohol in tea chloroplasts. Zeitschrift für Naturforschung 44:161–164

Hatzopoulos P, Banilas G, Giannoulia K, Gazis F, Nikoloudakis N et al (2002) Breeding, molecular markers and molecular biology of the olive tree. Eur J Lipid Sci Technol 104:574–586

Hernández ML, Mancha M, Martínez-Rivas JM (2005) Molecular cloning and characterization of genes encoding two microsomal oleate desaturases (*FAD2*) from olive. Phytochemistry 66:1417–1426

Hernández ML, Guschina IA, Martínez-Rivas JM, Mancha M, Harwood JL (2008) The utilization and desaturation of oleate and linoleate during glycerolipid biosynthesis in olive (*Olea europaea* L.) callus cultures. J Exp Bot 59:2425–2435

Hernández ML, Padilla MN, Mancha M, Martínez-Rivas JM (2009) Expression analysis identifies *FAD2-2* as the olive oleate desaturase gene mainly responsible for the linoleic acid content in virgin olive oil. J Agric Food Chem 57:6199–6206

Hyun Y, Choi S, Hwang HJ, Yu J, Nam SJ et al (2008) Cooperation and functional diversification of two closely related galactolipase genes for jasmonate biosynthesis. Dev Cell 14:183–192

Jones A, Davies HM, Voelker TA (1995) Palmitoyl-acyl carrier protein (ACP) thioesterase and the evolutionary origin of plant acyl-ACP thioesterases. Plant Cell 7:359–371

Kandler O, Hopf H (1982) Oligosaccarides based on sucrose (sucrosyl oligosaccharides). In: Loewus FA, Tanner W (eds) Encyclopedia of plant physiology, new series, vol 13. Springer, Berlin, pp 348–383

Kim I-S, Grosch W (1981) Partial purification and properties of a hydroperoxide lyase from fruits of pear. J Agric Food Chem 29:1220–1225

Knowles JR (1989) The mechanism of biotin-dependent enzymes. Annu Rev Biochem 58:195–221

Lessire R, Domergue F, Spinner C, Lucet-Levannier K, Lellouche JP et al (1998) Dehydration of 3-hydroxy icosanoyl-CoA and reduction of (E)-2,3-icosenoyl-CoA are required for elongation by leek microsomal elongase(s). Plant Physiol Biochem 36:205–211

Lorenzi V, Maury J, Casanova J, Berti L (2006) Purification, product characterization and kinetic properties of lipoxygenase from olive fruit (*Olea europaea* L.). Plant Physiol Biochem 44:450–454

Luaces P, Sanz C, Pérez AG (2007) Thermal stability of lipoxygenase and hydroperoxide lyase from olive fruit and repercussion on olive oil aroma biosynthesis. J Agric Food Chem 55:6309–6313

Matsui K (1998) Properties and structures of fatty acid hydroperoxide lyase. Belg J Bot 131:50–62

Matsui K, Toyota H, Kajiwara T, Kakuno T, Hatanaka A (1991) Fatty acid hydroperoxide cleaving enzyme, hydroperoxide lyase, from tea leaves. Phytochemistry 30:2109–2113

Matsui K, Shibutani M, Hase T, Kajiwara T (1996) Bell pepper fruit fatty acid hydroperoxide lyase is a cytochrome P450 (CYP74B). FEBS Lett 394:21–24

Matsui K, Ujitaa C, Fujimotoa S, Wilkinson J, Hiatt B et al (2000) Fatty acid 9- and 13-hydroperoxide lyases from cucumber. FEBS Lett 481:183–188

Morales MT, Aparicio R, Rios JJ (1994) Dynamic headspace gas chromatographic method for determining volatiles in virgin olive oil. J Chromatog A 668:455–462

Morales MT, Alonso MV, Rios JJ, Aparicio R (1995) Virgin olive oil aroma: relationship between volatile compounds and sensory attributes by chemometrics. J Agric Food Chem 43:2925–2931

Murphy DJ (2001) The biogenesis and functions of lipid bodies in animals, plants and microorganisms. Prog Lipid Res 40:325–438

Nikolau BJ, Oliver DJ, Schnable PS, Wurtele ES (2000) Molecular biology of acetyl-CoA metabolism. In: Harwood JL, Quinn PJ (eds) Recent advances in the biochemistry of plant lipids. Portland Press, London, pp 591–593

Noordermeer MA, Veldink GA, Vliegenthart JFG (1999) Alfalfa contains substantial 9-hydroperoxide lyase activity and a 3Z:2E-enal isomerase. FEBS Lett 443:201–204
Noordermeer MA, Veldink GA, Vliegenthart JFG (2001) Fatty acid hydroperoxide lyase: a plant cytochrome P450 enzyme involved in wound healing and pest resistance. Chem BioChem 2:494–504
Olías JM, Pérez AG, Rios JJ, Sanz LC (1993) Aroma of virgin olive oil: biogenesis of the "green" odor notes. J Agric Food Chem 41:2368–2373
Oliveira J, Tavares RM, Gerós H (2002) Utilization and transport of glucose in *Olea europaea* cell suspensions. Plant Cell Physiol 43:1510–1517
Padilla MN (2009) Caracterización bioquímica y molecular de la lipoxigenasa e hidroperóxido liasa de olivo y su relación con la biosíntesis del aroma del aceite de oliva virgen. Ph.D. thesis, Universidad de Sevilla, Sevilla
Padilla MN, Hernández ML, Sanz C, Martínez-Rivas JM (2009) Functional characterization of two 13-lipoxygenase genes from olive fruit in relation to the biosynthesis of volatile compounds of virgin olive oil. J Agric Food Chem 57:9097–9107
Padilla MN, Hernández ML, Sanz C, Martínez-Rivas JM (2010) Isolation, expression, and characterization of a 13-hydroperoxide lyase gene from olive fruit related to the biosynthesis of the main virgin olive oil aroma compounds. J Agric Food Chem 58:5649–5657
Pérez AG, Sanz LC, Olías JM (1993) Partial purification and some properties of alcohol acyltransferase from strawberry fruits. J Agric Food Chem 41:462–1466
Pérez-Gilabert M, Veldink GA, Vliegenthart JFG (1998) Oxidation of dilinoleoyl phosphatidylcholine by lipoxygenase 1 from soybeans. Arch Biochem Biophys 354:18–23
Poghosyan ZP, Giannoulia K, Katinakis P, Murphy DJ, Hatzopoulos P (2005) Temporal and transient expression of olive enoyl-ACP reductase gene during flower and fruit development. Plant Physiol Biochem 43:37–44
Phillips DR, Matthew JA, Reynolds J, Fenwick GR (1979) Partial purification and properties of a cis-3: trans-2-enal isomerase from cucumber fruit. Phytochemistry 18:401–404
Porta H, Rocha-Sosa M (2002) Plant lipoxygenases. Physiological and molecular features. Plant Physiol 130:15–21
Proietti P, Famiani F, Tombesi A (1999) Gas exchange in olive fruit. Photosynthetica 36:423–432
Ramli US, Baker DS, Quant PA, Harwood JL (2002a) Control mechanisms operating for lipid biosynthesis differ in oil-palm (*Elaeis guineensis* Jacq.) and olive (*Olea europaea* L.) callus cultures. Biochem J 364:385–391
Ramli US, Baker DS, Quant PA, Harwood JL (2002b) Control analysis of lipid biosynthesis in tissue cultures from oil crops shows that flux control is shared between fatty acid synthesis and lipid assembly. Biochem J 364:93–401
Ramli US, Salas JJ, Quant PA, Harwood JL (2005) Metabolic control analysis reveals an important role for diacylglycerol acyltransferase in olive but not in oil palm lipid accumulation. FEBS J 272:5764–5770
Ramli US, Salas JJ, Quant PA, Harwood JL (2009) Use of metabolic control analysis to give quantitative information on control of lipid biosynthesis in the important oil crop, *Elaeis guineensis* (oil palm). New Phytol 184:330–339
Rangel B, Platt KA, Thomson WW (1997) Ultrastructural aspects of the cytoplasmic origin and accumulation of oil in olive fruit (*Olea europaea*). Physiol Plant 101:109–114
Ridolfi M, Terenziani S, Patumi M, Fontanazza G (2002) Characterization of the lipoxygenases in some olive cultivars and determination of their role in volatile compounds formation. J Agric Food Chem 50:835–839
Ross B, Sánchez J, Millán F, Murphy DJ (1993) Differential presence of oleosins in oleogenic seed and mesocarp tissues in olive (*Olea europaea*) and avocado (*Persea americana*). Plant Sci 93:203–210
Rutter AJ, Sánchez J, Harwood JL (1997) Glycerolipid synthesis by microsomal fractions from *Olea europaea* fruits and tissue cultures. Phytochemistry 46:265–272
Sacchi R, Addeo F, Giudicianni I, Paolillo L (1992) Analysis of the positional distribution of fatty acids in olive oil triacylglycerols by high resolution 13C-NMR of the carbonyl region. Ital J Food Sci 2:117–123

Sachs MM, Freeling M, Okimoto R (1980) The anaerobic proteins of maize. Cell 20:761–767
Salas JJ (2004) Characterization of alcohol acyltransferase from olive fruit. J Agric Food Chem 52:3155–3158
Salas JJ, Sánchez J (1998) Alcohol dehydrogenases from olive (*Olea europaea*) fruit. Phytochemistry 48:35–40
Salas JJ, Sánchez J (1999a) Hydroperoxide lyase from olive (*Olea europaea*) fruits. Plant Physiol 143:19–26
Salas JJ, Sánchez J (1999b) The decrease of virgin olive oil flavor produced by high malaxation temperature is due to inactivation of hydroperoxide lyase. J Agric Food Chem 47:809–812
Salas JJ, Willams M, Harwood JL, Sánchez J (1999) Lipoxygenase activity in olive (*Olea europaea*) fruit. J Am Oil Chem Soc 76:1163–1168
Salas JJ, Sánchez J, Ramli US, Manaf AM, Williams M et al (2000) Biochemistry of lipid metabolism in olive and other oil fruits. Prog Lipid Res 39:151–180
Salas JJ, Sánchez C, García-González DL, Aparicio R (2005) Impact of the suppression of lipoxygenase and hydroperoxide lyase on the quality of the green odor in green leaves. J Agric Food Chem 53:1648–1655
Salas JJ, García-González DL, Aparicio R (2006) Volatile compound biosynthesis by green leaves from an *Arabidopsis Thaliana* hydroperoxide lyase knockout mutant. J Agric Food Chem 54:8199–8205
Salch YP, Grove MJ, Takamura H, Gardner HW (1995) Characterization of a C-5,13-cleaving enzyme of 13(S)-hydroperoxide of linolenic acid by soybean seed. Plant Physiol 108:1211–1218
Sánchez J (1994) Lipid photosynthesis in olive fruit. Prog Lipid Res 33:97–104
Sánchez J (1995) Olive oil biogenesis. Contribution of fruit photosynthesis. In: Kader JC, Mazliak P (eds) Plant lipid metabolism. Kluwer Academic, Dordrecht, pp 564–566
Sánchez J, Harwood JL (1992) Fatty acid synthesis in soluble fractions from olive (*Olea europaea*) fruits. J Plant Physiol 140:129–134
Sánchez J, Harwood JL (2002) Biosynthesis of triacylglycerols and volatiles in olives. Eur J Lipid Sci Technol 104:564–573
Sánchez J, Salas JJ (1997) Photosynthetic carbon metabolism of olives. In: Williams JP, Khan MU, Lem NW (eds) Physiology, biochemistry and molecular biology of plant lipids. Kluwer Academic, Dordrecht, pp 325–327
Sánchez J, de la Osa C, Harwood JL (1990) Effect of light and temperature on the biosynthesis of storage triacylglycerols in olive (*Olea europaea*) fruits. In: Harwood JL, Quinn PJ (eds) Plant lipid biochemistry, structure and utilization. Portland Press, London, pp 390–392
Sánchez J, del Cuvillo MT, Harwood JL (1992) Glycerolipid biosynthesis by microsomal fractions from olive fruits. Phytochemistry 31:129–134
Sánchez-Ortiz A, Pérez AG, Sanz C (2007) Cultivar differences on nonesterified polyunsaturated fatty acid as a limiting factor for the biogenesis of virgin olive oil aroma. J Agric Food Chem 55:7869–7873
Santinelli F, Damiani P, Christie WW (1992) The triacylglycerol structure of olive oil determined by silver ion high-performance liquid chromatography in combination with stereospecific analysis. J Am Oil Chem Soc 69:552–556
Sasaki Y, Nagano Y (2004) Plant acetyl-CoA carboxylase: structure, biosynthesis, regulation, and gene manipulation for plant breeding. Biosci Biotechnol Biochem 68:1175–1184
Scarpati ML, Lo Scalzo R, Vita G (1993) *Olea europaea* volatiles attractive and repellent to the olive fruit fly (*Dacus oleae*, Gmelin). J Chem Ecology 19:881–891
Shanklin J, Cahoon EB (1998) Desaturation and related modification of fatty acids. Ann Rev Plant Physiol Plant Mol Biol 49:611–641
Siedow JN (1991) Plant lipoxygenase: structure and function. Ann Rev Plant Physiol Plant Mol Biol 42:145–188
Sperling P, Heinz E (2001) Desaturases fused to their electron donor. Eur J Lipid Sci Technol 103:158–180

Stefanoudaki E, Koutsaftakis A, Harwood JL (2011) Influence of malaxation conditions on characteristic qualities of olive oil. Food Chem 127:1481–1486
Stoop JMH, Williamson JD, Pharr DM (1996) Mannitol metabolism in plants: a method for coping with stress. Trends Plant Sci 1:139–144
Stumpf PK (1987) The biosynthesis of saturated fatty acids. In: Stumpf PK, Conn EE (eds) The biochemistry of plants, vol 9. Academic Press, New York, pp 121–136
Vichi S, Pizzale L, Conte LS (2007) Stereospecific distribution of fatty acids in triacylglycerols of olive oils. Eur J Lipid Sci Technol 109:72–78
Vick BA, Zimmerman DC (1976) Lipoxygenase and hydroperoxide lyase in germinating watermelon seedlings. Plant Physiol 57:780–788
Vioque E, De la Maza MP (1973) Glicerolípidos de la aceituna. Grasas Aceites 24:226–235
Weselake RJ, Taylor DC, Rahman MH, Shah S, Laroche A et al (2009) Increasing the flow of carbon into seed oil. Biotechnol Adv 27:866–878
Williams M, Sánchez J, Hann C, Harwood JL (1993) Lipid biosynthesis in olive cultivars. J Exp Bot 44:1717–1723
Yamashita I, Nemoto Y, Yoshikawa S (1976) Formation of volatile alcohols and esters from aldehydes in strawberries. Phytochemistry 15:1633–1637
Zhang M, Fan J, Taylor DC, Ohlrogge JB (2009) DGAT1 and PDAT1 acyltransferases have overlapping functions in Arabidopsis triacylglycerol biosynthesis and are essential for normal pollen and seed development. Plant Cell 21:3885–3901
Zimmermann MH, Ziegler H (1975) List of sugars and sugar alcohols in sieve-tube exudates. In: Zimmermann MH, Milburn JA (eds) Encyclopedia of plant physiology, new series, vol 1. Springer, Berlin, pp 480–505

Chapter 5
Genetics and Molecular Biology of Olives

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Contents

5.1 Introduction

What makes olive oil exceptional? To borrow a phrase from Oscar Wilde, the pure and simple answer here is neither pure nor simple. To fully address this question, we need primarily to better understand various aspects of olive molecular physiology, genomics, and genetics. Combined with the wealth of knowledge from biochemical analyses, we may then explain why olive oil has such a unique chemical composition, characterized by a well-balanced fatty acid content along with desired pigments and

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties*,
DOI 10.1007/978-1-4614-7777-8_5, © Springer Science+Business Media New York 2013

a plethora of aroma and phenolic compounds. Most importantly, however, this knowledge is fundamental to our understanding of what factors might influence, and the extent to which they might influence, oil composition and yield and why olive oil quality may vary considerably among cultivars or orchards. Nevertheless, we should not underestimate the environment, an important factor that interplays with genetic information to shape oil quality. Such information will help breeders to develop agronomically favorable genotypes and growers to select proper cultivars or to achieve better cultivation management in optimal geographical regions and environments. Insofar as environmental conditions are predetermined, we need to address questions about the genetic information and stress our investigation into olive genetic variability. Due to the intrinsic handicaps of olive genetic crosses and progeny outcomes, an alternative route was necessary that borrowed tools from molecular-based approaches, thus resulting in an increasing interest in the steadily growing area of olive molecular biology. As new knowledge from olive biotechnology and holistic "omics" approaches accumulates, it is of immediate importance for breeders and professionals to become more familiar with those research fields in order to maximize benefits, bringing innovation back to the traditional oleoculture. This is actually one of the challenges in modern agriculture: to combine traditional quality with biotechnology tools via high-throughput strategies without adversely affecting the quality of the product or crop production. Today, to meet current consumer demand, it is advisable not to address questions on genetic transformation of olive crop for resistance or other improved traits. Nevertheless, scientists should have these basic methodologies at their disposal for possible use in the future, when genetically modified foods become more acceptable. Here we attempt to present the state of the art in olive molecular biology and genetics and to highlight recent advances and "omics" approaches, possible drawbacks, and future perspectives.

Most molecular biology research on olives so far has been mainly concentrated on two major disciplines: genome and genetic diversity studies or gene characterization and functional genomics. Given the increasing global interest in olive oil and its importance to the economy of many countries, especially those of the Mediterranean basin, it is not surprising that a major part of olive molecular biology and biochemistry studies has concentrated on the elucidation of regulatory points and controlling events in lipid biosynthesis and triacylglycerol (TAG) storage pathways. During the last decade, significant progress has been made in the isolation and characterization of key genes in the metabolic pathways of lipid synthesis, modification, and accumulation. The resulting knowledge has contributed to a thorough view of the complex molecular network governing olive oil production.

The geographical locations of early olive domestication and later genotype diffusion have been extensively studied using a number of molecular marker techniques. Biodiversity hotspots were also investigated as being of great importance for the sustainability of oleoculture as they constitute natural reservoirs for breeding programs. The developing high-throughput DNA technologies, like single nucleotide polymorphism (SNP) genotyping platforms, are quite promising for a thorough

characterization of the existing germplasm that could improve the detection of genotype-phenotype associations and the development of quantitative trait loci (QTL) markers. Molecular-marker-assisted developments paved the way for the authentication of olive oil, a procedure that is almost impossible using advanced analytical chemistry approaches. Functional genome sequence analysis may also lead to the elucidation of molecular mechanisms of olive tolerance to abiotic or biotic stress and to the isolation of key regulatory sequences or structural genes involved in important agronomical traits. These high-throughput approaches will also reveal key components in the biosynthesis of bioactive molecules and antioxidants, crucial constituents of exceptional olive oil quality products, and could be used to improve olive cultivation.

5.2 Olive Genetic Diversity Studies

Olive (*Olea europaea* L.) is a sclerophyllous evergreen species characteristic of Mediterranean flora, with a chromosome number of 2n = 46 (Breviglieri and Battaglia 1954). The wild variety, *O. europaea* var. *oleaster* (Hoffmanns & Link), or oleaster, is believed to be indigenous to the Mediterranean basin (Green 2002). Oleasters are similar to the cultivated forms (var. *europaea*), although they differ from the cultivated varieties in that they have spinescent juvenile shoots, smaller fruits with a higher stone/mesocarp ratio, and relatively low oil content and are characterized by a longer juvenile stage (Zohary and Spiegel-Roy 1975; Lumaret et al. 2004). Propagation of most cultivated varieties is achieved by grafting on specific rootstocks. This is necessary since olive trees grown from seeds usually show similar morphological traits specific to oleasters. This has caused much uncertainty in the discrimination between the two forms. The confusion has been even greater due to the high heterozygosity and genotypic diversity of genuine oleasters, which often overlap with those of cultivated olives or feral forms (clones that escaped from cultivation) (Banilas et al. 2009, and references therein). Allozyme polymorphism and the utilization of the first-generation molecular markers, including restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), and random amplified polymorphic DNA (RAPDs), have significantly improved our knowledge of the nuclear or mitochondrial genetic variation of oleasters. When oleasters from throughout the Mediterranean basin were analyzed, a clear genetic divergence between populations originating from eastern or western parts was observed (Besnard et al. 2001a, b; Lumaret and Ouazzani 2001; Bronzini de Caraffa et al. 2002; Lumaret et al. 2004). Oleasters from ancestors that thrived in the western Mediterranean refugium share the MOM and MCK mitotypes and the COM and CCK chlorotypes that are absent in oleasters from the ancestral populations occupying the eastern refugia. Oleasters in the Eastern Mediterranean share the ME1 and ME2 mitotypes and the CE1 chlorotype (Rugini et al. 2011).

The progenitor of domesticated olive is believed to be indigenous wild oleasters. The exact origin in time and space of olive domestication and diffusion across the

Mediterranean is not clear. According to archaeological and botanical data, early domestication most likely took place in the Middle East during the Early Bronze Age, in about the second half of the fifth millennium B.C. (Zohary and Spiegel-Roy 1975; Neef 1990; Liphschitz et al. 1991; Zohary and Hopf 1994). The domestication process is likely to have involved the selection of genotypes with desirable agronomic traits, mainly toward larger fruit size and a higher mesocarp/stone ratio or higher oil content. Domesticated olive gradually diffused from east to west (Terral et al. 2004), largely because of its multiple uses (e.g., oil, fruits, wood and in practical medicine). However, recent genetic analysis based on molecular markers suggests that independent domestication events (transiently or temporally) occurred in the western Mediterranean basin, further contributing to the richness and heterogeneity of the cultivated germplasm (Besnard et al. 2002a, b; Terral et al. 2004). Nowadays, more than 1,250 cultivar names have been reported around the world (Bartolini et al. 1998), although the exact number of distinct cultivars may be smaller due to the existence of synonyms (same clones with different denominations). For the purpose of better germplasm management, conservation, and sustainable use, numerous studies have been undertaken to determine more accurately the extent of existing genetic diversity in the cultivated and wild olive germplasms, to decipher true cultivars, and to unravel their intravarietal diversity and their characteristics.

The development of biochemical and polymerase chain reaction (PCR)-based molecular marker techniques has improved olive cultivar discrimination (Trujillo et al. 1995; Belaj et al. 2003). Ouazzani et al. (1993) used isozyme polymorphisms to analyze olive germplasm variability and to differentiate between wild plants and cultivars. RAPD and AFLP technologies have been extensively used for genotype fingerprinting and for the estimation of genetic similarities between or within olive cultivars from throughout the olive-producing countries. Both those molecular markers have been proven very useful because they do not require previous knowledge of the genome sequence, there is no need for marker or probe development, and they are not technically demanding procedures. They allow rapid analysis of genotypes with relatively low operational costs. Another advantage of using RAPDs in the analysis of olive germplasm variability and cultivar discrimination is the high level of polymorphism they generate probably due to the high degree of olive genetic variability (Belaj et al. 2003). RAPD-PCR is a size-based method utilizing a single oligomer (usually 10-mer) primer of arbitrary sequence in a PCR. It has been used to assess genomic DNA alterations including insertions, deletions, substitutions, and inversions. Wiesman et al. (1998) found that 14 RAPD primers were adequate to distinguish among 8 closely related variants of the Nabali cv., Mekuria et al. (1999) used only 6 primers to discriminate among 39 accessions, while 19 primers were required to discriminate 41 genotypes, mostly of Greek origin (Nikoloudakis et al. 2003).

Binary data from RAPD or AFLP analysis (band presence/absence) may be used to estimate genetic similarities or distances between genotypes and to construct informative dendrograms using appropriate algorithms, such as UPGMA clustering. However, they may not be applied to estimate phylogenetic relatedness since

they are dominant markers. Nevertheless, in a pioneering work using RAPDs in olives, Fabbri et al. (1995) showed that the cultivated germplasm is highly variable. Screening 17 cultivars from throughout the Mediterranean area, they also showed that most genotypes are clustered into groups according to their fruit size or usage, either small drupes for oil production or large ones for edible purposes. Interestingly, no apparent clustering according to geographic origin was detected. Similar results were obtained even when cultivars from a restricted area of the Mediterranean basin were analyzed (Nikoloudakis et al. 2003). There was a high degree of elite Greek cultivar clustering according to olive drupe usage. These results point to an extensive dispersal of elite clones in the past to different geographic areas and strong selection toward desired agronomic traits like oil content or fruit size. Belaj et al. (2004) also applied RAPDs to assess genetic similarities/differences and to study patterns of genetic variation among 82 Spanish olive cultivars. The dendrogram generated clustered most cultivars into two major groups from the southern and central parts of Spain, while clustering of genotypes from the Levante zone was also apparent.

RAPDs have also been successfully applied to assess the level of intravarietal genetic variability and discriminate among clones or genotypes within a variety. Gemas et al. (2000) used 20 RAPD primers to distinguish among genotypes of three cultivars grown in Portugal and found that there could have been genetic divergence among accessions according to their geographic location of origin. The same marker technology also uncovered a high degree of genetic diversity among selection clones of Ladolia, the main Cypriot cultivar, which was in agreement with the high degree of morphological and agronomic variation, suggesting that Ladolia likely originated from genetically distant land races (Banilas et al. 2003).

AFLPs have proven quite successful in olive genetic diversity studies and also equally or even more efficient in intravarietal genotype discrimination (Belaj et al. 2003). AFLPs allow for simultaneous sampling of multiple loci distributed throughout a genome. Angiolillo et al. (1999) introduced AFLP analysis in olive to study genetic variations within and among populations of cultivated olives from the most distant locations of the Mediterranean basin, including oleasters or other species of the genus *Olea*. They showed a clear structure between the cultivated and wild germplasms, which were separated into two distinct groups. Interestingly, similar levels of genetic variability were observed within each group. Except for the cultivars from Sicily, which showed high similarity and clustered together, the other varieties did not show any particular affinity based on their area of cultivation. More recently, Owen et al. (2005) applied a nonradioactive detection method in AFLP analysis of the cultivated olive germplasm in the eastern Mediterranean basin. The high discriminating capacity of AFLP markers allowed for the detection of a significant genetic distance between Greek and Turkish cultivars, while a close similarity between Turkish cultivars from those originating from the Middle East was also apparent. The group composed of the Greek cultivars also included those originating from the western Mediterranean. Recently, Albertini et al. (2011), using AFLP markers, showed that most relevant and old varieties cultivated in the Abruzzo region (central Italy) are clustered into seven groups.

Most importantly, their data point to the hypothesis that some ancestral population spread in central Italy through seed propagation and traditional varieties were selected as superior clones.

The isolation of simple sequence repeats (SSRs) or microsatellite markers in olive (Katsiotis et al. 1998; Cipriani et al. 2002) marked a new era in genetic diversity studies. Over the last decade, SSRs have dominated the field of olive genetic diversity because they provide several advantages over other size-based molecular markers for characterizing olive germplasms and for establishing genetic relationships in olives. Microsatellites are 1–6 nucleotide units repeated in tandem and randomly spread in most eukaryotic genomes. Unlike most RAPD and AFLP markers, microsatellites are codominant markers, allowing the identification of many alleles at a single locus. Therefore, they may be applied to study evolutionary relationships. In addition, they require a small amount of DNA, they are highly reproducible within and among laboratories, and the analysis can be semiautomated. The developed SSRs have been proven quite successful in cultivar identification and genetic diversity studies. Carriero et al. (2002) developed 10 primer pairs that are quite informative for the genetic characterization of 20 accessions belonging to 16 distinct olive varieties. Cipriani et al. (2002), after screening 30 primer pairs in a panel of cultivars, found that most of them yielded polymorphic SSRs. Most importantly, they detected frequent intracultivar polymorphisms by screening relatively few markers. De la Rosa et al. (2002) developed seven SSRs suitable for identification studies of olive germplasms, mapping, and paternity testing. These could also be useful in other oleaceous genera. The high discriminating power of SSRs in olive germplasms was shown by the fact that only 3 selected SSRs were adequate to differentiate among 118 cultivars from different countries around the Mediterranean basin (Sarri et al. 2006). More recently, Roubos et al. (2010) used SSR markers to analyze the genetic diversity of Greek olive germplasms. Although they did not detect any particular clustering of genotypes according to olive fruit size or usage, their dendrogram presented similarities with the dendrogram reported based on an AFLP-based study (Owen et al. 2005).

Belaj et al. (2003) compared the information provided by RAPDs, AFLPs, and SSRs in olive genetic diversity studies. They demonstrated that SSR markers produced the highest polymorphism resulting in high, but nevertheless expected, heterozygosity, while their discriminating capacity was at an intermediate level. Recently, 37 SSR loci were analyzed on a set of 21 olive cultivars resulting in a list of recommended markers and protocols for olive genotyping (Baldoni et al. 2009). Although this set of selected SSRs was unable to detect polymorphisms at the intracultivar level, it could be useful for the creation of a universal molecular database of olive cultivars.

Some researchers have used mitochondrial DNA (mtDNA) RFLPs to describe intraspecific diversity among important olive cultivars in Italy. The results also provided insights into the maternal lineages involved in the evolution of olives. More precisely, mtDNA RFLPs could assign 37 Italian cultivars to 3 distinct mitotypes: MIT1, MIT2, and MIT3, with MIT1 being the most common mitotype comprising elite cultivars grown for oil, edible fruits, or both (Rugini et al. 2011, and references therein).

In recent years, SNPs have become a popular genetic marker system. SNPs are the most abundant type of DNA sequence polymorphisms. A SNP is defined as a nucleotide site for which substitutions occur among individuals within a population. SNPs are usually highly abundant and, when combined with high-throughput platforms of allelic discrimination, they may be ideal markers for genotyping or other genetic analyses. However, the development of SNP markers requires deep knowledge of an organism's genome sequence, which is currently not the case for olive. As a result, a rather small number of SNPs have been reported in olive (Bracci et al. 2011). Most of them have been developed using a sequence-based approach of coding sequences that are available in the GenBank database (Reale et al. 2006; Muleo et al. 2009; Hakim et al. 2010).

All molecular marker techniques have verified that there is high heterozygosity and genotypic diversity within intra- and intervarietal cultivated olive trees. It is anticipated that the availability of the next generation of sequencing technologies will have a significant impact on the development of a large number of SNPs or other informative markers on olive that will make it possible to perform a genome-wide analysis of genetic variation. Nevertheless, it is of crucial importance for the scientific community to set a reference olive tree cultivar in order to identify SSRs, SNPs, or other molecular markers for olive cultivar discrimination, clustering, and genotype divergence.

5.3 Genetic Markers for Olive Oil Traceability

Marketable olive oils may have quite different organoleptic characteristics and quality depending on their varietal or geographical origin, as well as extraction techniques and practices that influence the quality and taste of the oil. This could result in price differences and varying market or consumer demands within a particular olive oil product category. Thus, the appearance of denominations and protected indications of origin have promoted labeling of oils according to those criteria. In order to protect agricultural products and foodstuffs originating from certain geographical areas, the European Council Regulations (EEC) Nos. 2081/92 and 510/2006 (EC 1992, 2006) have created systems of regional designation of origin covered by certification labels of protected designation of origin (PDO) or protected geographical indication (PGI). However, a major proportion of marketed virgin olive oil (VOO) is composed of blends of olive oils originating from different areas. This is to guarantee a particular quality of the product with typical organoleptic characteristics expected by the market or to lower the cost. However, the quality and price of a typical olive oil brand depend on the cultivar from which it originated. Although in such cases consumers must be informed about the origin of the product and whether it originates entirely from the zone covered by PDO or PGI, great variations in prices may lead consumers to doubt the originality and authenticity of different commercialized olive oil brands. Therefore, a dependable and creditable checking system that allows for monitoring of the traceability of olive oils

(geographical and botanical origin) is required to protect consumers against fraud and misdescription.

Advanced analytical chemical approaches are not yet able to discriminate cultivars and monitor olive oil traceability. This is because the oil or bioactive constituent contents are a result of the interplay between the genotype (cultivar) and environment (place of growth and particular biotic and abiotic parameters during seasonal growth). Recently, molecular markers capable of discriminating between cultivars have ameliorated such traceability systems for olive oil, especially in terms of investigating the botanical origin (Montealegre et al. 2010). They are becoming markers of choice to solve traceability- and provenance-related issues of olive oil since they are stable and not influenced by environmental or other factors, like chemical compositional markers often are. The most critical step in food genomics studies is the isolation of high-quality DNA from foodstuffs. The amount of DNA extracted, whatever technique was used to crush the olives to produce olive oil, is usually sufficient for PCR-based molecular marker techniques, but the major problem is the presence of high amounts of phenolic compounds that might inhibit PCR. Traditionally, the hexadecyltrimethylammonium bromide (CTAB) DNA extraction method (Murray and Thomson 1980) has been widely used with good success. The availability of commercial DNA extraction kits and published protocols that combine kits with other methods, like the phenol-chloroform or the hexane method, has made it possible to recover DNA of acceptable quality and quantity from olive oil that is adequate for PCR analysis (Spaniolas et al. 2008; Montealegre et al. 2010).

All the molecular markers that have been used to differentiate among cultivars have also been applied to ‘olive oil fingerprinting’. The idea is to use a data set of molecular marker profiles obtained from olive oil samples and to compare them with those obtained from the leaf DNA of the respective cultivars. Martins-Lopes et al. (2008) showed that RAPDs were less informative than the intersimple sequence repeats (ISSR) marker system for certification purposes in olive oil. However, most of the studies on olive oil traceability are based on microsatellite markers (SSRs). Pasqualone et al. (2004) evaluated SSRs to identify oil obtained from ten cultivars. Of the seven primer pairs used, six yielded amplified fragments of different lengths for each oil type considered that were capable of distinguishing among them. However, Breton et al. (2004), by analyzing the efficiency of SSR markers in order to distinguish and, ultimately, to identify olive oil of different origins in oil blends (mixed oils from different varieties), were partly successful. They showed that in controlled mixtures of oil samples from different cultivars, distinction and identification of the major olive variety used to elaborate an olive oil type was possible, but not of the minor ones. Extensive comparisons of SSR profiles of leaves versus oils for several varieties and markers conducted in three laboratories (Alba et al. 2009; Ayed et al. 2009; Vietina et al. 2011) revealed that a complete correspondence between SSRs of olive leaves and oils was only at 45 %, although the majority of alleles (approximately 70 %) was found both in oils and leaves. Such a discrepancy was mostly related to differences in allele sizes and to the presence of extra alleles or allele dropout (absence) in oil DNA. It seems likely that differences in allele sizes or numbers may be due to the lower quality of DNA extracted from olive oil as compared to leaves, while extra alleles are

probably due to accidental contamination with other oils present in the milling process. Since the pollinator contributes to the genetic constitution of seeds, the presence of unexpected alleles may also be caused by the seed oil that is often liberated during the crushing process (Agrimonti et al. 2011).

AFLPs have proven to be a reliable and reproducible means for olive genotyping. Therefore, they were among the first molecular markers tested in fingerprinting assays of olive oil. Busconi et al. (2003) reported that DNA extracted from monovarietal oil may be used for AFLP analysis, yielding banding profiles of high correspondence with the profiles obtained from DNA extracted from leaves of the respective cultivars. When the DNA extracted from oil is of adequate quality and free of inhibitors of restriction enzymes and DNA polymerase, a high correspondence between oil and plant AFLP markers (approximately 70 %) may be achieved (Pafundo et al. 2005). AFLP profiles obtained from the oil of single Italian olive varieties (monovarietal olive oil) were highly reproducible with all primer pairs tested and capable of detecting the varietal origin of the olive oil, although the bands were rather less intense than those obtained with the DNA extracted from leaves of the corresponding cultivars (Montemurro et al. 2008). To overcome difficulties encountered with degraded or contaminated oil DNA in AFLP technology, AFLP profiles of olive oil have been utilized in the development of sequence characterized amplified region (SCAR) markers. These markers have been used to determine the varietal composition of oil samples. Starting from AFLP profiles, Pafundo et al. (2007) developed SCARs to identify monovarietal olive oils derived from olive cultivars from throughout the Mediterranean basin. A single SCAR marker (CP-rpl16T) was able to divide 59 varieties into four groups; however, a marker alone is not discriminating enough to identify all varieties. Nevertheless, SCARs constitute a very promising marker system for identification and authenticity assays of olive oils, providing several advantages over AFLPs and SSRs, including simplicity, reproducibility, and easy scoring through ethidium bromide staining of agarose gels. This methodology could also be applied to multiplexed real-time PCR assays and microarray platforms. High-throughput technologies with next-generation sequencing platforms could be one of the best discriminating methods for authentication purposes. Once again, it should be stressed that the availability of decent quality DNA obtained from oils and the clarity of information obtained from mills or companies concerning olive oil products per se are important.

5.4 Molecular Biology of Olive Oil Biosynthesis

Olive oil has a well-balanced fatty acid (FA) composition, ideal for human consumption. It is characterized by low amounts of α-linolenic acid (ALA) (approximately 1 %) relative to its major unsaturated FAs, i.e., oleic acid (60–80 %) and linoleic acid (10–20 %). Over the last decade, significant progress has been made on the isolation and characterization of key genes/enzymes in FA modification and TAG storage in olives. Nevertheless, the molecular basis of lipid biosynthesis in

olive fruits has not been explored adequately. Most of our knowledge on the respective metabolic pathways comes from the model plant Arabidopsis or other oilseed crops. Briefly, FA biosynthesis mainly occurs in plastids, where acetyl-CoA carboxylase (ACCase) catalyzes the conversion of acetyl-CoA into malonyl-CoA. The pathway proceeds with condensation between acetyl-CoA and malonyl-ACP by β-ketoacyl-ACP synthase (KAS) III to form a 4-carbon unit called acetoacetyl-ACP, which is further reduced and dehydrated, resulting in the production of Δ2-trans-enoyl-ACP. This unsaturated intermediate molecule is then reduced again by enoyl-ACP reductase (*ENR*) to produce butyryl-ACP. Butyryl-ACP in turn reacts with malonyl-ACP in a second condensation reaction that is catalyzed by KAS I. The process is sequentially repeated by adding two carbons in every circle, until the growing chain reaches 16 carbons, i.e., palmitoyl-ACP. Further elongation of palmitoyl-ACP to produce stearoyl-ACP (C18) is catalyzed by KAS II (Harwood 1996) (Chap. 4).

Those FAs may be further modified, elongated, or desaturated. In vegetable oils, the level of desaturation is related to the C16/C18 FA ratio initially produced since the palmitate is the prevalent saturated FA (C16:0), while the C18 FAs are almost totally unsaturated. Stearoyl-ACP Δ9-desaturase is a very active enzyme in plastids and catalyzes the desaturation of stearic acid (C18:0) to produce the monounsaturated oleic acid (C18:1), the major FA in olives. TAGs are quantitatively the most significant storage lipids and the major component of olive oil. In both yeast and plants, TAGs may be produced via the acyl-CoA–independent transfer of FAs from phospholipids to the sn-3 position of diacylglycerol (DAG), catalyzed by the enzyme phospholipid: diacylglycerol acyltransferase (PDAT). Principally, however, it involves the sequential incorporation of FAs onto the glycerol backbone, a process commonly known as the glycerol-3-phosphate or Kennedy pathway. The first step in the process involves the acylation by GP acyltransferase (GPAT) of glycerol-3-phosphate (GP) at the sn-1 position to produce lysophosphatidic acid (LPA). LPA is further acylated at the sn-2 position by LPA acyltransferase (LPAT), resulting in the formation of the central metabolite phosphatidic acid (PA). The synthesis of PA is known to occur both in plastids and the endoplasmic reticulum (ER) via the so-called prokaryotic and eukaryotic pathways, respectively. Within the plastids, PAs may be further converted into glycerolipids or sulfolipids, whereas in the ER they are converted into phospholipids. In storage tissues, like maturing seeds, PA is predominantly dephosphorylated to produce DAG, which is further acylated by diacylglycerol acyltransferase (DGAT) to produce TAG. Subsequently, FAs incorporated into the glycerolipids are further modified by FA desaturases (FADs) localized either in the plastids or the ER membranes. The monounsaturated oleic acid may be further modified by the membrane-bound ω-6 (Δ12) FADs to produce linoleic acid (C18:2). Dienoic fatty acids may be converted into trienoic fatty acids, like ALA (C18:3), by the plastidial or the ER-localized *ω-3 FAD* (Chap. 4).

The ER and the plastidial counterpart enzymes are encoded by distinct genes, which generally produce highly similar polypeptides. However, the main enzymes involved in the last step of TAG formation are encoded by two sequence-unrelated genes: *DGAT1* (type 1) and *DGAT2* (type 2). The deduced DGAT polypeptides

encoded by the two genes are highly unrelated. In seeds of both olive and oilseed crops, TAGs accumulate within discrete subcellular particles called oil bodies or oleosomes (Murphy 1993; Hsieh and Huang 2004). Oil bodies are surrounded by a monolayer of phospholipids, in which structural proteins, principally oleosins, are embedded. However, oil droplets in the fleshy olive mesocarp are devoid of oleosins.

Olive fruit is a typical drupe consisting of an exocarp, a fleshy mesocarp, and a stony endocarp that surrounds the seed. In olive fruit, TAGs are formed and stored both in the mesocarp and the seed, two drupe compartments of distinct origin, physiological functions, and roles that also display differences in the mode of TAG accumulation. Storage TAGs in seeds (endosperm and embryo) provide energy-rich molecules to the embryo during the initial stages of germination, whereas in the mesocarp they are believed to attract animals for seed dissemination. Lignification of the endocarp takes place at approximately 11 weeks after flowering (WAF) and marks the launch of massive TAG synthesis and deposition in both the seed and the mesocarp. TAG accumulation in the mesocarp and seeds follows a typical sigmoid curve (Sánchez 1994). Accumulation of oil in seeds is relatively fast compared to the mesocarp, reaching a plateau at approximately 22 WAF. This could also be attributed to seed desiccation and senescence, vital biological processes controlled by different plant hormones such as abscisic acid (ABA) and ethylene, crucial for the survival of the embryo and species propagation. In the mesocarp, a significant proportion of oil starts to accumulate at around 16–19 WAF, depending on the environmental conditions and the cultivar, and oil biogenesis reaches a plateau during ripening (change in color) at approximately 28 WAF. Oil biosynthesis proceeds till very late in ripened drupes. The time of initial accumulation till the end of oil biosynthesis and accumulation depends on the cultivar and the environment (Sánchez and Harwood 2002; Alagna et al. 2009; Matteucci et al. 2011). The major genes implicated in fatty acid biosynthesis, modification, and TAG accumulation have been isolated, and their spatiotemporal expression during fruit development has been studied (Hatzopoulos et al. 2002 and references therein; Banilas et al. 2005, 2007, 2011; Poghosyan et al. 1999, 2005; Hernández et al. 2005; Giannoulia et al. 2007; Matteucci et al. 2011).

5.4.1 Fatty Acid Synthesis

The conversion of Δ2-trans-enoyl-ACP into butyryl-ACP by the enzyme enoyl-ACP reductase (*ENR*) completes the first sequence of reactions in FA synthesis. Since this enzymatic activity represents a distinct step in the integral cycle begun by KAS III and a subunit of a multicomplex enzyme, the study of a single component may give important insights into the regulation of the whole system at the gene-expression level. In order to study these early stages of FA synthesis in olive, Poghosyan et al. (2005) isolated a cDNA corresponding to a plastidial *ENR* gene from an olive drupe cDNA library, and its transcriptional regulation was monitored

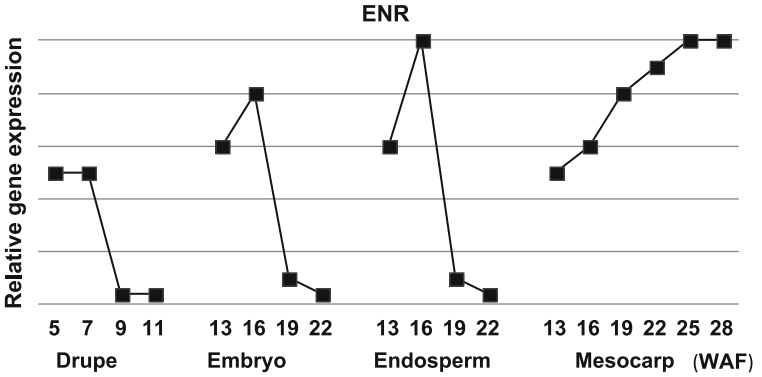

Fig. 5.1 Initial steps in FA synthesis. Relative expression of olive enoyl-ACP reductase (*ENR*) gene during fruit growth and in different drupe compartments, based on data presented previously (Poghosyan et al. 2005). Values are in arbitrary units in relative proportion to highest gene expression signal recorded

in young drupes (5 WAF) and during fruit development till ripening (28 WAF). The *ENR* gene was upregulated at very early stages of drupe development, when the embryo is at the globular (5 WAF) or heart (7 WAF) stage. Thereafter, transcripts started to decline, reaching down to almost undetectable levels at 11 WAF, at the time of endocarp lignification. Following that stage, differences in the pattern of transcript accumulation between mesocarp and seed tissues were observed. In seeds, *ENR* gene expression was relatively high at the early-torpedo-stage embryo, and the corresponding endosperm (13 WAF), peaked at 16 WAF (early to mid-torpedo embryos), and thereafter sharply declined to almost undetectable levels during the seed maturation and desiccation phases. In contrast, in the mesocarp, *ENR* transcripts gradually and constantly increased from 13 to 19 WAF and presumably remained high till 28 WAF (Fig. 5.1). It is likely that the relatively high levels of *ENR* transcripts in young drupes (5–7 WAF) are correlated with high demand for de novo lipid synthesis to furnish thylakoid membrane biogenesis in highly proliferating cells at those stages. In contrast, the sharp upregulation of *ENR* in the mesocarp at 19 WAF, or in seed at 16 WAF, is to provide FAs for lipid deposition. This was also supported by a similar pattern of stearoyl-ACP Δ9-desaturase transcript accumulation (Haralampidis et al. 1998), the enzyme that catalyzes the production of oleic acid (Fig. 5.2), and by lipid accumulation data (Sánchez 1995).

5.4.2 Fatty Acid Desaturation

The stearoyl-ACP Δ9-desaturase (*Δ9-des*) has a central role in the FA desaturation process, catalyzing the desaturation of stearic acid to produce the monounsaturated oleic acid, the most abundant FA in olive oil. The *Δ9-des* gene has been isolated and

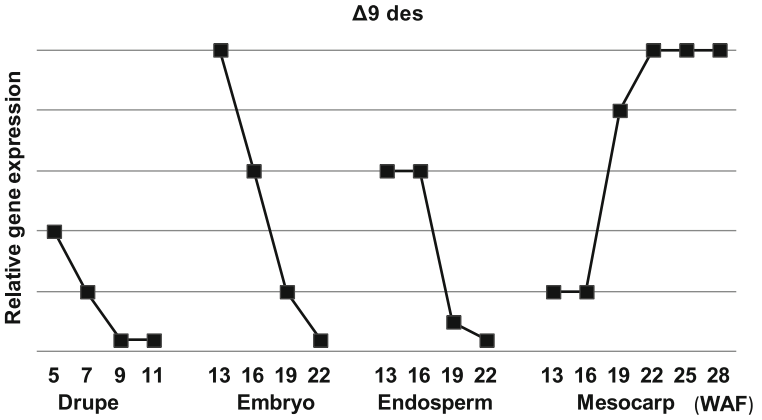

Fig. 5.2 Production of oleic acid through relative expression of olive stearoyl-ACP Δ9 desaturase gene during fruit development and in embryos, endosperms, and mesocarps, based on data presented previously (Haralampidis et al. 1998). Values are in arbitrary units in relative proportion to highest signal recorded

its expression characteristics during olive drupe development assessed (Haralampidis et al. 1998). At the early stages of drupe development (5 WAF) the gene is highly expressed, preferentially in embryos, but transcripts sharply decline soon after 7 WAF to almost undetectable levels at 9 or 11 WAF. *ENR* and *Δ9-des* genes share a similar transcript profile at those early stages of drupe growth, indicating an orchestrated regulation of lipid synthesis and initial steps of desaturation. Following endocarp lignification (11 WAF), when separation of the major oil accumulating drupe tissues from each other without injury becomes possible, embryo, endosperm, and mesocarp tissues were studied individually. This allowed Haralampidis et al. (1998) to detect that the expression of *Δ9-des* in embryo tissues at 13 WAF was at extreme high levels, while as the embryo developed further through the early-mid torpedo (16 WAF), mid-torpedo (19 WAF) toward the late torpedo stage (22 WAF), the transcript accumulation declined gradually to almost undetectable levels. As opposed to seed tissues, at the early stages of mesocarp growth (13–16 WAF), the respective expression was relatively low (Fig. 5.2). Those results suggest that the diminishing transcripts encountered at 9–11 WAF in intact drupes could be attributed rather to the high mesocarp/seed RNA ratio at those stages. Although a similar profile of transcript accumulation was detected in endosperms, the ratio of *Δ9-des* mRNA to total RNA was lower compared to embryos, and accumulation remained at high levels from 13 to 16 WAF. Thereafter, mRNA accumulation declined gradually to undetectable levels, as observed in embryos. Interestingly, the pattern of *Δ9-des* mRNA accumulation in mesocarp was quite distinct and showed expression for a longer time compared to seed tissues. The low level of transcription observed from 13 to 16 WAF was substituted by rapid mRNA levels that peaked at 19–22 WAF, coinciding with the time of massive oil accumulation in mesocarps (Sánchez

1994). This mRNA accumulation remained at high rates thereafter through fruit maturity (28 WAF). Transcript accumulation of *ENR* had a similar pattern in mesocarps or seed tissues to that observed for *Δ9-des* showing a coordinating type of developmentally regulated gene expression. Therefore, both the time and the level of *Δ9-des* mRNA accumulation in different compartments of the olive drupe during development coincide well with FA production and oil accumulation in the respective tissues. Haralampidis et al. (1998) also showed that ABA may regulate *Δ9-des* gene expression since exogenous application of ABA increased transcript levels in embryos but not in the respective endosperms when the exogenous application was for a short time period. However, prolonged exposure of immature embryos to ABA reduced transcript accumulation, suggesting a dose effect of ABA on the transient and temporal regulation of the *Δ9-des* gene. The initial low ABA content during embryo development could induce *Δ9-des* gene expression in embryos, while higher ABA content in the later stages of embryo growth, which signals the desiccation process, suppresses the *Δ9-des* gene, as was observed in in vitro cultivated embryos.

Oleic acid is incorporated into glycerolipids, either in plastids or in ER membranes, before being further modified by membrane-bound ω-6 (Δ12) desaturases, the enzymes responsible for the conversion of oleic acid into linoleic acid. There are two genes in Arabidopsis, *FAD2* and *FAD6*, that encode for the ER (microsomal) and the plastidial ω-6 desaturases, respectively. The two desaturases have different electron donor systems, i.e., NAD(P)H, ferredoxin: NAD(P) reductase, and ferredoxin for *FAD6*, and NADH, NADH:cytochrome b5 reductase, and cytochrome b5 for *FAD2*. By using degenerate primers two respective genes encoding *OeFAD2* and *OeFAD6* were isolated from the olive cultivar Koroneiki (Banilas et al. 2005), while Hernández et al. (2005) identified two different olive *FAD2* genes, designated *OepFAD2-1* and *OepFAD2-2*, in the genome of the Picual cultivar. Transcriptional analysis of the ER *OeFAD2* gene revealed that it is constitutively regulated in very young drupes (5–11 WAF) and in endosperm and embryo throughout seed development, reflecting its major role in desaturation for the biosynthesis of ER membrane lipids. Relatively higher transcript levels were detected in mesocarp as compared to seed tissues during fruit growth (13–28 WAF). Given that strong transcript upregulation of stearoyl-ACP Δ9-desaturase starts at 16 WAF, the relatively high transcript levels of *OeFAD2* in the mesocarps between 13 and 16 WAF could be attributed to the high demand for membrane biosynthesis rather than to the storage lipid desaturation. Supporting this, a high level of *OepFAD2-1* gene expression was detected in leaves, whereas *OepFAD2-2* was mostly expressed in the mature seed and the mesocarp, particularly at 31 WAF (Hernández et al. 2005). Besides its critical role in the production of linoleic acid in olive oil, the constitutive type of *OeFAD2* expression during the later stages of mesocarp growth (22–28 WAF) supports the notion that membrane biosynthesis/turnover is still required during that period, in which some mesocarpic cells are still photosynthetically active.

Similar to *OepFAD2-2*, but unlike *OepFAD2-1* and *OeFAD2*, *OeFAD6* is upregulated both in endosperms and embryos during fruit growth, showing a similar pattern of accumulation (Fig. 5.3). Although the expression is scarcely detectable at 13 WAF, upregulation starts at 16 WAF and gradually increases up to 22 WAF.

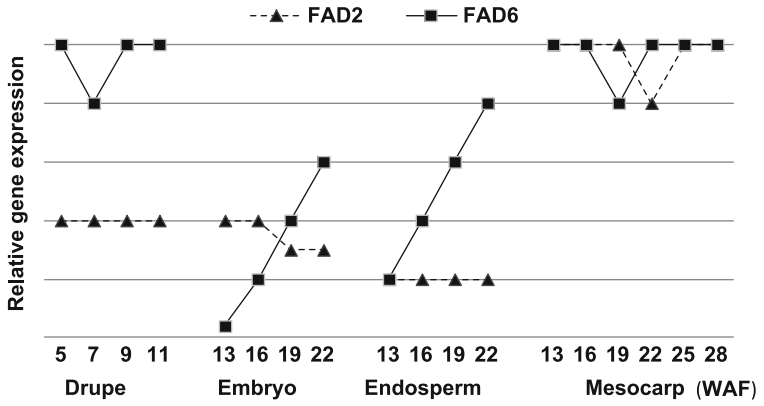

Fig. 5.3 Mid-stages of desaturation. Relative expression patterns of olive *FAD2* and *FAD6* genes during fruit growth and during embryo development, and endosperm and mesocarp growth, extracted from data presented previously (Banilas et al. 2005). Values are in arbitrary units with respect to highest signal detected for each gene individually. Thus, only expression patterns of individual genes may be compared and not their relative values to each other

These results together with the downregulation of *Δ9-des* in seed tissues from 13 up to 22 WAF show that the process of lipid poly-unsaturation, within the plastids of embryo or endosperm tissues does not parallel the process of oleic acid production. Nevertheless, the higher constitutive expression in mesocarps compared to seed tissues, of the plastidial *OeFAD6* from the very early developmental stages through 28 WAF is linked to the biosynthesis of photosynthetic membranous systems in the green drupes and mesocarps besides the modification of accumulated FAs. Developing olives contain active chloroplasts until approximately 28 WAF and olive fruit photosynthesis contributes to the carbon economy of developing olives and hence to oil biosynthesis (Sánchez 1995; Sánchez and Harwood 2002). The relatively higher level of *OeFAD6* mRNA accumulation in the endosperm at 22 WAF was comparable to the expression levels detected in mesocarps. Those results point to higher demand for linoleic acid production in endosperm rather than in embryo at the late stages of seed maturation, highlighting the implication of poly-unsaturated FAs in lipid catabolism during germination and seedling development. This notion is supported by the fact that a linoleate 13-lipoxygenase purified from oil bodies of olive endosperms can mainly oxygenate linoleic acid (Georgalaki et al. 1998). Even though the relative expression of *OeFAD2* and *OeFAD6* is at similar levels in mesocarps, showing that both are implicated in linoleic acid content, it has been indicated (Hernández et al. 2009) that *OepFAD2-2* seems to be the main gene responsible for the linoleic acid content in olive fruit mesocarp tissue.

OeFAD2 transcript accumulation in young drupes of Koroneiki cv. was lower as compared to mesocarp at later stages of drupe growth, while relatively high levels of *OeFAD6* expression was detected in young drupes and late mesocarpic tissues, suggesting that polyunsaturated FAs at the early stages of mesocarp development

(5–11 WAF) result mainly from *OeFAD6* activity (Fig. 5.3). Detailed analysis of Arabidopsis mutants have contributed to the unraveling of reversible exchange of FAs between plastidial and ER membranes. Polyunsaturated FAs produced by *FAD6* enzyme activity may be exported and subsequently incorporated into ER lipids and TAGs (Miquel and Browse 1998; Stoutjesdijk et al. 2002). The exocarp tissue surrounding the mesocarp consists of cells with a large number of photosynthetically active chloroplasts. The high level of *OeFAD6* transcript accumulation in young drupes could also be attributed to the presence of this high number of chloroplasts. Correa et al. (2002) showed that the olive exocarp is rich in lipids, specifically at the early stages of fruit growth, while the polyunsaturated linoleic acid accounts for a relatively high proportion (12 %) of the total FAs (Milosevic et al. 2002). Accumulated data show that developmental or hormonal signals could upregulate the expression of *FAD6* in embryos and endosperms, while these signals are unlikely to regulate the constitutive *FAD2* expression in a similar manner. The ER and the plastidial ω-6 desaturases were also abundantly expressed in other vegetative tissues and high transcript levels were detected in actively developing organs, like expanding leaves and shoot tips, most likely to furnish the requirement of polyunsaturated FAs in membrane biogenesis. By applying in situ hybridization, a strong signal for both genes was detected in olive reproductive tissues, which are not associated with membrane or storage lipid biosynthesis. This reflects the crucial role of the FA polyunsaturation pathway to meet the demands for flower development such as the production of linoleic acid as precursor to jasmonic acid (JA) and oxylipin biosynthesis for proper pollen development (McConn and Browse 1996; McConn et al. 1997). Recent results showed that low temperature affected the expression of oleate desaturase genes in a cultivar-specific manner. High temperature and dark decreased the expression levels of olive *OeFAD2* and *OeFAD6*. The expression of the *OeFAD2* gene was affected in response to wounding of olive fruit mesocarp (Hernández et al. 2011), implicating its potential role in jasmonate biosynthesis, a key regulatory molecule in plant defense against wounding or insects.

The ω-3 FA desaturases (FADs) are responsible for further desaturation of dienoic FAs to trienoic FAs, like ALA. Those enzymes are localized either in the chloroplast envelope membranes or the ER. Although olive oil is characterized by low amounts of ALA, its precise concentration influences its organoleptic properties and shapes the quality. Detailed analysis of Arabidopsis mutants contributed considerably to the understanding of the regulation of ω-3 desaturase function. The plastidial enzyme (*FAD7*) can catalyze either Cl6 or the Cl8 ω-3 trienoic fatty acids from their respective dienoic acid precursors, whereas its counterpart, ER-bound (*FAD3*), converts the Cl8 linoleic acid into ALA (Miquel and Browse 1998).

The plastidial (*OeFAD7*) and the ER-bound (*OeFAD3*) *ω-3 FAD* genes were isolated and characterized in order to determine the mechanisms that regulate and influence the ALA content in olive oil (Poghosyan et al. 1999; Banilas et al. 2007). Northern blot hybridization analysis showed that the *OeFAD7* gene is highly expressed in very young drupes (5 WAF), while transcript accumulation declines during further fruit growth till approximately 11 WAF, the stage at which lignification of the endocarp is apparent. The *OeFAD3* gene was also highly expressed in very

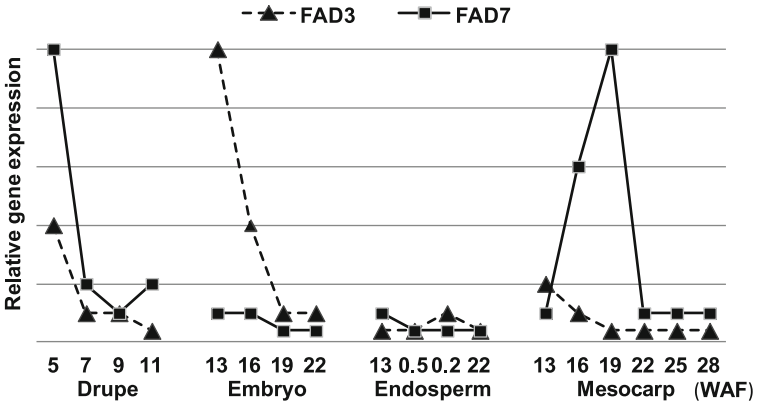

Fig. 5.4 Last stages of desaturation in olive oil. Relative expression patterns of olive *FAD3* and *FAD7* genes during fruit development and in different drupe compartments, gathered from data presented previously (Poghosyan et al. 1999; Banilas et al. 2007). Values are in arbitrary units with respect to highest signal detected for each gene individually. Thus, only expression patterns of individual genes may be compared and not their relative values to each other

young drupes and declined during further drupe growth, in a similar pattern to that observed for *OeFAD7*. At 13 WAF, only the ER *OeFAD3* gene was highly expressed in embryos (early torpedo stage). Thereafter, transcripts declined till embryo desiccation (22 WAF). However, the expression of *OeFAD3* in endosperms was steadily low from as early as 13 WAF till 22 WAF. The respective transcripts for *OeFAD7* were very low in both embryo and endosperm throughout seed development. The high levels of both gene transcripts in very young drupes could reflect the high demands in lipid biosynthesis for membrane biosynthesis in the drupe (embryo-endosperm-mesocarp). The decline of both gene transcripts thereafter and the high levels of *OeFAD3* transcripts in early to mid-torpedo stages indicate the crucial role of this gene in the biosynthesis of ALA for membrane biogenesis during the development of embryos via the ER-bound enzyme. These results are consistent with accumulating data suggesting that the ER-type desaturase is correlated with active membrane biogenesis in different organs where intensive cell division occurs (Matsuda et al. 2001; Banilas et al. 2007). These expression characteristics are in accordance with the low ALA content in olive seeds (Hernández et al. 2005). The pattern of expression of the ω-3 desaturase genes in young drupes resembles the one detected for other olive genes involved in lipid biosynthesis, such as *CYTb5*, *ENR*, or *Δ9-des* but very different from that of the ω-6 desaturase genes (Haralampidis et al. 1998; Martsinkovskaya et al. 1999; Poghosyan et al. 1999, 2005).

During mesocarp growth (13–28 WAF), the expression of *OeFAD7* followed a bell-shaped pattern, showing a peak at 19 WAF and drastically declining thereafter. In contrast, the *OeFAD3* gene was expressed at very low levels in mesocarps throughout development (Fig. 5.4). The results show that there was a high demand for *FAD7* activity transiently and at a narrow time window of mesocarp growth, particularly

between 16 and 22 WAF, when the drupes were still green and photosynthetically active (Poghosyan et al. 1999; Banilas et al. 2007). It is unknown whether such a demand is linked to specific physiological changes during fruit growth leading into maturation and ripening or whether the rapid and transient increase in transcript accumulation is a response to hormonal signal optima. Both ER *FAD2* and *FAD3* genes had an almost constitutive type of expression in both mesocarps and endosperms (constituting most mass in drupes); the relative proportion of dienoic and trienoic acids in both tissues are expected to be similar. This was corroborated by data showing a similar proportion of trienoic acids in both seed and mesocarp tissues. Since *FAD3* had a similar pattern of mRNA levels when compared to *Δ9-des* in embryos, it is anticipated that *FAD3* may also be regulated transiently by ABA. Irrespective of the signal(s) inducing the expression of ω-3 desaturase genes, *FAD7* showed a complex transcript accumulation opposite to that of *FAD3* in embryos and mesocarps, indicating that the genes are differentially regulated.

Besides the important contribution of ω-3 desaturases in the modification of storage lipids, they also have crucial roles in plant development. To elucidate such roles for the *OeFAD3* and *OeFAD7* genes, their spatiotemporal regulation was also studied in various vegetative or reproductive tissues of the olive tree (Poghosyan et al. 1999; Banilas et al. 2007). The ER-type *OeFAD3* was profoundly expressed in mitotically active vegetative tissues, such as young expanding leaves and shoot tips, whereas the respective expression of the plastidial type *OeFAD7* was rather low. In situ hybridization analysis showed that the sites of *OeFAD3* expression overlap perfectly with those of a mitotic-specific gene, an olive B-type cyclin, which has a prominent cell-cycle regulation of expression (Dewitte and Murray 2003). Therefore, it is suspected that in these cells, the demand of lipids for membrane biogenesis is furnished by *OeFAD3*. The sites of the plastidial-type expression were rather restricted in the apical meristem cells, the procambium, and the young leaf primordial. These results imply that *OeFAD7* expression furnishes thylakoid membrane lipid demands in those tissues/organs during the extensive proplastidial divisions in meristematically active cells such as leaf primordial, among others. The same type of analysis also showed that the ER-type *OeFAD3*, unlike *OeFAD7*, is highly expressed in pistils of floral buds close to anthesis, especially in vascular bundles and ovules, pointing to a role of the ER-type desaturase in ALA production in flowers. It is reasonable to accept that ALA serves as a precursor for JA biosynthesis (Creelman and Mullet 1995; Matsuda et al. 2001). The α-linolenate precursor for JA biosynthesis in Arabidopsis is particularly important for anther and pollen development. Arabidopsis *fad3 fad7 fad8* mutant containing negligible levels of trienoic fatty acids showed impaired anther development leading to male sterility, and no seeds were produced under normal conditions (McConn and Browse 1996). Vegetative growth and development were unaffected. Based on the accumulated data from the preceding results, from JA-deficient mutants in tomato (Li et al. 2001, 2004) and from JA-biosynthetic enzymes in flower organs, we assume a crucial role of *OeFAD3* in the development of reproductive tissues of olive, particularly the male gametophyte (Banilas et al. 2007).

5.4.3 Accumulation and Storage of Triacylglycerols

Olive oil is primarily composed of TAGs but also includes other quantitatively minor constituents like polyphenols, chlorophyll, tocopherols, and sterols. FA composition of TAGs in vegetable oils is strongly influenced by the GPAT, LPAT, and DGAT enzymes, three major classes of acyltransferases in the Kennedy pathway (Sánchez and Harwood 2002). Each enzyme's abundance and activity in a given stage of growth, along with substrate specificities and the available pool of acyl-CoAs, greatly influence the quality of the final product. Even though GPAT enzymes from different plant species usually prefer palmitoyl-CoA or oleoyl-CoA, in olives linoleic acid is preferentially found at the sn-1 position of TAGs (Santinelli et al. 1992). This is possibly due to the relatively high and constitutive expression of *OeFAD2* and *OeFAD6* genes in mesocarp, from the very early stages of growth till maturation (Banilas et al. 2005; Matteucci et al. 2011). *LPAT* has high enzyme specificity for unsaturated acyl-CoAs substrates, such as oleoyl-CoA or linoleoyl-CoA. Hence, saturated FAs are excluded from the sn-2 position (Sánchez and Harwood 2002), while in olive oil, oleic acid is the major FA at the sn-2 position.

DGAT enzyme activities catalyze the acylation of diacylglycerol at the sn-3 position, the last and most critical step in TAG biosynthesis of the Kennedy pathway (Ohlrogge and Browse 1995), a step marking the onset of lipid deposition and storage. This enzyme has broad substrate specificity. Therefore, the relative abundance of FA species in the acyl-CoA pool greatly influences FA composition at the sn-3 position (Harwood 1997). However, TAGs may also be produced via the transfer of acyl groups from phospholipids to diacylglycerols, an acyl-CoA-independent reaction catalyzed by PDAT (Dahlqvist et al. 2000; Stahl et al. 2004; Zhang et al. 2009).

In Arabidopsis there are at least three different *LPAT* genes. *AtLPAT1* encodes the plastid isoform essential for embryo development, and *AtLPAT2* encodes a ubiquitously distributed ER-localized enzyme (Kim and Huang 2004). The third gene, *AtLPAT3*, is predominantly expressed in pollen. Two other putative *LPAT* genes have been isolated from Arabidopsis (Kim et al. 2005). Recently, a cDNA has been isolated from olive mesocarp that shows strong homology to the *LPAT2* from Arabidopsis and *Brassica napus*, suggesting that this gene is probably an ER-type class of *LPAT*s (Hatzopoulos, unpublished data). Expression analyses showed that the olive *LPAT* gene was detected in various organs/tissues, and it is predominantly expressed in expanding leaves and flower buds. It is also highly expressed in mesocarps at the time of *Δ9-des* upregulation (19–22 WAF). However, later on its expression declines rapidly, while *Δ9-des* upregulation, and thus oleic acid production, remains high till fruit maturation (28 WAF) (Fig. 5.5). Those results suggest that most of the oleic acid incorporation at the sn-2 position of TAGs should take place at 19–22 WAF, while oleoyl-CoA produced at the later stages of mesocarp growth-maturation should also be incorporated at the sn-3 position by DGAT activity. It also implies that by 28 WAF most DAGs have been synthesized de novo by the Kennedy pathway in the olive drupe.

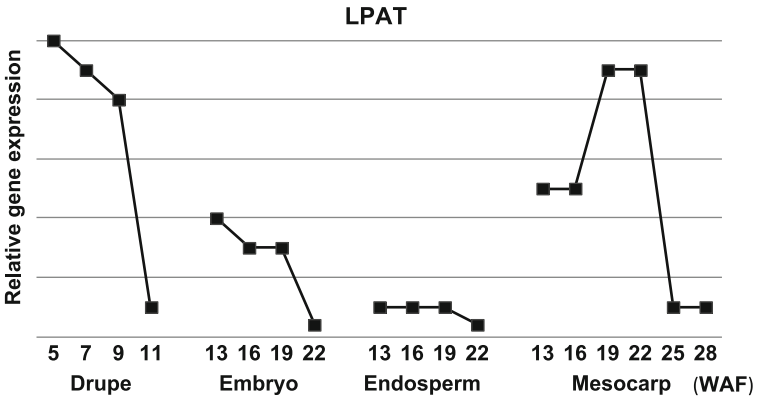

Fig. 5.5 Relative expression of olive LPAT gene during fruit growth and in different drupe compartments, based on unpublished data of Hatzopoulos et al. (2002). Values are in arbitrary units in relative proportion to highest signal detected

DGAT1 and *DGAT2* represent the main types of genes encoding DGAT activities, both of which are ER-localized (Cases et al. 1998; Lardizabal et al. 2001). They have very low sequence similarity to each other, even though they possess similar enzymatic properties. Several type 1 *DGAT* genes have been isolated and characterized from various plant species. However, the physiological functions of type 2 *DGAT*s have been much less determined as of yet (Shockey et al. 2006). Research so far on oilseed crops that contain unusual FAs shows a prominent role of *DGAT2* in developing seeds of epoxy and hydroxy FA accumulating plants rather than in soybeans or Arabidopsis (Li et al. 2010). Both *DGAT1* and *DGAT2* genes have been isolated from olive (Koroneiki cv.) and their organ- and tissue-specific expression patterns investigated (Giannoulia et al. 2000; Banilas et al. 2011). Results suggest that *DGAT1* and *DGAT2* are developmentally regulated and share an overall overlapping but distinct transcription pattern in various tissues during vegetative growth. *DGAT1* gene expression was relatively high during seed development, both in embryo and endosperm tissues, while the respective transcript levels of *DGAT2* were negligible (Fig. 5.6). Both genes were expressed at low levels in the early stages of drupe growth, 5–11 WAF. Olive is among a few exceptions of commercially important oil-producing crops characterized by the production of its oil in mesocarpic cells before the onset of ripening, when the drupe is still photosynthetically competent. As mentioned earlier, oil accumulation during drupe growth follows a typical sigmoid curve (Sánchez 1994). The major proportion of oil accumulation starts at approximately 16–19 WAF and reaches a plateau around 28 WAF; thereafter oil accumulation is still active. Olive *DGAT* presumably exerts significant flux control when the rate of TAG biosynthesis is high (Ramli et al. 2005). Transcriptional analysis suggests that both *DGAT*s are implicated in TAG accumulation at the early stages (16–19 WAF) of mesocarp growth. However, and most importantly for oil accumulation, a more prominent role of *DGAT2* was apparent at

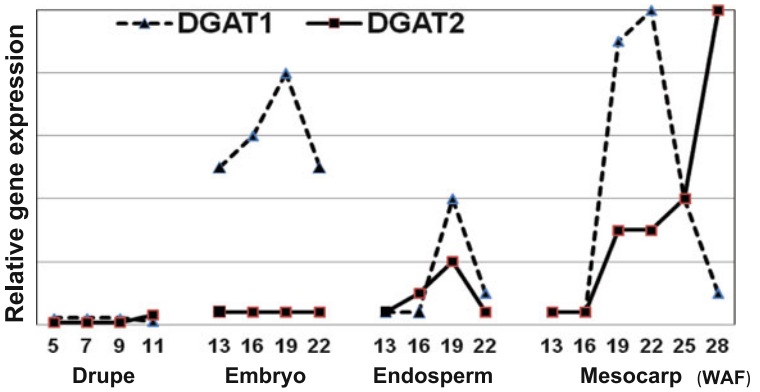

Fig. 5.6 Accumulation of TAGs monitored by relative expression patterns of olive *DGAT1* and *DGAT2* genes. During early fruit development expression is minimal, but nevertheless, the genes are highly expressed during embryo development and endosperm and mesocarp growth as determined by data presented previously (Banilas et al. 2011). Values are in arbitrary units with respect to highest signal detected for each gene individually. Thus, only expression patterns of individual genes may be compared and not their relative values to each other

the time of the onset of ripening. While both *DGAT1* and *DGAT2* genes are upregulated at 19–22 WAF, thereafter *DGAT1* expression declines, whereas *DGAT2* mRNA accumulates further till 28 WAF, following a sigmoid-type expression pattern in mesocarps (Fig. 5.6). It is not yet clear how the upregulation of *DGAT2* at the later stages of olive fruit maturation might determine FA composition or deposition of TAGs. It is well known that the increase in oil droplet size in olive mesocarpic cells is facilitated by the coalescence of smaller lipid droplets. During the later ripening drupe stages the cellular milieu starts to be depleted of water and steadily increases toward a lipidic (hydrophobic) environment. The enzymatic stability of the *DGAT2* and, most importantly, its substrate specificity and activity should be assessed in this peculiar cellular environment. However, it has also been proposed that during olive ripening oil biosynthesis may continue, together with an increase in dry matter, albeit at a slower rate than in previous phases (Conde et al. 2008). Thus, it is suspected that olive *DGAT2* may have a role in lipid droplet enlargement or TAG synthesis in mesocarpic cells during olive maturation. Matteucci et al. (2011) proposed an interesting alternative scenario, in which the olive *DGAT2* may be active within senescing chloroplasts, as *DGAT* is in Arabidopsis chloroplasts, where it plays a role by sequestering FAs de-esterified from galactolipids into TAGs. Next, plastoglobuli exuded from chloroplasts and chromoplasts might provide oil droplets with TAGs in the cytoplasm at mesocarp maturation stages. In agreement with this, Banilas et al. (2011) showed that *DGAT2*, but not *DGAT1*, is strongly expressed in senescing organs, such as cotyledons, mature leaves, and flowers. Irrespective of the FA composition of TAGs, total *DGAT* gene transcription activity remains high from

19 WAF till maturity, pointing to the fact that the most important step in modulating enzymatic activity in TAG synthesis is at the transcriptional level.

The maturation process at the latest stages of development of the olive drupe has a limited effect on the FA profile of olive oil, in which the linoleic acid (C18:2) increases slightly (Gutiérrez et al. 1999; Ayton et al. 2001). This can be explained by the fact that both the Δ-9 des gene, the enzyme for oleic acid (C18:1), and ω-6 desaturase genes, the enzymes converting the former into linoleic acid, are expressed highly through 28 WAF in mesocarp (Haralampidis et al. 1998; Banilas et al. 2005). The contents of the other major fatty acids, palmitoleic acid (C16:1), stearic acid (C18:0), and linolenic acid (C18:3), remain relatively stable and low during the normal commercial harvesting period.

Even though we cannot compare the band intensities (mRNA levels) detected on different northerns or semiquantitative RT-PCRs using respective probes/primers of a particular gene, the signal detected using *Δ9-des* probe or primers was the most intense. We imagine that the vast accumulation of *Δ9-des* mRNAs is the key factor in the high levels of oleic acid in olive oil. However, mRNA levels do not always agree with enzyme activity. This should be further justified by measuring the respective enzyme activity.

TAGs in olives, as in other oleogenic seed crops, accumulate in embryo- and endosperm-located subcellular spherical entities called oilbodies or oleosomes (Murphy 1993; Hsieh and Huang 2004). Oilbodies are surrounded by a monolayer of phospholipids in which structural proteins are embedded, most of them termed oleosins. They completely cover the surface of the oleosomes. Oleosins are small (15–26 kDa) basic proteins characterized by a unique, highly conserved, and uncommonly long central hydrophobic domain. This central domain contains a relatively hydrophilic proline knot motif that plays a crucial role in targeting the protein into oilbodies and giving the structural conformation and folding of this domain (Abell et al. 1997). Based on a highly conserved sequence similarity of oleosins, oligonucleotide primers were designed and used in RACE-PCR reactions to amplify an olive oleosin cDNA, referred to as *OeOLE* (Giannoulia et al. 2007). Like other oleosins isolated from different plant species, *OeOLE* exhibits a very long central hydrophobic domain, surrounded by N- and C-terminal amphipathic domains. Trancriptional analysis revealed that the *OeOLE* is spatiotemporally expressed in seed tissues but not in mesocarpic cells. Embryos and endosperms share a similar bell-shaped expression profile during seed development. Transcription starts in young drupes at 11 WAF; nevertheless, this signal derives from the embryo or endosperm tissues. Upregulation of transcription starts at 13 WAF, peaks at 16–19 WAF in both seed tissues, and thereafter transcript accumulation decreases gradually (Fig. 5.7). This shows that the oleosin gene is under a strictly regulated expression in olive seed tissues. These results strongly suggest that oleosin protein synthesis is regulated at the transcriptional level, coinciding with oil accumulation in seed tissues. Interestingly, the onset of oleosin gene expression coincides well with the upregulation of *ENR*, *Δ9-des*, and *DGAT1*, supporting the notion of an orchestrated type of FA synthesis, modification, and storage in seed tissues regulated at the transcriptional level. Even though lipid biosynthesis, modification, and

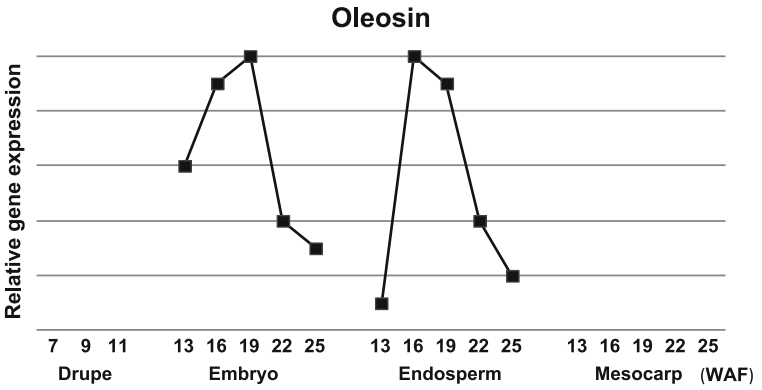

Fig. 5.7 Storage of TAGs in oil bodies. Relative expression of olive oleosin gene during embryo and endosperm development is apparent but absent in young drupe and mesocarpic tissues, as determined by data presented previously (Giannoulia et al. 2007). Values are in arbitrary units in relative proportion to highest signal recorded

deposition in olive fruits follow a similar pathway, mesocarp tissues do not contain detectable oleosins, indicating that the accumulated TAGs in oilbodies or oil droplets serve different purposes.

5.5 Molecular Biology of Olive Oil Aroma

The quality of olive oil is greatly dependent on its flavor and aroma compounds (Chaps. 8 and 9). It is well known that different cultivars yield oils of significantly different aroma profiles due to differences in their genomes. However, the effect of geography, geology, and climate of the growing region, collectively known as the terroir effect, may also affect the array of aroma compounds and eventually shape the typical character of olive oil (Chap. 12). Those environmental variables may alter oil organoleptic properties due to changes in gene expression. Therefore, it is important to study the molecular basis of olive oil aroma biosynthesis in combination with the repertoire of terroir.

The most important volatile compounds in olive oil are the C6 unsaturated aldehydes, both quantitatively and qualitatively, for shaping the aroma profile of VOO (Aparicio et al. 1996; Morales et al. 1999; Salas et al. 2000; Angerosa et al. 2000; Sánchez and Harwood 2002), particularly through adding "green notes" to its sensory characteristics (Olías et al. 1993; Morales et al. 1996: Aparicio and Morales 1998) (Chap. 8). These volatile compounds are produced through the lipoxygenase (*LOX*) pathway (Morales et al. 1996; Salas et al. 2000) (Chaps. 4 and 8). This metabolic pathway involves a series of enzymatic degradations of the polyunsaturated fatty acids (PUFAs) linoleic and ALAs into C6 or C9 carbonyl fragments, which

may be further modified to produce an array of aroma compounds. The aroma profile in a given olive oil is thus strongly influenced by the activity and characteristics of the key genes/enzymes in the *LOX* pathway. These enzymes include *LOXs*, which catalyze the dioxygenation of PUFAs into FA hydroperoxides, and hydroperoxide lyase (*HPL*), which catalyzes the cleavage of FA hydroperoxides to produce volatile aldehydes and oxoacids. It is important to mention that the enzymatic actions in the *LOX* pathway are initiated in the presence of oxygen, after the olives are crushed during the oil extraction process (Morales et al. 1999) (Chap. 8).

Recently, Palmieri-Thiers et al. (2009) isolated an olive *LOX* gene from mesocarp RNA of 30 WAF ripen Leccino olives. It was classified as a type 1 *LOX* (*LOX*1), due to the absence of chloroplast transit peptides, and was shown to have dual 9-*LOX* and 13-LOX positional specificities toward linoleic acid. Both gene expression and activity were induced at the veraison (when the drupe started to change color from green to purple) stage (approximately 22 WAF) and reached maximum levels at the black stage (30 WAF). Padilla et al. (2009) isolated two different olive *LOXs* by utilizing RNA from Picual cv. mesocarp at 13 or 28 WAF. Both enzymes were classified as type 2 *LOXs* (chloroplastic) and exhibited 13-LOX activity, as do all members of the type 2 subfamily (Feussner and Wasternack 2002). *LOX2-1* gene expression was highest in young drupes and leaves, while during fruit development till ripening it was rather constitutively expressed. In contrast, *LOX2-2* was upregulated at approximately 24 WAF, both in Picual and Arbequina cvs., reaching maximal levels of expression at 28 WAF. Based on these results, it is plausible to suggest a prominent role of the olive *LOX2-2* in the production of VOO aroma compounds.

Padilla et al. (2010) also isolated and characterized an *HPL* gene from olive. In plants there are three *HPL* classes: the 13- or 9-HPLs, which cleave exclusively either the 13- or the 9-hydroperoxides, and the 9/13-HPLs that cleave both 9- and 13-hydroperoxides (Mita et al. 2005; Matsui 2006). The recombinant olive *HPL* was specific for 13-hydroperoxide derivatives of α-linolenic and linoleic acids. Transcriptional analysis showed that the gene was highly expressed in ripening Picual mesocarp (28 WAF), suggesting a possible implication of this gene in the shaping of the aroma profile of olive oils.

5.6 Large-Scale Transcriptomics of Olive Fruit

Recently, steps toward understanding gene networks controlling major metabolic pathways during olive fruit development and ripening have been initiated. Next-generation sequencing technology (454 pyrosequencing) was carried out to define the transcriptome of olive drupes. The aim was the identification of expressed sequence tags (ESTs) involved in phenolic and lipid metabolism during fruit development (Alagna et al. 2009). Four cDNA libraries from epi-mesocarp tissues from two olive cultivars (Coratina and Tendellone) were constructed. Two developmental stages were analyzed and compared: completed fruit sets at approximately 6–7

WAF and mesocarp at approximately 19 WAF, the veraison stage. Coratina is characterized by high oleuropein (a main terpene secoiridoid) content, while Tendellone is an oleuropein-lacking natural variant. A total of 22,904 clusters of ESTs were produced and assembled into tentative consensus sequences (TCS). Approximately 13,000 TCS showed significant similarities to proteins. The majority of the enzyme-related uniquenes encode for transferases (3,982), followed by hydrolases (2,628) and oxidoreductases (1,895), while approximately 1,000 TCS were found to be involved in lipid biosynthesis (mainly in the biosynthesis of steroids, glycerolipid, or FA metabolism) and 761 TCS in the biosynthesis of secondary metabolites (mainly in alkaloid, phenylpropanoid, terpenoid, or flavonoid biosynthesis). However, it should be noted that the same enzymatic function may be redundantly encoded by several unigenes, while different TCS may cover non-matching fragments of the same gene (Alagna et al. 2009). In both genotypes, the majority of transcripts expressed at higher levels at the early stage of fruit development as compared to the veraison stage correspond to genes that are involved in photosynthesis or steroid synthesis or that encode for structural proteins. In contrast, most transcripts that are upregulated at the veraison stage include genes engaged in lipid and vitamin biosynthesis and carbohydrate metabolism. Unfortunately, our current knowledge of polyphenol or triterpenoid metabolism and oleuropein biosynthesis is very limited (Galla et al. 2009).

Another study used suppression subtractive hybridization (SSH) libraries from pericarp mRNA of Leccino fruits at three developmental stages: approximately 4 WAF (fruit set), 13 WAF (completed pit hardening), and 19 WAF (veraison) (Galla et al. 2009). This resulted in a great contribution to the available olive ESTs, which currently total approximately 4,000 sequences. By using the Blast2GO software (Conesa et al. 2005; Aparicio et al. 2006), a tool for functional annotation of (novel) sequences and the analysis of annotation data, annotation of ESTs was accomplished according to terms of the Gene Ontology (GO) project (Genome Group of the Gene Ontology Consortium 2009). A total of 1,132 genes, of which 642 were unique sequences, were differentially expressed during olive fruit development. Among them, it is worth mentioning the upregulation during early fruit development of genes involved in starch and sucrose metabolism, the pentose phosphate pathway, glycolysis, gluconeogenesis, or the synthesis of pyruvate. Genes encoding key enzymes in the synthesis of short chain FAs were upregulated at the early fruit development, while gene expression levels of precursors of palmitoleic or oleic acid synthesis increased later in development, which agreed with previous results (Haralampidis et al. 1998; Poghosyan et al. 2005). Interestingly, a number of genes involved in FA metabolism were significantly upregulated at the veraison stage. Key genes controlling flavones, flavonol, or anthocyanin biosynthesis were also upregulated following the completion of pit hardening, suggesting an increased accumulation of related metabolites late in fruit development. Two key genes in ABA synthesis were upregulated throughout fruit development, while genes involved in auxin biosynthesis and metabolism were downregulated during the course of development. Interestingly, following veraison, genes involved in jasmonate metabolism and brassinosteroid biosynthesis were up- and downregulated, respectively, suggesting possible roles of these newer hormone classes in the olive maturation process (Galla et al. 2009).

5.7 Future Trends and Challenges

Worldwide olive oil demand has witnessed a continual increase in the past decade (García-González and Aparicio 2010). To meet current needs, oleoculture is currently expanding to new regions, like Latin America, South Africa, Australia, and China. As cultivation expands to diverse environments and given the ongoing climate changes, like global warming, further emphasis should be placed on stress-tolerant and insect-/disease-resistant genotypes using genome selection approaches. In this respect, the magnitude of genetic variability, as uncovered through extensive genetic analysis over the last decades, may constitute promising plant material for exploration and exploitation in breeding programs. However, the respective studies are incomplete in that they are mostly restricted to accessions from germplasm collections or to major cultivars. Further exploration, description, and agronomic evaluation of olive germplasm are needed, including more accessions and minor or underutilized domestic varieties and oleasters (genuine wild olives). Molecular biology tools used in agricultural biotechnology, such as marker-aided genetic analysis and marker-assisted selection, will help to evaluate in great detail the extent of existing genetic diversity. In addition, they will help to identify genes and other loci or developmental circuits, important parameters in exploring elite traits and necessary functions, and, thus, to explain phenotypic plasticity. This knowledge is fundamental for direct improvement or selection strategies. There is a remarkable genetic variation that could be utilized through nontransgenic breeding methods if methodologies are developed for selecting superior genotypes more rapidly and efficiently, thereby improving the decision-making process. For purposes of conservation and sustainable use, it is important to determine more accurately the extent of existing genetic diversity in cultivated and wild germplasms, to estimate intravarietal diversity, and to better depict genotype characteristics. The developing next-generation DNA technologies, like SNP genotyping platforms, are quite promising for deeper characterization of existing germplasms that will ameliorate the detection of genotype-phenotype associations and the development of QTL markers. Functional genome sequence analysis, coupled with genetics, is needed to better elucidate the molecular mechanisms of olive tolerance to abiotic and biotic stresses and to characterize genes or molecular mechanisms and key regulatory components involved in important biological and agronomical traits.

In view of the lack of knowledge about the detailed control of olive oil synthesis, we propose the sequencing of DNA from two distinct varieties that have high commercial value, one used for table consumption and the other for oil production. Both should be cultivated widely throughout the Mediterranean basin and have excellent characteristics and high quality. Such reference genomes are required in identifying key regulatory DNA elements, present or absent in different cultivars, providing the necessary means to understand the regulation of valuable constituents of oil synthesis and characteristics of the exceptional quality of olive oil. Similarly, in grapes two different genomes of a single highly used cultivar (Pinot Noir) were chosen for sequencing a red wine grape variety, which confer that variety's organoleptic

characters and flavors to both Burgundy and Champagne wines (Jaillon et al. 2007; Velasco et al. 2007).

Further functional genomics research may uncover roles for genes likely to be involved in important metabolic pathways and, most importantly, may help to understand how key genes might control quality traits in olive fruits, such as enzymes catalyzing bioactive or antioxidant molecule biosynthesis. It is interesting to note that additional "omics" strategies are required in order to acquire a clear picture of the perplexing events leading to drupe growth and development. Genechip and Illumina sequencing approaches will verify the transcriptional regulation of most of the genes induced/suppressed during this process. Proteomics will define the level of protein/enzyme availability at certain developmental stages. The necessity of having different tissues, e.g., mesocarp versus seed tissues, may be instrumental in shedding light on the complexity of the proteome. This will also determine whether regulation occurs at the transcriptional or at the translational level or at both. Finally, the functionality of proteins/enzymes should be determined using metabolomics. It is anticipated that such holistic and multidiscipline approaches may require transnational collaborations. Since such approaches are within the purview of the scientists working in the field of olive biochemistry and molecular biology, and many of these technologies are at present relatively low cost, it is of immediate importance for the future sustainability of olive agriculture to set up such international collaboration networks.

References

Abell BM, Holbrook LA, Abenes M, Murphy DJ, Hills MJ et al (1997) Role of the proline knot motif in oleosin endoplasmic reticulum topology and oil body targeting. Plant Cell 9:1481–1493

Agrimonti C, Vietina M, Pafundo S, Marmiroli N (2011) The use of food genomics to ensure the traceability of olive oil. Trends Food Sci Tech 22:237–244

Alagna F, D'Agostino N, Torchia L, Servili M, Rao R et al (2009) Comparative 454 pyrosequencing of transcripts from two olive genotypes during fruit development. BMC Genomics 10:399

Alba V, Sabetta W, Blanco A, Pasqualone A, Montemurro C (2009) Microsatellite marker to identify specific alleles in DNA extracted from monovarietal virgin olive oils. Europ Food Res Technol 229:375–382

Albertini E, Torricelli R, Bitocchi E, Raggi L, Marconi G et al (2011) Structure of genetic diversity in *Olea europaea* L. cultivars from Central Italy. Mol Breed 27:533–547

Angerosa F, Mostallino R, Basti C, Vito R (2000) Virgin olive oil odour notes: their relationships with the volatile compound from the lipoxygenase pathway and secoiridoid compounds. Food Chem 68:283–287

Angiolillo A, Mencuccini M, Baldoni L (1999) Olive (*Olea europaea* L.) genetic diversity assessed by amplified fragment length polymorphisms. Theor Appl Genet 98:411–421

Aparicio R, Morales MT (1998) Characterization of olive ripeness by green aroma compounds of virgin olive oil. J Agric Food Chem 46:1116–1122

Aparicio R, Morales MT, Alonso MV (1996) Relationship between volatile compounds and sensory attributes by statistical sensory wheel. J Am Oil Chem Soc 73:1253–1264

Aparicio G, Gotz S, Conesa A, Segrelles D, Blanquer I et al (2006) Blast2GO goes grid: developing a grid-enabled prototype for functional genomics analysis. Stud Health Technol Inform 120:194–204

Ayed RB, Grati-Kamoun N, Moreau F, Rebai A (2009) Comparative study of microsatellite profiles of DNA from oil and leaves of two Tunisian olive cultivars. Europ Food Res Technol 229:757–762

Ayton J, Mailer RJ, Robards K (2001) Changes in oil content and composition of developing olives in a selection of Australian cultivars. Aust J Exp Agric 41:815–821

Baldoni L, Cultrera N, Mariotti R, Ricciolini C, Arcioni S et al (2009) A consensus list of microsatellite markers for olive genotyping. Mol Breed 24:213–231

Banilas G, Minas J, Gregoriou C, Demoliou C, Kourti A et al (2003) Genetic diversity among accessions of an ancient olive variety of Cyprus. Genome 46:370–376

Banilas G, Moressis A, Nikoloudakis N, Hatzopoulos P (2005) Spatial and temporal expressions of two distinct oleate desaturases from olive (*Olea europaea* L.). Plant Sci 168:547–555

Banilas G, Nikiforiadis A, Makariti I, Moressis A, Hatzopoulos P (2007) Discrete roles of a microsomal linoleate desaturase gene in olive identified by spatiotemporal transcriptional analysis. Tree Physiol 27:481–490

Banilas G, Korkas E, Kaldis P, Hatzopoulos P (2009) Olive and grapevine biodiversity in Greece and Cyprus. In: Lichtfouse E (ed) Sustainable agriculture reviews, vol 2. Springer, Berlin/Heidelberg, pp 401–428

Banilas G, Karampelias M, Makariti I, Kourti A, Hatzopoulos P (2011) The olive DGAT2 gene is developmentally regulated and shares overlapping but distinct expression patterns with DGAT1. J Exp Bot 62:521–532

Bartolini G, Prevost G, Messeri C, Carignani G, Menini UG (1998) Olive germplasm. Cultivars and world-wide collections. FAO, Rome

Belaj A, Satovic Z, Cipriani G, Baldoni L, Testolin R et al (2003) Comparative study of the discriminating capacity of RAPD, AFLP and SSR markers and of their effectiveness in establishing genetic relationships in olive. Theor Appl Genet 107:736–744

Belaj A, Satovic Z, Trujillo I, Rallo L (2004) Genetic relationships of Spanish olive cultivars using RAPD markers. Hort Sci 39:916–1156

Besnard G, Baradat PH, Breton C, Khadari B, Bervillé A (2001a) Olive domestication from structure of oleasters cultivars using nuclear RAPDs and mitochondrial RFLPs. Genet Sel Evol 33:251–268

Besnard G, Baradat P, Chevalier D, Tagmount A, Bervillé A (2001b) Genetic differentiation in the olive complex (*Olea europaea* L.) revealed by RAPDs and RFLPs in the rRNA genes. Genet Res Crop Evol 48:165–182

Besnard G, Khadari B, Baradat P, Bervillé A (2002a) *Olea europaea* (Oleaceae) phylogeography based on chloroplast DNA polymorphism. Theor Appl Genet 104:1353–1361

Besnard G, Khadari B, Baradat P, Bervillé A (2002b) Combination of chloroplast and mitochondrial DNA polymorphisms to study cytoplasm genetic differentiation in the olive (*Olea europaea* L.) complex. Theor Appl Genet 105:139–144

Bracci T, Busconi M, Fogher C, Sebastiani L (2011) Molecular studies in olive (*Olea europaea* L.): overview on DNA markers applications and recent advances in genome analysis. Plant Cell Rep 30:449–462

Breton C, Claux D, Metton I, Skorski G, Berville A (2004) Comparative study of methods for DNA preparation from olive oil samples to identify cultivars SSR alleles in commercial oil samples: possible forensic applications. J Agric Food Chem 52:531–537

Breviglieri N, Battaglia E (1954) Ricerche cariologiche in *Olea europaea* L. Caryologia 6:271–283

Bronzini de Caraffa V, Maury J, Gambotti C, Breton C, Bervillé A et al (2002) Mitochondrial DNA variation and RAPD mark oleasters olive feral olive from Western and Eastern Mediterranean. Theor Appl Genet 104:1209–1216

Busconi M, Foroni C, Corradi M, Bongiorni C, Cattapan F et al (2003) DNA extraction from olive oil and its use in the identification of the production cultivar. Food Chem 83:127–134

Carriero F, Fontanazza G, Cellini F, Giorio G (2002) Identification of simple sequence repeats (SSRs) in olive (*Olea europaea* L.). Theor Appl Genet 104:301–307

Cases S, Smith SJ, Zheng YW, Myers HM, Lear SR et al (1998) Identification of a gene encoding an acyl CoA: diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. Proc Natl Acad Sci USA 95:13018–13023

Cipriani G, Marrazzo MT, Marconi R, Cimato A, Testolin R (2002) Microsatellite markers isolated in olive (*Olea europaea* L.) are suitable for individual fingerprinting and reveal polymorphism within ancient cultivars. Theor Appl Genet 104:223–228

Conde C, Delrot S, Geros H (2008) Physiological, biochemical and molecular changes occurring during olive development and ripening. J Plant Physiol 165:1545–1562

Conesa A, Götz S, Garcìa-Gòmez JM, Terol J, Talòn M, Robles M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21:3674–3676

Correa MJP, Rodrıquez-Garcia MI, Arnal AO (2002) Caracterizacion histoquimica de la etapa temprana del desarrollo del fruto del olivo (*Olea europaea* L.). Acta Bot Bras 16:77–82

Creelman RA, Mullet JE (1995) Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. Proc Natl Acad Sci USA 92:4114–4119

Dahlqvist A, Stahl U, Lenman M, Banas A, Lee M, Sandager L, Ronne H, Stymne S (2000) Phospholipid: diacylglycerol Acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. Proc Natl Acad Sci USA 97:6487–6492

De la Rosa C, James M, Tobutt KR (2002) Isolation and characterization of polymorphic microsatellites in olive (*Olea europaea* L.) and their transferability to other genera in the Oleaceae. Mol Ecol Notes 2:265–267

Dewitte W, Murray JA (2003) The plant cell cycle. Annu Rev Plant Biol 54:235–264

European Communities (EC) (1992) Official journal of the commission of the European communities. Regulation No. 2081/92, L 208, 14 July 1992

European Communities (EC) (2006) Official journal of the commission of the European communities. Regulation No. 510/06, L93/12, 31 March 2006

Fabbri A, Hormaza JI, Polito VS (1995) Random amplified polymorphic DNA analysis of olive *(Olea europaea* L.) cultivars. J Am Soc Hort Sci 120:538–542

Feussner I, Wasternack C (2002) The lipoxygenase pathway. Annu Rev Plant Biol 53:275–297

Galla G, Barcaccia G, Ramina A, Collani S, Alagna F, Baldoni L, Cultrera NG, Martinelli F, Sebastiani L, Tonutti P (2009) Computational annotation of genes differentially expressed along olive fruit development. BMC Plant Biol 9:128

García-González DL, Aparicio R (2010) Research in olive oil: challenges for the near future. J Agric Food Chem 58:12569–12577

Gemas VJ, Rijo-Johansen MJ, Tenreiro R, Fevreiro P (2000) Inter- and intra-varietal analysis of three *Olea europeaea* L. cultivars using the RAPD technique. J Hortic Sci Biotech 75:312–319

Genome Group of the Gene Ontology Consortium (2009) The gene ontology's reference genome project: A unified framework for functional annotation across species. PLoS Comput Biol 5(7): e1000431

Georgalaki MD, Bachmann A, Sotiroudis TG, Xenakis A, Porzel A et al (1998) Characterization of a 13-lipoxygenase from virgin olive oil and oil bodies of olive endosperms. Fett/Lipid 100:554–560

Giannoulia K, Haralampidis K, Poghosyan Z, Murphy DJ, Hatzopoulos P (2000) Differential expression of diacylglycerol acyltransferase (DGAT) genes in olive tissues. Trans Biochem Soc 28:695–697

Giannoulia K, Banilas G, Hatzopoulos P (2007) Oleosin gene expression in olive. J Plant Physiol 164:104–107

Green PS (2002) A revision of Olea L. Kew Bull 57:91–140

Gutiérrez F, Jimenez B, Ruíz A, Albi MA (1999) Effect of olive ripeness on the oxidative stability of virgin olive oil extracted from the varieties Picual and Hojiblanca and on the different components involved. J Agric Food Chem 44:121–127

Hakim RI, Grati-Kammoun N, Makhloufi E, Rebai A (2010) Discovery and potential of SNP markers in characterization of Tunisian olive germplasm. Diversity 2:17–27

Haralampidis K, Milioni D, Sánchez J, Baltrusch M, Heinz E et al (1998) Temporal and transient expression of stearoyl-ACP carrier protein desaturase gene during olive fruit development. J Exp Bot 49:1661–1669

Harwood JL (1996) Recent advances in the biosynthesis of plant fatty acids. Biochim Biophys Acta 1301:7–56

Harwood JL (1997) Plant lipid metabolism. In: Dey PM, Harbourne JM (eds) Plant biochemistry. Academic, New York, pp 237–272

Hatzopoulos P, Banilas G, Giannoulia K, Gazis F, Nikoloudakis N et al (2002) Breeding, molecular markers and molecular biology of the olive tree. Eur J Lipid Sci Technol 104:574–586

Hernández ML, Mancha M, Martínez-Rivas JM (2005) Molecular cloning and characterization of genes encoding two microsomal oleate desaturases (FAD2) from olive. Phytochemistry 66:417–1426

Hernández ML, Padilla MN, Mancha M, Martínez-Rivas JM (2009) Expression analysis identifies FAD2-2 as olive oleate desaturase gene mainly responsible for the linoleic acid content in virgin olive oil. J Agric Food Chem 57:6199–6206

Hernández ML, Padilla MN, Sicardo MD, Mancha M, Martínez-Rivas JM (2011) Effect of different environmental stresses on the expression of oleate desaturase genes and fatty acid composition in olive fruit. Phytochemistry 72:178–187

Hsieh K, Huang AHC (2004) Endoplasmic reticulum, oleosins, and oils in seeds and tapetum cells. Plant Physiol 136:3427–3434

Jaillon O, Aury JM, Noel B, Policriti A, Clepet C et al (2007) The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 449:463–467

Katsiotis A, Hagidimitriou M, Douka A, Hatzopoulos P (1998) Genomic organization, sequence interrelationship, and physical localization using in situ hybridization of two tandemly repeated DNA sequences in the genus *Olea*. Genome 41:527–534

Kim HU, Huang AHC (2004) Plastid lysophosphatidyl acyltransferase is essential for embryo development in Arabidopsis. Plant Physiol 134:1206–1216

Kim HU, Li Y, Huang AHC (2005) Ubiquitous and endoplasmic reticulum-located lysophosphatidyl acyltransferase, LPAT2, is essential for female but not male gametophyte development in Arabidopsis. Plant Cell 17:1073–1089

Lardizabal KD, Mai JT, Wagner NW, Wyrick A, Voelker T et al (2001) DGAT2 is a new diacylglycerol acyltransferase gene family: purification, cloning, and expression in insect cells of two polypeptides from *Mortierella ramanniana* with diacylglycerol acyltransferase activity. J Biol Chem 276:38862–38869

Li L, Li C, Howe GA (2001) Genetic analysis of wound signaling in tomato, evidence for a dual role of jasmonic acid in defence and female fertility. Plant Physiol 127:1414–1417

Li L, Zhao Y, McCaig BC, Wingerd BA, Wang J et al (2004) The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defence responses, and glandular trichome development. Plant Cell 16:126–143

Li R, Yu K, Hildebrand DF (2010) DGAT1, DGAT2, and PDAT expression in seeds and other tissues of epoxy and hydroxy fatty acid accumulating plants. Lipids 45:145–157

Liphschitz N, Gophna R, Hartman M, Biger G (1991) The beginning of olive (*Olea europaea*) cultivation in the old world: a reassessment. J Arch Sci 18:441–453

Lumaret R, Ouazzani N (2001) Ancient wild olives in Mediterranean forests. Nature 413:700

Lumaret R, Ouazzani N, Michaud H, Vivier G, Deguilloux MF et al (2004) Allozyme variation of oleaster populations (wild olive tree) (*Olea europaea* L.) in the Mediterranean basin. Heredity 92:343–351

Martins-Lopes P, Gomes S, Santos E, Guedes-Pinto H (2008) DNA markers for Portuguese olive oil fingerprinting. J Agric Food Chem 56:11786–11791

Martsinkovskaya AI, Poghosyan ZP, Haralampidis K, Murphy DJ, Hatzopoulos P (1999) Temporal and spatial gene expression of cytochrome B5 during flower and fruit development in olives. Plant Mol Biol 40:79–90

Matsuda O, Watanabe C, Iba K (2001) Hormonal regulation of tissue-specific ectopic expression of an Arabidopsis endoplasmic reticulum-type ω-3 fatty acid desaturase (FAD3) gene. Planta 213:833–840

Matsui K (2006) Green leaf volatiles: hydroperoxide lyase pathway of oxylipin metabolism. Curr Opin Plant Biol 9:274–280

Matteucci M, D'Angeli S, Errico S, Lamanna R, Perrotta G et al (2011) Cold affects the transcription of fatty acid desaturases and oil quality in the fruit of *Olea europaea* L. genotypes with different cold hardiness. J Exp Bot 62:3403–3420

McConn M, Browse J (1996) The critical requirement for linolenic acid is pollen development, not photosynthesis, in an Arabidopsis mutant. Plant Cell 8:403–416

McConn M, Creelman RA, Bell E, Mullet JE, Browse J (1997) Jasmonate is essential for insect defence in Arabidopsis. Proc Natl Acad Sci USA 94:5473–5477

Mekuria GT, Collins GG, Sedgley M (1999) Genetic variability between different accessions of some common commercial olive cultivars. J Hort Sci Biotech 74:309–314

Milosevic J, Ashton DS, Cocksedge MJ (2002) Lipids present in olive skin. Int J Food Sci Technol 37:523–526

Miquel MF, Browse JA (1998) Arabidopsis lipids: a fat chance. Plant Physiol Biochem 36:187–197

Mita G, Quarta A, Fasano P, De Paolis A, Di Sansebastiano GP et al (2005) Molecular cloning and characterization of an almond 9-hydroperoxide lyase. A new CYP74 targeted to lipid bodies. J Exp Bot 56:2321–2333

Montealegre C, Marina Alegre ML, García-Ruiz C (2010) Traceability markers to the botanical origin in olive oils. J Agric Food Chem 58:28–38

Montemurro C, Pasqualone A, Simeone R, Sabetta W, Blanco A (2008) AFLP molecular markers to identify virgin olive oils from single Italian cultivars. Eur Food Res Technol 226:1439–1444

Morales MT, Aparicio R, Calvente JJ (1996) Influence of olive ripeness on the concentration of green aroma compounds in virgin olive oil. Flavor Fragrance J 11:17–25

Morales MT, Angerosa F, Aparicio R (1999) Effect of the extraction conditions of virgin olive oil on the lipoxygenase cascade: chemical and sensory implications. Grasas Aceites 50:114–121

Muleo R, Colao MC, Miano D, Cirilli M, Intrieri MC et al (2009) Mutation scanning and genotyping by high resolution DNA melting analysis in olive germplasm. Genome 52:252–260

Murphy DJ (1993) Structure, function and biogenesis of storage lipid bodies and oleosins in plants. Prog Lipid Res 32:247–280

Murray MG, Thomson WF (1980) Rapid isolation of high weight plant DNA. Nucl Acids Res 8:4321–4325

Neef R (1990) Introduction, development and environmental implications of olive culture: the evidence from Jordan. In: Bottema S, Entjes-Nieborg G, van Zeist W (eds) Man's role in the shaping of the Eastern Mediterranean Landscape. A.A. Balkema, Rotterdam, pp 295–306

Nikoloudakis N, Banilas G, Gazis F, Metzidakis N, Hatzopoulos P (2003) Discrimination and genetic diversity among cultivated olives of Greece using RAPD markers. J Am Soc Hort Sci 128:741–746

Ohlrogge J, Browse J (1995) Lipid biosynthesis. Plant Cell 7:957–970

Olías JM, Pérez AG, Rios JJ, Sanz C (1993) Aroma of virgin olive oil: biogenesis of the green odor notes. J Agric Food Chem 41:2368–2373

Ouazzani N, Lumaret R, Villemur P, Di Giusto F (1993) Leaf allozyme variation in cultivated and wild olive trees (*Olea europaea* L.). J Hered 84:34–42

Owen CA, Bita EC, Banilas G, Hajjar SE, Sellianakis V et al (2005) AFLP reveals structural details of genetic diversity within cultivated olive germplasm from the Eastern Mediterranean. Theor Appl Genet 110:1169–1176

Padilla MN, Hernández ML, Sanz C, Martinez-Rivas JM (2009) Functional characterization of two 13-lipoxygenase genes from olive fruit in relation to the biosynthesis of volatile compounds of virgin olive oil. J Agric Food Chem 57:9097–9107

Padilla MN, Hernández ML, Pérez AG, Sanz C, Martínez-Rivas JM (2010) Isolation, expression, and characterization of a 13-hydroperoxide lyase gene from olive fruit related to the biosynthesis of the main virgin olive oil aroma compounds. J Agric Food Chem 58:5649–5657

Pafundo S, Agrimonti C, Marmiroli N (2005) Traceability of plant contribution in olive oil by amplified fragment length polymorphisms. J Agric Food Chem 53:6995–7002

Pafundo S, Agrimonti C, Maestri E, Marmiroli N (2007) Applicability of SCAR markers to food genomics: olive oil traceability. J Agric Food Chem 55:6052–6059

Palmieri-Thiers C, Canaan S, Brunini V, Lorenzi V, Tomi F et al (2009) A lipoxygenase with dual positional specificity is expressed in olives (*Olea europaea* L.) during ripening. Biochim Biophys Acta 1791:339–346

Pasqualone A, Montemurro C, Caponio F, Blanco A (2004) Identification of virgin olive oil from different cultivars by analysis of DNA microsatellites. J Agric Food Chem 52:1068–1071

Poghosyan ZP, Haralampidis K, Martsinkovskaya AI, Murphy DJ, Hatzopoulos P (1999) Developmental regulation and spatial expression of a plastidial fatty acid desaturase from *Olea europaea*. Plant Physiol Biochem 37:109–119

Poghosyan ZP, Giannoulia K, Katinakis P, Murphy DJ, Hatzopoulos P (2005) Temporal and transient expression of olive enoyl-ACP reductase gene during flower and fruit development. Plant Physiol Biochem 43:37–44

Ramli US, Salas JJ, Quant PA, Harwood JL (2005) Metabolic control analysis reveals an important role for diacylglycerol acyltransferase in olive but not in oil palm lipid accumulation. FEBS J 272:5764–5770

Reale S, Doveri S, Diaz A, Angiolillo A, Lucentini L et al (2006) SNP-based markers for discriminating olive (*Olea europaea* L.) cultivars. Genome 49:1193–1205

Roubos K, Moustakas M, Aravanopoulos FA (2010) Molecular identification of Greek olive (*Olea europaea*) cultivars based on microsatellite loci. Genet Mol Res 9:1865–1876

Rugini E, De Pace C, Gutiérrez-Pesce P, Muleo R (2011) Olea. In: Kole C (ed) Wild crop relatives: genomic and breeding resources, temperate fruits. Springer, Berlin/Heidelberg, pp 79–117

Salas JJ, Sánchez J, Ramli US, Manaf AM, Williams M et al (2000) Biochemistry of lipid metabolism in olive and other oil fruits. Prog Lipid Res 39:151–180

Sánchez J (1994) Lipid photosynthesis in olive fruit. Prog Lipid Res 33:97–104

Sánchez J (1995) Olive oil biogenesis: contribution of fruit photosynthesis. In: Kader J-C, Mazliak P (eds) Plant lipid metabolism. Kluwer Academic, Dordrecht, pp 564–566

Sánchez J, Harwood JL (2002) Biosynthesis of triacylglycerols and volatiles in olives. Eur J Lipid Sci Technol 104:564–573

Santinelli F, Damiani P, Christie WW (1992) The triacylglycerol structure of olive oil determined by silver ion high-performance liquid chromatography in combination with stereospecific analysis. J Am Oil Chem Soc 75:552–556

Sarri V, Baldoni L, Porceddu A, Cultrera NGM, Contento A et al (2006) Microsatellite markers are suitable tools for discriminating among olive cultivars and assigning them to geographically defined populations. Genome 49:1606–1615

Shockey JM, Gidda SK, Chapital DC, Kuan JC, Dhanoa PK et al (2006) Tung (*Vernicia fordii*) DGAT1 and DGAT2 possess different affinities for eleostearic acid-containing substrates and are localized to different subdomains of the endoplasmic reticulum. Plant Cell 18:2294–2313

Spaniolas S, Bazakos C, Awad M, Kalaitzis P (2008) Exploitation of the chloroplast trnL (UAA) intron polymorphisms for the authentication of plant oils by means of a Lab-on-a-chip capillary electrophoresis system. J Agric Food Chem 16:6886–6891

Stahl U, Carlsson AS, Lenman M, Dahlqvist A, Huang B et al (2004) Cloning and functional characterization of a phospholipid: diacylglycerol acyltransferase from Arabidopsis. Plant Physiol 135:1324–1335

Stoutjesdijk PA, Singh SP, Liu Q, Hurlstone CJ, Waterhouse PA et al (2002) hpRNA-mediated targeting of the Arabidopsis FAD2 gene gives highly efficient and stable silencing. Plant Physiol 129:1723–1731

Terral JF, Alonso N, Capdevila RB, Chatti N, Fabre L et al (2004) Historical biogeography of olive domestication (*Olea europaea* L.) as revealed by geometrical morphometry applied to biological and archaeological material. J Biogeogr 31:63–77

Trujillo I, Arús P, Rallo L (1995) Identifying olive cultivars by isozymes analysis. J Am Soc Hort Sci 120:318–324

Velasco R, Zharkikh A, Troggio M, Cartwright DA, Cestaro A et al (2007) A high quality draft consensus sequence of the genome of a heterozygous grapevine variety. PLoS One 2(12):e1326

Vietina M, Agrimonti C, Bonas U, Marmiroli M, Marmiroli N (2011) Applicability of SSR markers to the traceability of monovarietal olive oils. J Sci Food Agric 8:1381–1391

Wiesman Z, Avidan N, Lavee S, Quebedeaux B (1998) Molecular characterization of common olive varieties in Israel and the West Bank using randomly amplified polymorphic DNA (RAPD) markers. J Am Soc Hort Sci 123:837–841

Zhang M, Fan J, Taylor DC, Ohlrogge JB (2009) DGAT1 and PDAT1 acyltransferases have overlapping functions in Arabidopsis triacylglycerol biosynthesis and are essential for normal pollen and seed development. Plant Cell 21:3885–3901

Zohary D, Hopf M (1994) Olive: *Olea europaea*. In: Domestication of plants in the old world. Clarendon Press, Oxford, UK, pp 137–143

Zohary D, Spiegel-Roy P (1975) Beginnings of fruit growing in the old world. Science 187:319–327

Chapter 6
Chromatographic Methodologies: Compounds for Olive Oil Traceability Issues

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Contents

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties*,
DOI 10.1007/978-1-4614-7777-8_6, © Springer Science+Business Media New York 2013

6.1 Introduction

Olive oil, like other oils and fats, is made up of a high number of compounds, which results in a highly complex matrix. Thus, numerous analytical methods have been proposed to characterize olive oil. Analytical chemists have found, however, a powerful tool in chromatographic techniques, which have been mainly used for the individual quantification of olive oil compounds. In current regulations (IOC 2010a; EC 2011), most of the methods applied to the analysis of olive oil, with the aim of its authentication and characterization, are actually based on liquid or gas chromatographic techniques.

Because the components of olive oil are so many, the number of analytical methods employed is also large. Thus, to organize the analysis of such a heterogeneous group of compounds, in this chapter, the chemical composition of olive oil has been clustered into major and minor compounds when describing them. The group of major compounds is primarily made up of triacylglycerols (TAGs) or glycerol esters of fatty acids (FAs), which are formed by FAs of 16–24 carbon atoms, though only 6 are major compounds: palmitic (6.30–20.93 %), palmitoleic (0.32–3.52 %), stearic (0.32–5.33 %), oleic (55.23–86.64 %), linoleic (2.7–20.24 %), and linolenic (0.11–1.52 %). In fact, fatty acids contribute 94–96 % of the total weight of TAGs and follow a pattern whereby those in the 2-position are unsaturated, with linolenic acid being favored more than oleic and linoleic acids. Twenty TAGs have been identified and independently quantified, but only five are present in significant proportions: OOO (27.53–59.34 %), POO + SOL (12.42–30.57 %), OOL + LnPP (4.14–17.46 %), POL + SLL (2.69–12.31 %), and SOO + OLA (3.17–8.39 %). The wide ranges of TAG and FA contents show the broad variability of the olive oil chemical composition that is largely dependent on cultivar and geographical origin and, to a certain extent, on olive ripeness and extraction system. In the case of unsaturated FAs, the *cis* geometric configuration appears naturally, although the *trans* form occasionally appears as a result of certain manipulations (León-Camacho 1997). Other compounds are partial glycerides, mainly diacylglycerols. An incomplete TAG biosynthesis and hydrolytic reactions in olives are the reasons of the presence of diacylglycerols (DAGs) in the virgin olive oil polar fraction (1–3 %). They are found as 1,2- and 1,3-isomers. 1,2-DAGs are attributed to the incomplete biosynthesis of TAGs (Kennedy pathway), whereas 1,3-DAGs are attributed to TAG hydrolysis (Pérez-Camino et al. 1996); in fact, the amount of 1,3-TAGs is higher in cloudy VOOs. Other compounds are free nonesterified FAs and monoacylglycerols (MAGs) that are present in lower quantities ($\leq$0.25 %), and their major constituents are glycerol oleate, linoleate, and palmitate.

The broad and heterogeneous set of minor compounds includes several series of chemical compounds. Compounds with a lipid structure (e.g., phospholipids, esters of FAs with saturated fatty alcohols of linear chains, known as waxes), compounds that are not related to lipids from a chemical-structure point of view (e.g., pigments, volatiles) (described in Chaps. 7–9), and compounds that have the common

characteristic of being obtained from unsaponifiable matter. By unsaponifiable matter we mean the set of natural or accidental constituents that fail to react with NaOH or KOH to produce soaps while remaining soluble in classic fat solvents (hexane, ether) after saponification. These compounds rarely represent more than 2 % of total olive oil composition but include many substances of a very different nature and chemical structure, up to the point where it is thought to be the olive oil fingerprint. Thus, it is very useful in olive oil authentication, characterization of varietal virgin olive oils (VOOs), identification of olive oil geographical origin, and so forth, as described in Chaps. 12 and 16. The main constituents of this fraction, by increasing order of polarity, are as follows:

1. Hydrocarbons, squalene being the main one (approximately 4,000–5,000 ppm); linear chain saturated hydrocarbons, predominantly those with odd chains; hydrocarbons with terpenic structure; hydrocarbons with steroid structure, formed by the dehydration of certain sterols upon refining and oil manipulation;
2. 4,4-dimethylesterols, or triterpenic alcohols; cycloartenol and 24-methylene-cycloartanol are present at higher concentrations;
3. 4-monomethylsterols; obtusifoliol and gramisterol are the most representative;
4. 4-demethylsterols; β-sitosterol and Δ^5-avenasterol are the main ones;
5. Triterpenic dialcohols; erythrodiol and uvaol are most typical.

Therefore, the methodologies used to analyze these compounds are described in the preceding order. The methods include not only those described in various national and international regulations but those that might be useful to the analyst to further the study and knowledge of such a unique product as olive oil. Tables 6.1 and 6.2 summarize the sample preparation and the main chromatographic characteristics of the most common methods used in the analysis of the aforementioned series of chemical compounds.

6.2 Major Compounds

6.2.1 Fatty Acids

The knowledge of the composition, both in quantity and quality, of olive oil FAs has always been one of the main issues because of its importance in the description and determination of possible olive oil adulterants. The ways of isolating and deriving these compounds and the selection of the most appropriate techniques for their determination have been discussed in many classic books. This chapter will focus on gas chromatographic analyses of these compounds and how they should be prepared for such analyses.

Table 6.1 Most common methods for quantifying acyl lipids and fatty acids

Compounds	Technique	Preparation	Chromatographic characteristics
Triacylglycerols	HPLC-RI	0.12 g oil in 0.5 mL hexane is charged into column (SPE-cartridge: 1 g of Si) and solution pulled through and then eluted with 10 mL hexane-diethylether (87:13v/v)	Mobile-phase flow rate (0.6–1.0 mL/min) Oven temperature: 20 °C Mobile phase: propionitrile Column: RP-18 (4 μm) Detector: RI
	HPLC-RI	0.5 g oil in 10 mL acetone or acetone/chloroform (1:1 v/v)	Mobile phase flow-rate (0.6–1.0 mL/min) Oven temperature: 25 °C Mobile phase: acetone/acetonitrile (1:1 v/v) Column: RP-18 (4 μm) Detector: RI
	GC-FID	Sample solved in hexane or heptane (according injection method)	Column: capillary 30 m × 0.25 mm × 0.10–0.15 μm Phase stationary 65 %phenyl + 35 %methylsiloxane Carrier gas: hydrogen Operation conditions: temperature gradient Injection mode: on-column or split
Fatty acids	GC-FID	Total fatty acids: Methylation with cold methanolic solution of KOH or double Methylation in a methanolic medium alkaline and acid catalysis	Column: capillary 25–100 m × 0.2–0.8 mm × 0.1–0.2 μm Phase: stationary (polyglicol, polyester, or cyanopropylsilicone)
	GC-FID	*Trans* fatty acids: Methylation with cold methanolic solution of KOH	Carrier gas: hydrogen
	GC-FID	Fatty acid in 2-position: Hydrolysis with pancreatic lipase before methylation in a methanolic medium with alkaline and acid catalysis	Operation conditions: temperature gradient Injection mode: split

2-glyceryl monopalmitate (%)	GC-FID	Hydrolysis with pancreatic lipase Separation by GLC or SPE Requires silanization	Column: capillary (12 m × 0.32 mm × 0.10–0.30 μm) Phase: methylpolisiloxane or 5 % phenylmethyl-polysiloxane Carrier gas: hydrogen Operation conditions: temperature gradient Injection mode: on-column
Monoacylglycerol Diacylglycerols	GC-FID	Requires silylation	Column: capillary 15–25 m × 0.25–0.35 mm × 0.1–0.2 μm Phase: SP-2100 or OV-3 Carrier gas: hydrogen Operation conditions: temperature gradient Injection mode: split or on-column
Waxes	GC-FID	Isolation on LC Si-column	Column: capillary 12–15 m × 0.25–0.32 mm × 0.1–0.3 μm Phase: 5 % phenylmethyl-polysiloxane Carrier gas: hydrogen Operation conditions: temperature gradient Injection mode: split or on-column

Note: *GC* gas chromatography, *FID* flame ionization detector, *HPLC* high-performance liquid chromatography, *RI* refractive index, *SPE* solid-phase extraction

Table 6.2 Most common methods for determining minor compounds

Chemical series	Technique	Preparation	Chromatographic characteristics
Sterols[a]	GC-FID	Unsaponifiable-matter isolation TLC or HPLC. Requires silylation	Column: capillary (25–30 m × 0.25–0.32 mm × 0.15–0.30 μm) Phase: 5 % phenylmethylpolysiloxane Carrier gas: hydrogen Operation conditions: isothermal Injection mode: split
Erythrodiol + uvaol	GC-FID	Unsaponifiable-matter isolation TLC or HPLC. Requires silylation	Column: capillary (25–30 m × 0.25–0.32 mm × 0.15–0.30 μm) Phase: 5 % phenylmethylpolysiloxane Carrier gas: hydrogen Operation conditions: isothermal Injection mode: split
Aliphatic and terpenic alcohols	GC-FID	Unsaponifiable-matter isolation TLC or HPLC. Requires silylation	Column: capillary (25–30 m × 0.25–0.32 mm × 0.15–0.30 μm) Phase: 5 % phenylmethylpolysiloxane Carrier gas: hydrogen Operation conditions: temperature gradient Injection mode: split

Aliphatic hydrocarbons and sterenes	GC-FID	Unsaponifiable-matter isolation on LC Si-column	Column: capillary (25–30 m × 0.25–0.32 mm × 0.15–0.30 μm) Phase: 5 % phenylmethylpolysiloxane Carrier gas: hydrogen Operation conditions: temperature gradient Injection mode: split
Tocopherols	GC-FID	Extraction of unsaponifiable-matter fractionation by TLC. Requires silylation	Column: capillary (25–30 m × 0.25–0.32 mm × 0.15–0.30 μm) Phase: 5 % phenylmethylpolysiloxane Carrier gas: hydrogen Operation conditions: isothermal Injection mode: split
	HPLC-FD (Si column)	0.10 g oil in mL n-hexane	Mobile phase flow rate: 1.0 mL/min Oven temperature: 20 °C Mobile phase: n-hexane/isopropanol (99:1 v/v) Detector: λ_{emi}: 295 nm; λ_{abs}: 330 nm

[a]See Mariani et al. (2006) for the methodology of free and esterified sterols

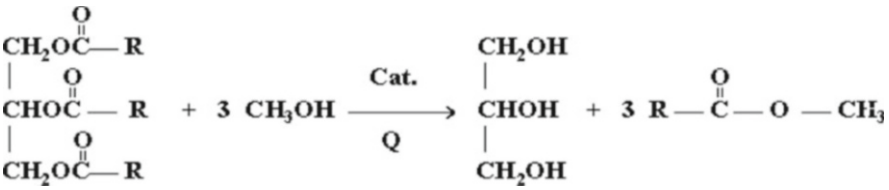
Note: *GC* gas chromatography, *FID* flame ionization detector, *HPLC* high-performance liquid chromatography, *TLC*: thin-layer chromatography

Fatty acids, with a few rare exceptions, are the main components of any oil or fat. Most of the time, however, they are not as free fatty acids (FFAs); when they are, they occur only in small amounts. They usually form esters, most often with glycerol, to produce glycerides (mono-, di-, and triacylglycerols) and phospholipids. They can also form esters with aliphatic alcohols with a linear structure (waxes) or terpenic structure (terpene and sterol esters).

Gas liquid chromatography (GLC) of FFAs is an unusual technique that does not yield good results, and these compounds must be analyzed as volatile derivatives.

6.2.1.1 Derivatization of Fatty Acids

The usual way to derivatize FAs is by forming their methyl esters since they are more volatile and nonpolar than free acids, and this makes them easier to elute through chromatographic columns. The reaction that takes place can be expressed as follows:



This interesterification reaction involves slow kinetics. The presence of water slows down the reaction ratio and diminishes the output (Ulberth and Henninger 1992). The reaction must be accelerated by means of catalysis or by increasing the temperature to be efficient. The most usual methods for preparing the FA methyl esters FAMEs are in methanolic medium with alkaline catalysis, with acid catalysis, with alkaline and acid catalysis, or with diazomethane (IOC 2001a; Christie 2011a).

Methylation in a Methanolic Medium with Alkaline Catalysis

Methylation with sodium methylate is the most widely used method. A solution of sodium methylate in methanol, prepared by direct reaction of metallic sodium with methyl alcohol, is added to the olive oil sample. Reflux heating, for a certain amount of time, will clean and homogenize the solution, dissolving the methyl esters in the reaction medium. They are then extracted by adding a solvent such as hexane or diethyl ether and an aqueous solution of 10 % sodium chloride to separate both phases without their emulsionizing. This method presents some drawbacks. Even though the reaction takes place with high yield, there is always the danger that the sodium salts of FAs (soaps) might form in the alkaline medium. In addition to this, FFAs do not methylate properly when this procedure is used.

To overcome this disadvantage, the fatty matter can be treated with sodium methylate, in a methanol solution inside a closed tube and heated. Because of the increased pressure inside the tube, the temperature rises above the boiling point of methanol up to 85–90 °C. This increases the reaction yield. The heating must take place for a period of no less than 2 h.

Another procedure that yields good results is methylation with potassium hydroxide. It consists of treating a hexane solution of olive oil with a KOH 2N methanol solution, shaking it, and letting it decant. It is rapid and has the advantage of not needing heating, which entails the elimination of the volatilization losses of acids of chains lower than C12 (IUPAC 1987a). This method is widely used to avoid isomerization of unsaturated FAs, but free acids do not derivatize well when this technique is applied; therefore, its use is not advisable for high-acidity olive oils.

Methylation in a Methanolic Medium with Acid Catalysis

Transesterification with methanol can also be catalyzed in an acidic medium. A common procedure consists in making olive oil react with hydrochloric acid solution in methanol in a proportion of 2–3 %. The acid must have been previously dried in a closed tube at 100 °C for about an hour. Also with this procedure, it has been observed that the reaction is not complete. The esters are extracted with hexane before being injected (EC 1991; IOC 2001a).

A variation of the method consists in adding a sulfuric acid solution in methanol and hexane and then subsequently heating by reflux for 90 min. This method increases the output of the reaction thanks to the dehydrating action of the sulfuric acid, thereby shortening the time used in preparing the sample (EC 1991; IOC 2001a).

Lewis's acidic catalysis is another procedure that efficiently uses methylation with methanol that has been catalyzed with boron trifluoride (IUPAC 1987a). The sample is reflux-heated with a methanolic solution of sodium hydroxide until it is homogenized (complete formation of soaps). Next, a methanolic solution of boron trifluoride is added and the heating continues for a few moments. The esters are extracted by adding a saline solution with heptane. The saponification process is not necessary for FAs.

Methylation in a Methanolic Medium with Alkaline and Acid Catalysis

As noted earlier, methylation in an alkaline medium, besides being incomplete, has the disadvantage that olive oil can produce soaps (to saponify), and free acids do not methylate well in an alkaline medium. On the other hand, glycerides do not methylate properly in an acidic medium. Thus, a widespread method to improve the yield of the methylation reaction consists in combining both catalyses.

Usually, the sample undergoes a first alkaline methylation with reflux heating. The medium is then neutralized and then acidified by means of the methanolic solution of sulfuric acid before being reheated by reflux. This process guarantees the

hydrolysis of the soaps that might have formed at the first step and the esterification of the free acids.

Another procedure consists in first treating the sample with a methanolic solution of potassium hydroxide, heated by reflux. Then dimethyl sulfate is added and the solution is shaken and heated again. The reaction is monitored using bromocresol green as an indicator. When the reaction is complete, the indicator changes from blue to yellow. Finally, a methanolic solution of sulfuric acid is added to separate the phases. The extract of the methyl esters can be injected after purification with alumina (EC 1991).

Methylation with Diazomethane

The apparently fast procedure of methylation with diazomethane has been used for free acids (Christie 2011a). The sample, after being solved in methanol, is treated with an ethereal solution of diazomethane, which is prepared at the very moment of the reaction and under a nitrogen stream. The process is fast but requires the advance preparation of the diazomethane from N-methyl-N-nitroso-p-toluene-sulfonamide (Diazal^{M}, Aldrich Chemical, Milwaukee, WI, USA) and 2-(2-ethoxyethoxy) ethanol in an alkaline medium. Diazomethane is toxic and explosive; therefore, some safety measures must be taken.

Formation of Methyl Esters Without Previous Extraction of the Fat

In some cases, the analyst might be in trouble when undertaking the study of the composition of FAs, either because the amount of available oily material is minimal or because of extraction selectivity. Another factor to be considered is the possibility that the olive oil alteration during the study itself can induce the analyst to make serious mistakes in his or her conclusions. Some techniques have been developed to carry out the interesterification of some oily materials without having to extract the fat (Garcés and Mancha 1993). Even though this method can also be useful for olive oil transmethylation, its main advantage lies in the fact that it can be directly applied to fresh tissues containing a considerable amount of water, such as olives, without the need for them to be previously dried as in the aforementioned methods. In this method, both methylation with methanol and acid catalysis are carried out in a dissolving phase. The following reactive mixtures have been successfully used:

1. Methanol:heptane:benzene:2,2-dimethoxypropane:sulfuric acid (37:36:20:5:2, by volume);
2. Methanol:heptane:toluene:2,2-dimethoxypropane:sulfuric acid (39:34:20:5:2, by volume);
3. Methanol:heptane:tetrahydrofurane:2,2-dimethoxypropane:sulfuric acid (31:42:20:5:2, by volume).

The working temperature is 80 °C and the process takes place in a closed tube. Both extraction and transmethylation are carried out in a single phase because the working temperature is the same for both. After the reaction takes place, the sample is cooled at room temperature and two different phases occur. The organic (upper) phase containing the methyl esters is now ready for chromatographic analysis. This method is extremely interesting and allows for many possibilities, such as system automation when there are many samples to be analyzed.

6.2.1.2 GLC Analysis of Methyl Esters of Fatty Acids

Once FAMEs have been obtained by means of any of the aforementioned methods, gas chromatography (GC) analysis can be undertaken with different aims. The first focus, however, is on a study of the different types of chromatographic columns and an analysis of the results that can be obtained using each of them.

The material used to prepare packed columns is the main factor in the nature of the separations that can be achieved. Liquid phases, such as SE-30, OV-1, JXR, or OF-1, separate FAs by their molecular weight; when the amount in the stationary phase is low, however, FAs can also be separated by their unsaturation degree. Such phases as Apiezón-L separate by unsaturation for the same chain length, with prior elution of the unsaturated components.

The polyester liquid phases are quite appropriate as they allow for separation of esters of the same chain length from 0 to 6 double bonds, eluting the unsaturated components after their corresponding saturated compound. These phases can be classified into three groups:

1. High-polarity phases, such as polymers of ethylene glycol succinate (EGS), diethylene glycol succinate (DEGS), copolymers of the former with a methylsilicone (EGSS-X), CP-Sil 84, and CP-Sil 88;
2. Medium-polarity phases, such as polyethylene glycol adipate, polybutanediol succinate, and EGS copolymers with a high proportion of methylsilicone (EGSS-Y);
3. Low-polarity phases, such as polyneopenthylglycol succinate, EGS with a phenyl-silicone (EGSP-Z), Carbowax 20M, and Silar 5CP.

EGSS-X, EGSS-Y, and newer phases of equivalent polarity are widely accepted as the most useful representatives of the first and second groups respectively because of their relatively high thermal stability, particularly in work with packed columns. The phases from the group of highest polarity are stable at temperatures above those possible with EGSS-X and they run to excellent separations of polyunsaturated FAs. Figure 6.1 shows the analysis using columns of 15 % of EGSS-Y in a 100–120 mesh silanized and acid-washed support.

The low-polarity phases are mainly utilized in wall-coated open tubular (WCOT) and support-coated open tubular (SCOT) capillary columns because the saturated and monoenoic compounds of the same chain length are separated rather poorly

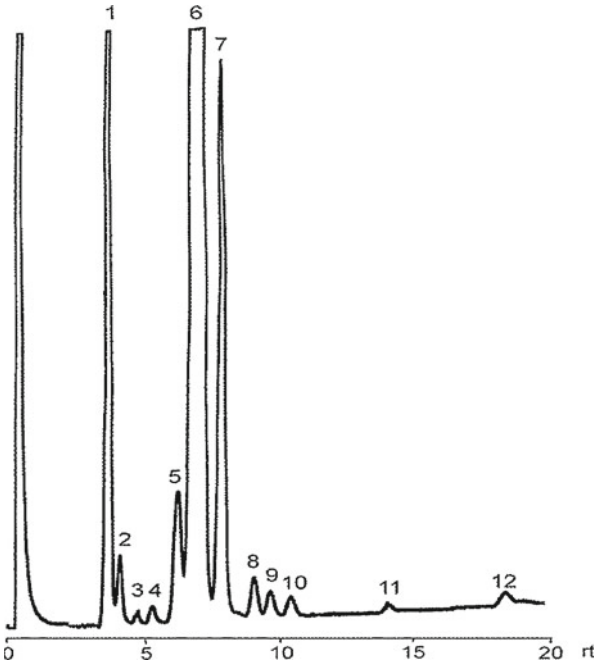


Fig. 6.1 Chromatogram showing fatty acid methyl esters of an olive oil sample. Packed column: 15 % de EGSS-X 100–120 mesh silanyzed and acid-washed support. *1*: palmitic; *2*: palmitoleic; *3*: margaric; *4*: margaroleic; *5*: stearic; *6*: oleic; *7*: linoleic; *8*: α-linolenic; *9*: arachidic; *10*: gadoleic; *11*: behenic; *12*: lignoceric (*Source*: Authors)

when these phases are used in packed columns; at the same time, the quality and nature of the separations are related to the quantity applied to the stationary phase support. For example, low polyester levels are advisable to elute the methylesters of long-chain polyunsaturated FAs at low temperatures. The relative retention time of FA esters (generally 16:0 or 18:0) tends to shorten as the amount of liquid phase in the support decreases as well. Unfortunately, variation of retention times for the esters of FAs is inevitable when the column ages with use as the stationary phase polymerizes further from the column.

The nature of the support also influences the quality of the separations. Silanized and acid-washed materials are inert, and the separations are controlled only by means of the liquid phase. A minute particle size (160–120 mesh) is the best for analytical columns.

Some losses of esters of polyunsaturated acids partly respond to the use of very active supports or aged columns and to the transesterification by polyester liquid phase, which is caused by the remnants of the catalyzer used in the fabrication of the polyester.

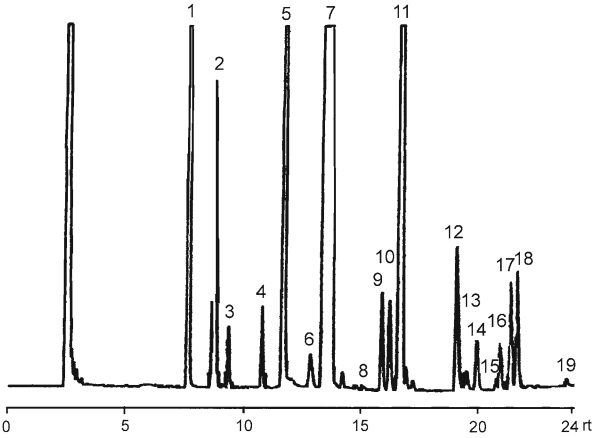


Fig. 6.2 Chromatogram showing fatty acid methyl esters of an olive oil sample. Capillary column SP-2380, 60 m, 0.25 mm i.d., 0.25 μm film thickness. *1*: palmitic; *2*: palmitoleic; *3*: margaric; *4*: margaroleic; *5*: stearic; *6*: elaidic; *7*: oleic; *8*: 18:2(9t, 12t); *9*: 18:2(9c, 12t); *10*: 18:2(9t, 12c); *11*: linoleic; *12*: arachidic; *13*: 18:3(9t, 12c, 15t); *14*: 18:3(9c, 12c, 15t); *15*: 18:3(9c, 12t, 15c); *16*: 18:3(9t, 12c, 15c); *17*: α-linolenic; *18*: gadoleic; *19*: behenic (Camacho and Cert (1994), with permission of Grasas y Aceites)

The use of WCOT columns of fused silica introduced remarkable improvements in separations with higher resolution, higher precision in analysis both in quality and quantity, higher sensitivity, a shortening in analysis time, reduction in the preparation of analysis procedures, etc. Polyglycol phases based on Carbowax 20M, FFAP, Supelcowax-10, and SP-1000 seem to have been the most utilized in recent years. Figure 6.2 shows a chromatogram of olive oil FAs carried out in an SP-2380 capillary column (60 m, 0.25 mm, i.d. 0.25 μm film thickness).

Stationary phases are the same as those used in packed columns; the only difference is that, instead of impregnating a support, they wet the interior wall of the capillary tube. Most phases are now chemically bonded to tube walls, which decreases loss in the column and, in turn, increases duration and temperature resistance and improves the resolution. Table 6.3 shows the basic characteristics of packed and capillary columns.

6.2.1.3 Determination of *Trans*-Fatty Acids

Trans-fatty acid (TFA) isomers should be almost completely absent in VOOs (maximum 0.05 %) (Table 16.6) because they are formed during technological treatments such as partial catalytic hydrogenation; the content and distribution of the *trans* isomers depend on the hydrogenation parameters (i.e., temperature, hydrogen

Table 6.3 Basic differences between packed and capillary columns

	Column	
Characteristics	Packed	Capillary
Length (m)	0.5–4	5–200
Internal diameter (mm)	2–4	0.2–0.7
Specific permeability	1–10	10–10,000
Flow rate (ml/min)	10–60	0.5–15
Pressure (psi)	10–40	3–40
Efficiency (plates/m)	2,000	2,000
Total efficiency (TPN)	4,000	100,000
Film thickness (μm)	1–10	0.1–1
Charge capacity (ng)	10,000	50

Table 6.4 Effect of injection temperature and methylation method on C18:1(9t) and C18:2(9c, 12t) acid content of a virgin olive oil

	Method A		Method B		Method C	
Injection T (°C)	200	300	200	300	200	300
C18:1(9t) (%)	0.026	0.042	0.017	0.030	0.016	0.024
C18:2(9c, 12t) (%)	0.012	0.090	0.012	0.013	0.015	0.013

Note: Method A: methylation in a methanolic medium with alkaline and acid catalysis; method B: methylation with sodium methylate; method C: methylation with potassium hydroxide, with permission of Grasas y Aceites)

pressure, and kind of catalyst). Thus, TFA quantification is of great relevance for olive oil authentication.

TFA determination has been carried out by infrared spectroscopy (IR) (Chap. 10). However, today the use of (GC) with great-length capillary columns allows the analyst to determine the percentage of TFAs (as with IR) and to separate individual FA esters of a given chain length that differ by degree of unsaturation and in the positions of the double bonds (e.g., two isomers of 18:1 and of 18:3 are separated). In the analysis of the methyl esters of polyunsaturated FAs in olive oil it is important to note that the shorter the distance between the last double bond and the end of the molecule, the longer the retention time of the isomer. A good correlation exists between the data supplied by GC and infrared instruments (Tena et al. 2009).

To carry out TFA determination by GC, FAMEs should be prepared by means of any procedure mentioned above; however, it has been observed that, for samples with low *trans* isomer contents, methylation methods using heating produce an increase in those isomers. Therefore, for this determination, cold methylation with methanolic potassium hydroxide or diazomethane is recommended. Also, different injection temperatures have been studied (León-Camacho 1997) because there is always the possibility that *cis* FA isomerization might appear in the chromatographic injector. An analysis of a VOO with low *trans* isomer content (Table 6.4)

Table 6.5 Effect of mass discrimination on fatty acid methyl esters of a virgin olive oil as affected by amount of sample injected

	No. 1	No. 2
C16:1	0.24	0.24
C18:0	3.60	3.69
C18:1(ω9)	76.00	76.85
C18:2 (ω9, ω12)	10.44	10.44
C18:3 (ω9, ω12, ω15)	0.73	0.73

Note: No. 1 arachidic acid peak up to full scale; No. 2 arachidic acid peak between 20 % and 50 % full scale. Values expressed in percentages of total fraction of fatty acid methyl esters. (Camacho and Cert (1994), with permission of Grasas y Aceites)

showed that results are similar for methods B and C, whereas method A yields results that are slightly higher in elaidic acid (18:1t), which is in agreement with a preference for isomerization in an acidic medium. On the other hand, a remarkable increase in elaidic acid as the injection temperature increases has been observed as well.

After numerous injections, the analyst might also observe an increase in the amount of elaidic acid that results from the catalytic action of the residues accumulated in the injector. In contrast, the cleaner the injector, the lower the amount of acid that will result; consequently, cold methylation, with a solution of potassium hydroxide (KOH) in methanol at a temperature no higher than 225 °C and cleanliness control, by means of injecting a previously contrasted VOO (with a minimum oleic acid content of 60 % and certified of free of TFAs), is recommended (IOC 2001c).

Regarding GC analysis, the methods indicate that the amount of injected sample must be such that it might yield a peak of arachidic acid (C20:0) so as to represent over 20 % of the full scale (EC 1992a), although it must not be greater than 50 %. It has been proved that overlapping between the peaks corresponding to both elaidic and oleic acid and a quantitative distortion, either through discrimination or saturation of the detector, can occur (Table 6.5).

The columns to be used by GC analysis do have a chromatographic profile similar to that presented in Fig. 6.3a, with the linolenic acid at a slightly lower retention time than the eicosenoic acid. The use of columns of lesser polarity produces the appearance of peaks that interfere in the determination of *trans* isomers of linolenic acid. These peaks can be compounds such as ethyl or other esters (Fig. 6.3b). The position of the *trans* isomers therefore must be precisely determined by analyzing a refined oil sample deodorized at high temperature (260 °C) for 5–8 h, as shown in Fig. 6.3, or by analyzing an oil sample spiked with a hydrogenated soybean oil (Grandgirard et al. 1984).

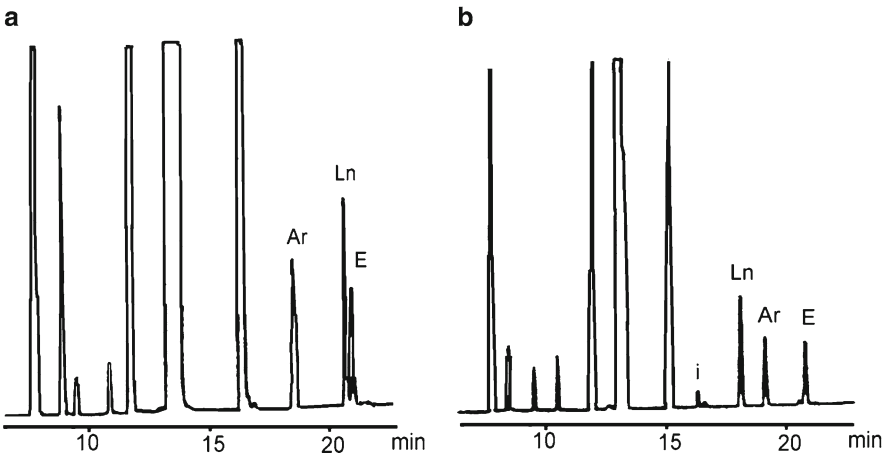


Fig. 6.3 Comparison of chromatographic profiles of fatty acid methyl esters: (**a**) higher polarity column: linolenic acid appears at Rt slightly lower than eicosenoic acid. (**b**) Lower polarity column: Rt of linolenic and arachidic acid are inversed; peaks of zone i interfere with *trans*-linolenic acid determination. Note: *E*: eicosanoic acid; *Ar*: arachidic acid; *Ln*: linolenic acid (Camacho and Cert (1994), with permission of Grasas y Aceites)

An HP-88 capillary column – coated with 88 % cyanopropyl aryl siloxane (100 m × 0.2 mm i.d. × 0.2 μm film thickness) – or a Varian Chrompack CP-Sil 88 column – coated with cyanopropyl polysiloxane (100 m × 0.25 mm i.d. × 0.2 μm film thickness) – can provide an accurate quantitation of TFA isomers previously derivatized as methylesters. Better peak resolution is obtained if the carrier gas is hydrogen instead of helium. Furthermore, no silver-ion prefractionation of TFA (by TLC, SPE, or HPLC) is required prior to the GC analysis (Nimal Ratnayake et al. 2006).

6.2.2 Triacylglycerols

Nearly all the commercially important oils of plant origin consist almost exclusively of the simple lipid class triacylglycerols (TAGs), named triglycerides in the older literature. Synthesized by enzyme systems, they consist of a glycerol moiety with each hydroxyl group esterified to a FA. Thus, the determination of those FAs that esterify the different positions of glycerol has been established as a measurement of olive oil purity (Chap. 16). This is so because of the specificity of the TAG compositions to different types of oils and fats (Aparicio and McIntyre 1998).

The determination of TAGs is quite arduous but of the utmost importance for olive oil fraud control. The procedure is as follows: (1) chemical hydrolysis to determine the percentage of every FA (referred to the total FAs); (2) enzymatic

hydrolysis using pancreatic lipase to estimate the percentage of the β-position FAs in TAGs; (3) enzymatic hydrolysis using phospholipase to distinguish the FAs in the first and third positions of TAGs. Furthermore, and exclusively from an analytical viewpoint, the recovery of TAGs, and thus their correction factors, depends on several chromatographic parameters of the GLC and HPLC techniques, e.g., injection procedure, column quality, carrier gas flow rate, or the stationary and mobile phases and the elution time, among many others. On the other hand, the optimization of operational parameters must take into account not only the compromise between resolution and analysis time but also the delicate balance between separation efficiency and recovery.

6.2.2.1 High-Performance Liquid Chromatography Analysis

High-performance liquid chromatography (HPLC) was started as an alternative method to FA GLC. Thus far, chemists or biochemists have found few advantages from using this technique instead of GLC, although great progress has been made in developing HPLC. The situation is completely different, however, when it comes to determining the TAG composition. In this case, not until later was GLC able to solve the problem because no phases resisted the high temperatures (approximately 350 °C) needed to volatilize TAGs (Kalo and Kemppinen 2012). This made it necessary to resort to the hydrolysis mentioned previously. Different HPLC methods have been developed in recent decades, thanks to the work of chemists and biochemists specialized in fats. TAGs are separated according to the number of carbon atoms and to their unsaturation by different sample preparation procedures and a wide variety of stationary and mobile phases, columns, and detectors. All these parameters can be grouped into two categories:

1. Partition chromatography to separate TAGs (if incompletely);
2. Distribution chromatography in nonaqueous reverse phase, which uses chemically bound C-18 hydrocarbon columns.

Because good resolution of TAGs has been possible by reversed-phase high-performance liquid chromatography (RP-HPLC) for many years, few papers have been published using GLC. Table 16.12 summarizes the RP-HPLC conditions (column dimension, stationary and mobile phases, kind of detector, and flow rate) used for the determination of an olive oil TAG profile.

Columns

The number of stationary phases that can be used is limited. Phases of the octadecylsilyl type are among the most universally used, although columns with a high-carbon loading seem to be better. This is so because a stationary phase similar in

chain length to fatty acyl chains increases the interactions and, as a consequence, produces the maximum efficiency.

The five most frequently used columns and their micron packing are μBondapak C18 (10 μm), RP-18 (4 μm), Zorbax-ODS (6 μm), ODS2 C18 (5 μm), and silicagel columns soaked in silver ion.

RP-18 is, however, the most commonly used, whatever the edible oil, because it shows the greatest resolution of TAG homologous series. The resolving potential of this column with regard to particle diameter is well known. For this purpose, the habitual column of 250 × 4.6 mm × 5 μm packing, which is used with an acetonitrile 1:1 acetone mobile phase and a refraction index (RI) detector, is being currently replaced by 100 × 3 mm × 3 μm packing with no loss of resolution and savings of time and solvents. In fact, an increase in the olive oil TAG resolution has been observed as the particle size diminishes. ECN42 triacylglycerides, which are of interest in olive oil, are the first ones to elute from the column, and hence the use of columns with a smaller particle size minimizes potential problems in the integration and quantification of the chromatographic peaks. Today, excellent results – in terms of speed, resolution, and accuracy – are attained with methods based on ultraperformance liquid chromatography-evaporative light scattering detection (UPLC-ELSD), which uses columns of 100 × 2.1 mm × 1.7 μm packing (Ross et al. 2011).

Column temperature is another parameter that affects the separation of TAGs. Thus, analyses under ambient temperature are not suggested as it varies over the course of a year, and retention times will vary as well. A thermostated oven for the column is recommended because it allows reproducible retention times. The optimum oven temperature must be determined by empirical studies.

Mobile Phases

Acetonitrile is the main component of the most used mobile phases, with a further solvent added to modify its elution properties with the objective of improving the solubility of TAGs in the mobile phase and hence to optimize the separation of the chromatographic peaks. The four most frequently used mobile phases are (1) acetonitrile:acetone, (2) acetonitrile:isopropanol, (3) acetonitrile:dichloromethane, and (4) acetonitrile:ethanol:hexane. Depending on the type of detection used and on the degree of separation required, any one of these phases can be used, although acetonitrile:acetone mixtures give good results analyzing the TAGs of olive oil. Recently, however, propionitrile (HPLC grade) has been suggested as mobile phase (Fig. 6.4) for a method focused on the detection of the presence of hazelnut oil in olive oil (García-González et al. 2007). Although it is expensive, and highly toxic, its results are better for the cited application than the methods based on an acetonitrile:acetone mixture as mobile phase as it reduces dramatically the baseline drift due to the low volatility of the solvent (Aparicio 2004).

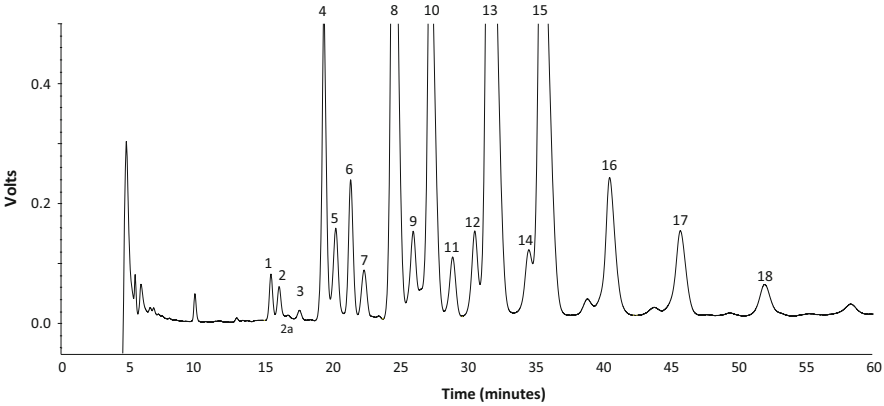


Fig. 6.4 HPLC chromatogram of triacylglycerold using propionitrile as mobile phase. Note: *1*: LLL; *2*: OLLn + PoLL; *3*: PLLn; *4*: OLL; *5*: OOLn + PoOL; *6*: PLL + PoPoO; *7*: POLn + PPoPo + PPoL; *8*: OOL + LnPP; *9*: PoOO; *10*: SLL + PLO; *11*: PoOP + SPoL + SOLn + SPoPo; *12*: PLP; *13*: OOO + PoPP; *14*: SOL; *15*: POO; *16*: POP; *17*: SOO; *18*: POS + SLS (*Source*: Authors)

The elution methods of the mobile phase are isocratic, if the mobile phase remains constant throughout the analysis, and gradient, if the mobile phase does not remain constant. The latter elution method can be subdivided into flow gradient and elution gradient.

Isocratic elution methods, for example, are used in those detectors where the variation of the composition of the mobile phase affects the resulting signal, as in the case of refractive index (RI) detectors. Although RI detectors can theoretically be used with a gradient elution method, by having a reference column in parallel with the main one, it is very difficult to get a stable baseline. Christie (1992) suggests equating detector response directly with the mass of components because accuracy improves if careful calibration and calculation of response factors are carefully carried out.

Detectors

Detectors are the accessories of the HPLC instrument that help in identification and quantification of compounds of the sample injected. Detectors work according to many different scientific principles, but all of them have been designed to work with certain properties like:

1. To be inert to the samples injected and the mobile phases passing through;
2. To be preferably nondestructive to the sample;
3. To produce quantitative responses as well as uniform, reliable, and reproducible analytical data;
4. To have good sensitivity because the sample quantity may be low enough.

Despite those properties, one of the major problems of the HPLC technique is the lack of a simple and universal detector that shows a wide range of linearity. The three most common detectors used for quantifying olive oil TAGs are RI, ultraviolet (UV), and evaporative light-scattering detectors (ELSDs).

The RI detector, which is nearly universal, is based on the measure of the RI difference between the eluent and the thermolabile phase in a continuous way. This detector is greatly affected by temperature and by the composition of the mobile phase. Therefore, it is suggested to work with thermostated systems and isocratic elution. Although it also has the drawbacks of low sensitivity and of different responses toward saturated and highly unsaturated TAGs, it seems to be the most appropriate detector for quantitative analysis of olive oil TAGs.

With respect to practical aspects, RI detectors should not be placed near sunlit windows, and the analyst should take care of possible gas bubbles in solvents, leaks in the system, back pressure, and pulsations of HPLC pumps as they affect the baseline stability (Christie 1992). In the case of olive oil and olive-pomace oil analyses, the International Olive Council (IOC) has adopted a method to determine TAGs by means of HPLC that uses a column of 250 mm length × 4.5 mm internal diameter packed with 5 μm diameter particles of silica with 22–23 % carbon in the form of octadecylsilane (e.g., Lichrosorb RP-18 and Lichrosphere 100 CH18 columns), an isocratic elution system with two possible mobile phases (acetone + acetonitrile or propionitrile) and a RI detector (IOC 2010b). In determining raw olive-pomace oils, it suggests passing the presolved acetone sample through a 0.2 μm pore size filter to remove possible precipitates that might shorten the effective life of the chromatographic column.

To eliminate oxidized compounds that interfere in the trilinolein (LLL) determination, samples can be purified by using aluminum oxide columns or by means of a silicagel SEP-PAD (EC 1992b). By using both procedures, selective triglyceride retention according to polarity is possible; however, this implies checking the composition of the FAs in the oil after its purification in a long, tedious process during which mistakes can be made. It has been proven that the technique described for the analysis of polar components in oils (IUPAC 1987b) is very useful for oil purification. Therefore, the flowing of oil through a silicagel column, eluting with hexane:diethyl ether 87:13 (v/v), makes it possible to obtain the purified triglycerides. Further elution of the column with diethyl ether leads to the isolation of the polar fraction where the interfering compounds appear (Fig. 6.5).

The UV detector has not been very widely used because it presents problems with isomerization and conjugation of double bonds. However, it has a good sensitivity and allows the use of elution gradients that improve the resolution. Different wavelengths have been tried, though those between 200 and 230 nm have been reported to give good quantitative data because that range includes the region where the ester chromophore absorbs. The wavelength at 220 nm is generally accepted to avoid interferences from unsaturated TAGs. Another problem is that some solvents such as acetone and chloroform cannot be used because they absorb in that range of wavelength and, hence, overlap with the detection region of TAGs. Also, quantification may present a problem because the response is linear in a range of 50–250 μg.

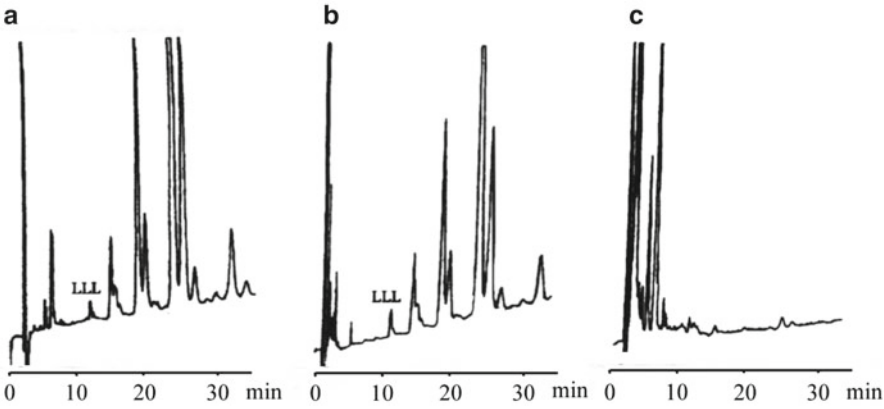


Fig. 6.5 HPLC chromatograms of olive-pomace oil triglycerides. C-18 column, 4 μm particle size, 1.15 ml/min acetone-acetonitrile 50:50 (**a**) oil sample without purification; (**b**) oil sample purified through silicagel column; (**c**) polar fraction retained on silicagel (Camacho and Cert (1994), with permission of Grasas y Aceites)

Thus, the analyst should estimate the TAG response factors before proceeding with the analysis.

Although the bromination of TAGs, prior to their separation, allows for a better separation of certain TAGs with a UV detector, the alternative is the use of ELSDs. An ELSD is both simple and universal, and in these detectors the eluent of the column is transported by means of an air current in a nebulizer. Later on, the aerosol heats up, and this produces the evaporation of the solvent. Only the tiny solute drops remain, which go through a light beam that is reflected and refracted. The amount of light is measured and related to the concentration of the eluted component. ELSD is compatible with gradient elution, and no baseline drift for nonaqueous volatile solvents has been detected (Carelli and Cert 1993), although the response tends to decrease as peaks widen because of the nature of the detection system. Although apparently the result should not be influenced by the chemical nature of the compound, it has been proven that some influences actually occur. In fact, the sample must be less volatile than the solvent, and the lack of linearity of the response at very low and high concentrations may represent a problem in quantitative studies. The viscosity, density, and flow rate of the mobile phase and the design of the nebulizer seem to be the cause of its poor linearity. Newer designs, however, seem to have been ameliorated (Cuhna and Oliveira 2006).

Mass Spectrometry

Analysis of TAG composition of oil samples by HPLC mass spectrometry (MS) is a widespread method because MS supplies identification of non- or partially

resolved HPLC peaks (Cozzolino and De Giulio 2011). In fact, MS provides detailed information about the FA composition of TAG molecules, though only a few ionization techniques are suitable for coupling with HPLC. Thus, electrospray ionization (ESI) is an excellent technique because it enables the identification of the individual acyls without the need for authentic reference standards, while APCI coupled to HPLC is very effective because it allows for identifying the positional isomers (Holčapek et al. 2003), and in addition to the peaks corresponding to the molecular mass of the TAGs, there are other signals at lower masses related to DAG fragments (Jakab et al. 2002). Studies based on results from a HPLC APCI-MS have allowed the identification of TAGs that are not present in olive oils but are suspected of having been adulterated (Fasciotti and Netto 2010).

Recently, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is being used for a rapid differentiation of edible oils based on the molecular weight and FA composition of TAGs. The main advantage of this technique is the fast and rapid sample preparation and the absence of analyte purification, chemical modification, or derivatization. More recently, a new approach based on laser desorption ionization (LDI) coupled TOF MS has been used with success for the detection of adulteration of olive oil with sunflower oil (Calvano et al. 2005).

Although these techniques cannot be applied in routine analyses, advances in HPLC MS instrumentations have allowed the development of rapid analytical approaches that at present show excellent sensitivity and accuracy.

6.2.2.2 Gas–Liquid Chromatography Analysis

GLC offers attractive possibilities for the analysis of complex compound mixtures with a wide range of molecular weight, as in the case of acylglycerols. The development of the technique, since 1962 when the first analysis of TAGs by GLC was published (Kuksis and McCarthy 1962) till today, has allowed improving on separation efficiency, quantitative recovery, reproducibility, analysis time, and lifetime of chemically bonded stationary phases, among other parameters up to the point that at present it competes with success with HPLC technique. GLC technique, however, is not exempted from problems such as, for example, the injection system and the column deterioration.

Sample Preparation

An olive oil sample is extracted with hexane, then filtered, and later centrifuged to clarify the solution. Chloroform-methanol mixtures are used when extraction is carried out from olives. The sample does not require either a complex advance preparation or derivatization unless a structural study is to be undertaken or the analyst attempts to resolve the lipids with free OH groups, such as silanized mono- and diglycerides (Pérez-Camino et al. 1996).

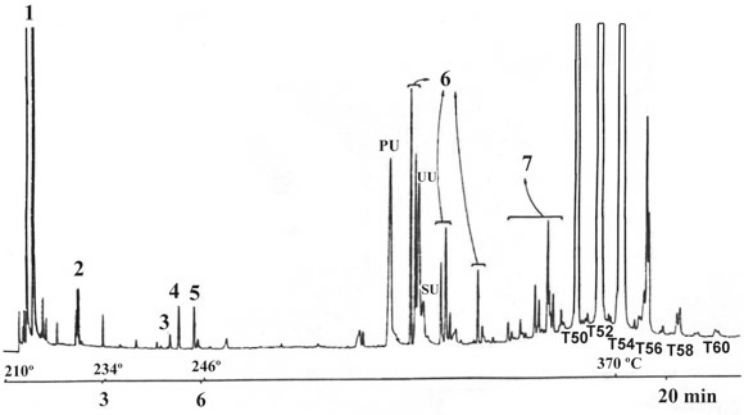


Fig. 6.6 Triglyceride profiles of oils from raw coffee beans and from fully hydrogenated palm kernel oils. (A) methyl-silicone column. Notes: *1*: free fatty acids; *2*: diterpenes; *3*: campesterol; *4*: stigmasterol; *5*: β-sistosterol; *6*: diterpene esters; *7*: sterol esters; T_{CN}, triacylglycerol; CN, Carbon number (*Source*: Geeraert (1987), with permission of Elsevier)

Columns

The chemical nature of the stationary phase determines the selectivity in the GLC technique. Packed columns with thermostable nonpolar stationary phases of the dimethyl polysiloxane type (OV-1, OV-3, SE-52, SE-54, SP-2100) were employed in early works because they can endure temperatures above 370 °C for long periods of time. The selectivity of columns packed with silicone stationary phases is conditioned solely on differences in vapor pressure so that the chromatogram shows the peaks according to the molecular masses of the compounds. However, the rapid progress of capillary column technology has meant that GLC packed columns are rarely used now.

The elution sequence in capillary columns coated with methyl silicone is FAs, monoglycerides, diterpenes, sterols, diglycerides, diterpene esters, sterol esters, and triglycerides, separated by carbon atom number (Geeraert 1987) (Fig. 6.6). However, columns coated with polar stationary phases, such as cyanopropyl silicone, are capable of resolving triglyceride mixtures better than nonpolar phases (Fig. 6.7). This kind of column does not reach temperatures over 300 °C, but there are stable polymeric polar phases that are able to reach temperatures far from 400 °C.

The stationary phases that have been most successfully used in the separation of TAGs are the polarizable phenyl-methyl-silicone phases, capable of enduring temperatures of approximately 360–370° for a long time. These phases, which are currently used, separate triglycerides both by carbon atom number and by unsaturation (Geeraert and Sandra 1985, 1987) (Fig. 6.8).

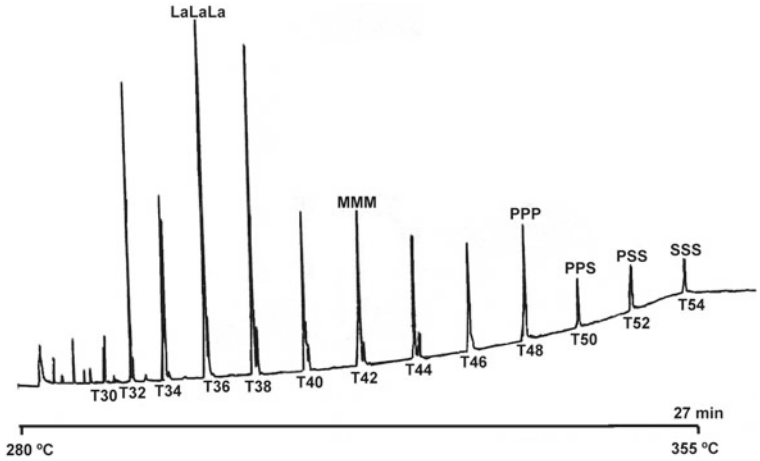


Fig. 6.7 Triglyceride profiles of oils from raw coffee beans obtained by gas–liquid chromatography. (B) Cyanopropyl-silicone column. Notes: *La*: lauric acid; *M*: miristic acid; *P*: palmitic acid; *S*: stearic acid; T_{CN}, triacylglycerol; CN, Carbon number (*Source*: Geeraert (1987), with permission of Elsevier)

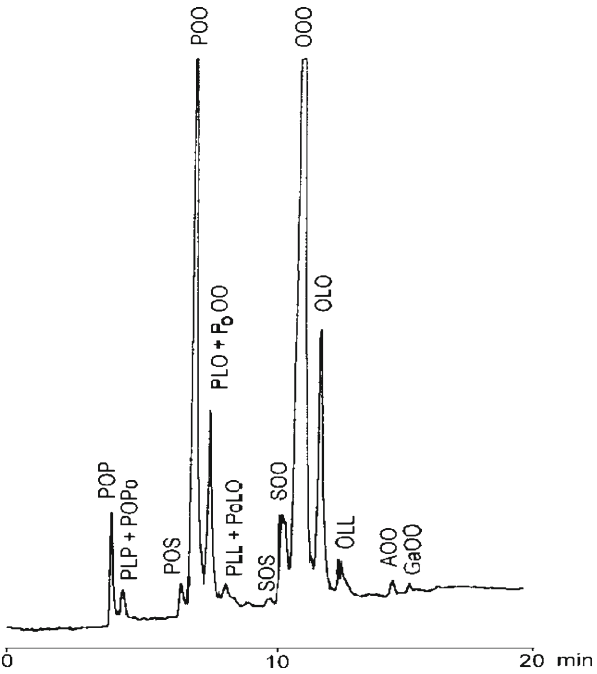
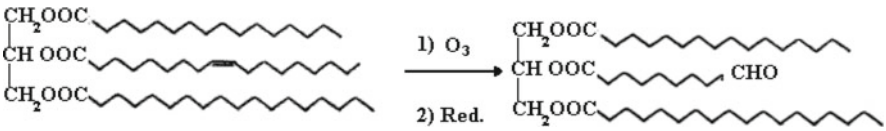


Fig. 6.8 Triglyceride profile of olive oil obtained by gas–liquid chromatography with a phenyl-methyl-silicone column. Note: *P*: palmitic acid; *O*: oleic acid; *L*: linoleic acid; *Po*: palmitoleic acid; *S*: stearic acid; *A*: arachidic acid; *Ga*: gadoleic acid (*Source*: Authors)

The resolution for these columns, under certain efficiency conditions, is as follows. Each peak corresponding to a given carbon number using polar columns is now split into various peaks. For CN 48–56, this splitting is given by the number of unsaturated FAs (NUFA). For example, for CN 54, four peaks can be obtained in the following order: UUU, SUU, SUS, and SSS, where U and S are the monounsaturate and the saturate, respectively, for a FA with 18 carbon atoms. Positional isomers, such as SUS and SSU, cannot be separated by means of this technique; instead, derivatization must be carried out in advance. Ozonolysis, followed by a reduction, is the most widely used procedure (Geeraert and Schepper 1982). Prior to injecting the sample, the latter undergoes ozonolysis according to the following diagram:



For this purpose, O_3 is passed through the sample, which has been dissolved in isooctane, at room temperature for a few minutes. O_3 excess is eliminated with nitrogen at 120 °C for a few seconds, and the sample is reduced with trimethylphosphine at 120 °C for 20 min, then concentrated and injected.

As stated earlier, a good separation, together with a high resolution, can be obtained only using capillary columns. The columns are made of silicagel and their length varies between 5 and 30 m depending on the type of sample and the desired level of resolution. Columns of 5 m have been successfully used to separate TAG mixtures formed by low molecular weight FAs.

To obtain high efficiency and increase the column theoretical plate number (TPN), columns with 250 μm internal diameter are used. To obtain resolution in a satisfactory period of time and at a reasonable temperature, film thickness is normally between 0.1 and 0.12 μm because of the scantiness of the stationary phase; although it is not chemically bonded, the column presents little bleed. The columns are frequently covered with polyamide; however, as the columns are exposed to high temperatures, the polymer deteriorates and the columns become brittle. This problem is avoided by using aluminum or stainless-steel-coated columns because they are more durable.

Injection Systems

All existing studies of GLC analysis of olive oil TAGs suggest the use of cold-on-column injection to reduce or eliminate thermal decomposition, mass discrimination, and other undesirable effects associated with classical split/splitless injection. The reason can be the wide range of molecular weights and volatility differences among triglycerides.

Table 6.6 Triolein relative response factors (r_f) of various triacylglycerols (TAGs)

TAG	r_f	μg/ml
PPP	533	0.53 ± 0.02
PoPoPo	582	0.64 ± 0.02
POP	421	0.59 ± 0.02
POO	206	0.77 ± 0.04
SSS	510	0.87 ± 0.02
OOO	557	1
LLL	551	1.34 ± 0.07
NNN	495	1.27 ± 0.09

Abbreviations: *L* linoleic acid, *O* oleic acid, *P* palmitic acid, *Po* palmitoleic acid, *S* stearic acid (Camacho and Cert (1994), with permission of Grasas y Aceites)

This technique allows injecting the sample directly into the capillary column; to do this, the injector temperature must be lower than the solvent boiling point. Afterwards, the sample is heated at oven temperature since all the components volatilize without suffering any loss. There are several cold-on-column systems, all of them having the aim of fast heating of the injector and a fast and efficient cooling system. Because of these conditions, the injection system has two disadvantages: (1) analysis takes longer, and retention times are not reproduced as easily as in the split-injection system; (2) the time that TAGs are inside the column is considerably increased, which, as retention time increases, results in both resolution loss and peak widening. The system, however, avoids mass discrimination problems and the pyrolysis of the most unsaturated TAGs in the injector.

The development of split/splitless injectors in which the sample path is longer and more tortuous and minimizes mass discrimination loss. As a result, it is possible to apply this technique to TAG analysis with results identical to those obtained by using cold-on-column injection.

Detectors

Detection is carried out by using a flame ionization detector (FID), and the response factors depend to a certain extent on the ratio of the carrier gas; when hydrogen is used, the varying flow rate during the temperature-programmed operation may affect FID response factors.

Standard solution analysis with split injection produces different relative response factors, according to results yielded by the use of a cold-on-column injector and a column with phenyl-methyl-silicon phase. Results are shown in Table 6.6.

Except for LLL, the obtained factor shows a linear relation with TAG retention times with a variation coefficient of 0.99. This decrease in the response with elution time has been attributed to quenching, which is due to the level of loss of the stationary phase used. In our experiments, however, we have observed that there may be a contribution of the mass discrimination effect. The low LLL response corresponds to the loss of highly unsaturated TAGs described in the literature, which suggests an alteration of the compounds during analysis (Carelli and Cert 1993).

6.3 Minor Components

As stated previously, TAGs represent the main lipid fraction of foods, whereas the set of minor components (MCs) is constituted by compounds that can derive from TAGs and by other liposoluble compounds. The group clusters are as follows: diacylglycerols (DAGs), monoacylglycerols (MAGs), free fatty acids (FFAs), oxygenated fatty acids (OFAs), cyclic fatty acids, nonlinear FAs (branched FAs), dimeric FAs, and a set of compounds derived from the unsaponifiable matter in addition to phenols, pigments, and volatiles. The total of these components, though, can be present in relatively high quantities does not exceed, in general, 2–5 % of the total lipid composition. The analytical methods for determining pigments, volatiles, and phenols are described in Chaps. 7–9.

6.3.1 Diacylglycerols and Monoacylglycerols

Diacylglycerols (DAGs) are present in a range of 1–3 % in VOOs and are found as 1,2- and 1,3-isomers. The 1,2-isomers are attributed to the incomplete biosynthesis of TAGs, which are the main lipids inside the drupe vacuole of olives, whereas the 1,3-isomers are attributed to enzymatic or chemical hydrolysis of TAGs occurring before or during the oil extraction process. Thus, a VOO freshly made from sound olive fruits contains almost exclusively 1,2-DAGs, while virgin olive oils from poor-quality fruits contain high quantities of 1,3-isomers. Also, VOOs stored for a long time show a decrease in 1,2-DAGs and an increase in 1,3-DAGs that some authors ascribe to the isomerization of the former toward the latter. Thus, the 1,3-/1,2-DAG isomer ratio has been suggested for assessing the genuineness of VOOs with low acidities during the early stages of storage (Pérez-Camino et al. 2001). Some authors have also suggested this ratio as a parameter for determining VOO quality (Gertz and Fiebig 2006) despite the fact that its relationship with sensory quality is merely casual with the appearance of a causal relation in the case of VOO defects resulting from hydrolytic processes (e.g., fusty, vinegary).

The method for determining the composition and content of DAGs by capillary gas chromatography in vegetable oils, with lauric acid content below 1 %, is described in an IOC method (IOC 2011). The internal standard (dinonadecanoine) and silylating reagent is added to olive oil sample and the mixture is solved in a suitable reagent and directly injected in the gas chromatograph (capillary column of 8–12 m length × 0.25–0.32 mm i.d. coated with SE52 or SE54). DAGs are separated by their carbon atom number; a peak of 1,2-DAGs (saturated and unsaturated) appears before 1,3-DAGs.

In the analysis of DAGs, it is advisable to extract and purify them (by column chromatography or SPE) and to add a suitable internal standard (e.g., 1,3-dipalmitoyl rac-glycerol, dinonadecanoine) prior to the purification step. The German Joint Committee for the Analysis of Fats, Oils, Fatty Products, Related Products and Raw Materials (GA Fett) has developed a method for the determination of isomeric DAGs in VOOs (Gertz and Fiebig 2006), while it is also the method already described for the determination of the composition and content of DAGs by GC with a lauric acid content below 1 %. The main differences concern the analytical procedure used for the separation of the fraction containing nonpolar lipids. Pérez-Camino et al. (2001), however, propose to charge the silica solid-phase extraction (SPE) cartridge with an aliquot of 500 μL of the dehydrated olive oil solution in hexane (0.2 mg/mL) and 200 μL of the internal standard. With this method, the purificaction comprises two steps. The first is carried out by passing a solution of hexane/methylene chloride/diethyl ether (89:10:1) through the column, which is discarded. The fraction of the second step, which is obtained by passing a solution of chloroform/methanol (2:1), is evaporated until dryness in a rotary evaporator under reduced pressure, and the residue is derivatized to the corresponding trimethylsilyl (TMS) derivatives of 1,2-DAGs and 1,3-DAGs by treating the residue with a silylating reagent (e.g., 9 mL of anhydrous pyridine, 3 mL of hexamethyldisilazane, and 1 mL of trimethylchlorosilane) for a few minutes. 1 mL of the solution is subjected to GC analysis so that the pairing of the FAs in each DAG can be determined. The GC instrument should be equipped with a FID and thermostable GC columns coated with medium polarity (e.g., 65 % phenyl- and 35 % dimethylpolysiloxane) (Frega et al. 1993) or with 65 % methyl phenylsilicone (Pérez-Camino et al. 2001).

Monoacylglycerols (MAGs) are present at trace levels in olive oils, and their analytical determination is carried out with a procedure similar to that used with DAGs, TMS derivatization and FID-GC analysis with nonpolar capillary columns.

6.3.2 Free Fatty Acids

Free fatty acids (FFAs) are usually determined by titration, although determination can also be carried out by GC as FA methyl esters (FAMEs). FFA methyl esters are prepared by derivatization with trimethylsilyldiazomethane because methylation

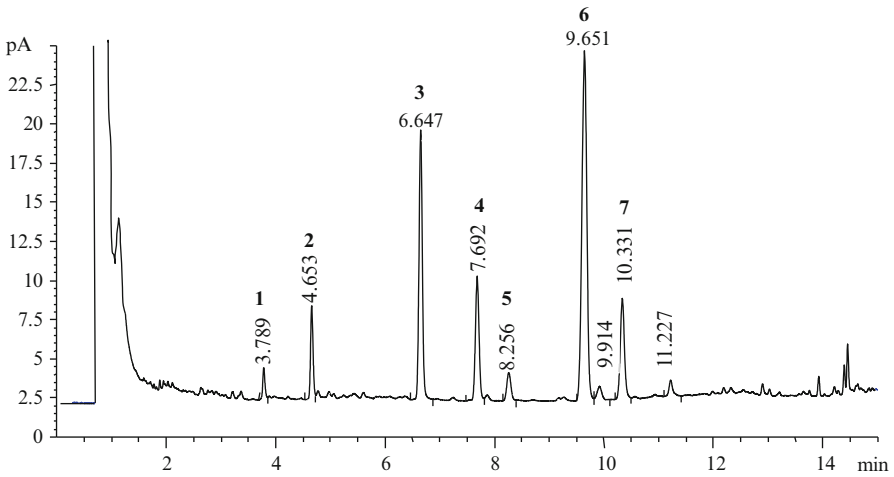


Fig. 6.9 Chromatogram of methyl and ethyl esters. Note: *1*: methyl ester C16:0; *2*: ethyl ester C16:0; *3*: ethyl ester C17:0 (IS); *4*: methyl ester c18:1; *5*: methyl ester C18:2; *6*: ethyl ester C18:1; *7*: ethyl ester C18:2 (Courtesy of Dr. Pérez-Camino; Instituto de la Grasa-CSIC, Sevilla, Spain)

enhances the volatility and reduces activity of FFAs, although it can be omitted if a highly polar capillary column (e.g., coated with 88 % cyanopropyl-aryl-siloxane or 100 % cyanopropyl polysiloxane) is used. The procedure also requires the addition of an internal standard (e.g., tridecanoic or nonadecanoic FAs) and a purification step by SPE filled with NH_2. Medium-polarity thermostable columns (e.g., coated 65 % phenyl-35 % dimethyl-polysiloxane) have been used with success.

6.3.3 Fatty Acid Alkyl Esters

Fatty acid alkyl esters (FAAEs) present in olive oil are formed by esterification of FFAs with low molecular weight alcohols. In fact, alkyl esters are made up of methyl and ethyl esters of FAs previously present in their free form. Inappropriate practices in the harvesting and storing of the olives prior to olive oil extraction can result in the rupture of the olive drupe and its contact with microorganisms (yeasts) and lipolytic and pectolytic enzymes.

Quantification can be done by isolation with a silicagel SPE cartridge (1,000 mg) previously conditioned with hexane (Pérez-Camino et al. 2008). The cartridge is firstly eluted with a solvent mixture of hexane/toluene (85:15) and rejected, while the second fraction, resulting from passing 10 mL of the same mixture at a flow rate of 1 mL/min, is collected and then evaporated in a rotary evaporator. The residue is dissolved in n-heptane and an aliquot of the solution injected into GC equipped with a programmed temperature vaporizer (PTV) injector using a polar capillary column (30 m×0.25 mm i.d. coated with 35 % dimethyl-65 % diphenylpolysiloxane). Figure 6.9 shows the chromatogram of FAAEs from an olive oil sample.

The standard method proposed by IOC (IOC 2010c) allows the simultaneous determination of alkyl esters and waxes. The method consists of separation by column chromatography and gas chromatographic analysis. In the first step, 500 mg of sample is weighted and the internal standard, which depends on the category, is added, e.g., 0.1 mg of lauryl arachidate in the case of olive oil and 0.25–0.50 mg in the case of olive-pomace oil. The resulting mixture is transferred to the chromatographic column with the aid of two 2-mL portions of n-hexane. The method suggests allowing the solvent to flow to 1 mm above the upper level of the absorbent and then to add n-hexane/diethyl ether (99:1) and collect 220 ml at a flow of about 15 drops every 10 s. An aliquot of 10 mL of the solution is injected on-column into a gas chromatograph (8–12 m length × 0.25–0.32 mm i.d. coated with SE52 or SE54). The method describes the operating conditions that can be slightly modified to obtain satisfactory peak separations of alkyl esters.

6.3.4 Waxes

The term wax has been used by analysts for an large variety of plant products containing several kinds of fatty materials. The biosynthesis pathways of wax components can be an acyl reduction, which yields primary alcohols and wax esters, or a decarbonylation pathway that synthesizes secondary alcohols, aldehydes, alkanes, and ketones. Wax esters are, however, the most common form of waxes and consist of FAs esterified to long-chain alcohols with similar chain lengths (Christie 2011b).

Because waxes are synthesized in the epidermal cells of olives from saturated very-long-chain FAs, their presence in olive oil is of interest because their concentration differs among the olive oil categories, and information about its presence can be used as an indicator of both quality and purity. The main waxes found in olive oils are esters of an even number of carbon atoms from C36 to C46. IOC trade standards (IOC 2010a) and EU directives (EC 2011) have regulated the maximum amount of waxes (C40 + C42 + C44 + C46) in the different olive oil categories (Table 16.6), although, as described in Chap. 16, differences in the content of waxes cannot be used as a unique criterion to detect the presence of olive-pomace oil in olive oil but together with the amount of erythrodiol and uvaol. The reasons for this can be found in the fact that researchers have proven that changes in the wax concentration in olive oil occur naturally because in processes where triglyceride hydrolysis is produced, an increase in FFAs takes place, and this provokes an increase in the esterification reaction rate. Thus, the increase in wax content takes place in VOO samples in storage. Such an increase depends on both reactive species concentration and storage conditions (Mariani and Venturini 1996).

The content of waxes also seems to help the characterization of VOOs because the content of C36 and C38 waxes are usually higher than C40, C42, C44, and C46 waxes in this designation, in contrast to the concentration of waxes in refined and olive-pomace oils.

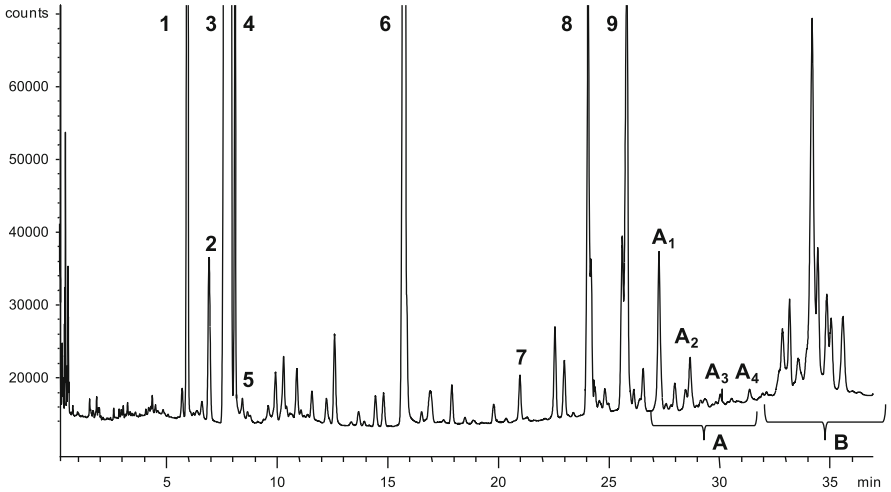


Fig. 6.10 Gas chromatogram of wax fraction of an olive oil. Abbreviations: *1*: methyl C16; *2*: ethyl C16; *3*: methyl heptadecanoate (IS); *4*: methyl C18; *5*: ethyl C18; *6*: squalene; *7*: lauryl arachidate (S); *8*: phytol ester; *9*: geranylgeraniol ester; *A1*: C40 esters; *A2*: C42 esters; *A3*: C44 esters; *A4*: C46 esters; *B*: Sterol esters and triterpene alcohols (*Source*: Authors although based on IOC trade standards)

Basically, all the methods for the determination of waxes consist of a separation from other lipid constituents using silicagel chromatography and subsequent quantification using gas chromatography. The official analytical method determines the waxes by separating them according to the number of carbon atoms. After the addition of the internal standard (lauryl arachidate), the fractionation step is carried out by chromatography on a hydrated silicagel column. The eluted fraction is recovered under the test conditions and then injected on-column in a GC capillary column. SE-54 or SE-52 semipolar columns are used, although greater resolution can be obtained with phenyl-methyl-silicone columns, which can endure higher temperatures. It is advisable to condition the column when using it for the first time by a gradual heating until a temperature of 350 °C is reached.

Although there is a specific method for the determination of waxes (IOC 2007), a new method that allows the simultaneous determination of waxes, FA methyl esters, and FA ethyl esters by capillary gas chromatography (IOC 2010c) is widely applied in laboratories as it saves time when the objective is to determine the olive oil genuineness. Thus, this method is recommended for distinguishing between olive oil and olive-pomace oil, for the detection of the presence of lower-quality oils (ordinary, lampante) in extra VOOs, and for the detection of the fraudulent addition of some deodorized oils to extra virgin olive oils. Figure 6.10 shows a chromatogram of the wax fraction of an olive oil by the method COI/T.20/Doc. No 28/Rev.1 (IOC 2010c).

SPE cartridges (1 g of silicagel) are replacing silicagel columns for sample purification because they require smaller amounts of sample and a reduced volume of elution solvent. Thus, Nota et al. (1999) proposed a method based on a purification step by SPE silicagel cartridge, the use of carbon tetrachloride as eluent, and GC analysis with programmed temperature vaporizer injector and a 65 % phenyl-methyl-silicone capillary column as an alternative to a former EC regulation (EC 1993). More recently, a new method based on the isolation of alkyl esters and waxes by SPE and analysis by capillary column (DB5; 5–12 m long) gas chromatography with on-column injector and FID detector has been proposed (Cert et al. 2011). The method is more rapid than current standards (IOC 2007, 2010c) with lower consumption of organic solvents, and no difference concerning the recovery factors, although there is no information about the precision and limits of quantification yet.

Another analytical alternative is based on the use of a Through Oven Transfer Adsorption Desorption (TOTAD) interface (Aragón et al. 2011). The oil, with an internal standard (C32 wax ester) diluted in n-heptane, is injected directly with no sample pretreatment step other than filtration. Normal-phase liquid chromatography (NPLC) separates the wax ester fraction from the TAGs (Biedermann et al. 2008) and the TOTAD interface transfers it to the GC to be analyzed. The method allows an automatic analysis of different wax esters, and it is simpler and faster than IOC methods (IOC 2007, 2010c), which are tedious and time-consuming, although its resolution is poorer.

Biedermann et al. (2008) also proposed that samples were prepared by NPLC but the precolumn was attached to the inlet of the column in the GC×GC instrument by means of a press-fit connector. The first dimension is performed with a PS-255 column (20 m×0.25 mm i.d.) and the second-dimension with a SOP-50 (50 % phenyl polysiloxane) (1.5 m×0.15 mm i.d.).

6.3.5 Other Minor Components: A Miscellany

Oxidation process at high temperatures (e.g., deep frying) gives rise to dimeric FAs, which exhibit a wide variety of structures with an oxygen atom (epoxy or keto or hydroxy derivatives) in their molecules (Márquez-Ruiz and Dobarganes 2006). Dimeric FAs can also be produced from TAG dimers, after the transesterification or the saponification treatments of the refining process (Chap. 19), and they are made up of the dimeric form with two FAs attached by a carbon-carbon bond in the central part of the hydrocarbon chains.

GC-MS analysis with a nonpolar column (5 % phenyl-95 % dimethylpolysiloxane) is a good method for determining dimeric FAs, although high-performance size exclusion chromatography (HPSEC) coupled to a RI detector is preferred by analysts (Dobarganes and Márquez-Ruiz 2007; Tena 2010).

Olive-pomace oil contains several classes of oxygenated acids amounting to more than 10 % of the component FAs, with *trans-9:10-epoxyoctadecanoic* acid,

which constitutes approximately 2 % of the mixed acids of the oil, the major component (Vioque et al. 1961). During the malaxation process, weak oxidation of unsaturated FAs can also take place that results in oxyacids, which are classified as bioactive compounds such as antifungal agents (Heo et al. 2009). Today the determination is performed by reversed-phase HPLC coupled to a UV-diode array detector.

The concentration of phospholipids is low enough in olive oils. Thus, concentrations in a range of 21–124 mg/kg have been reported in the literature (Koidis and Boskou 2006). Phosphatidylglycerol is the major phospholipid detected in olive oil samples by LC-MS (Boukhchina et al. 2004), although other phospholipids like phosphatidylcholine and phosphatidylinositol are also present in olive oils.

6.4 Unsaponifiable Fraction

By unsaponifiable matter we mean the set of natural or accidental constituents that fail to react with soda and potassium hydroxide to produce soaps but remain soluble in classic solvents (e.g., hexane, petroleum ether) after saponification. All fats contain unsaponifiable matter in varying amounts. The amount is generally 1–2 %, although the percentage can rise as high 99 % in shark liver.

Because the composition of the unsaponifiable fraction of fats can be quite varied, it is often used in the study of the characteristics and genuineness of an oil or fat. This section covers the most important analytical methods for the study of the unsaponifiable matter and of each of its previously mentioned components (6.1. Introduction).

6.4.1 Determining Unsaponifiable Matter

The method for determining unsaponifiable matter involves olive oil saponification and unsaponifiable matter separation by means of extraction with an appropriate solvent, such as diethyl or petroleum ether. The most satisfactory methods are based on extraction by a humid process (i.e., extraction of unsaponifiable matter from an aqueous or alcoholic solution of soap). The Balton–Renis method, sometimes mentioned as a dry extraction process, is based on the extraction of unsaponifiable substances from a solid soap. According to this method, the fat is saponified with a solution of sodium hydroxide in 95 % alcohol, followed by alcohol evaporation until a doughy mass is obtained. The soap is then mixed with sand and sodium bicarbonate and the mixture is dry-heated. The desiccated mass is extracted with petroleum ether to separate the unsaponifiable matter (Mehlenbacher 1960).

Although the determination of unsaponifiable matter seems simple, this type of analysis still presents problems because of the lack of accuracy and precision in the

results. The general dissatisfaction with old methods has led to new proposals and modifications of established techniques. Essentially, all of the new proposals and modifications address variations in the amount of alkali and solvents used and, most especially, in operational details rather than in any significant change in principle. The specific difficulties previously met include the following:

- Impossibility of extracting all of the unsaponifiable matter,
- Formation of emulsions that are hard to break down,
- Soap hydrolysis,
- Loss of unsaponifiable matter during solvent drying and evaporation, and
- Incomplete saponification.

The impossibility of extracting all of the unsaponifiable matter is more likely to occur when petroleum ether is used as a solvent because its extraction rate is much slower than that of diethyl ether. This problem does not occur during studies of animal and vegetable fats and oils but appears during studies of sea animal oils and other fats that contain a relatively high quantity of unsaponifiable matter. Most of the methods used to determine unsaponifiable matter specify that between three and seven extractions are necessary, even though it is not unusual for seven extractions to be insufficient. This shortcoming cannot be foreseen; it can be discovered only by evaporating the solvent from the final extraction and determining the residue gravimetrically. The amount of unsaponifiable matter obtained in the final extraction should not exceed 1 mg.

Although diethyl ether is the preferred solvent, it has a number of limitations. Annoying emulsions are usually formed that are not easily separated. Soaps can pass into the solvent together with the unsaponifiable matter. In this case, it is wise to separate the soaps by washing the ether extract with an aqueous solution of sodium hydroxide, which can provoke soap hydrolysis and liberate acids. Therefore, the most appropriate method appears to be using distilled water and alternating it with an aqueous alkaline solution to carry along the free acids that remain in the ether extract.

After solvent evaporation, it is necessary to dry the unsaponifiable residue in order to ensure the elimination of any traces of solvent and water. Given the possible presence of volatile substances in the unsaponifiable fraction, measures must be taken to prevent extreme drying conditions. Water elimination is usually performed by treating the unsaponifiable extract with water-free sodium sulfate and then filtering it. Some of the unsaponifiable matter can also be distilled during solvent evaporation, but this can be prevented by evaporating at a low temperature and reduced pressure. Finally, any remaining moisture traces are eliminated by vacuum drying with acetone or benzene.

Incomplete saponification will yield erroneous results because the saponifiable portion of triacylglycerols is soluble in ether and hexane. No universally applicable saponifying method exists; however, if the steps prescribed in the normalized methods are carefully followed (Mehlenbacher 1960), there should be no serious difficulties except for a few fats that resist saponification. When incomplete saponification is suspected, the unsaponifiable residue containing the nonsaponified segment must

be resaponified and reextracted in exactly the same way established for the initial saponification and extraction.

6.4.2 *Unsaponifiable Separation*

Although several procedures can directly determine some of the unsaponifiable components, they are usually separated in fractions. Fractionation of the unsaponifiable components into several groups of constituents (e.g., hydrocarbons, tocopherols, and sterols) is an essential introduction to any thorough study of its composition. Separation of the unsaponifiable constituents can be carried out by innumerable procedures, most of them chromatographic, including column and thin-layer chromatography.

- *Column chromatography*: This method is extremely long for complete separation of unsaponifiables. For this reason, the current trend is to use it exclusively to study those constituents that are more readily eluted by absorption chromatography or that appear at very low concentrations because a great quantity of material is needed and thus the columns have a greater load capacity than do thin layers. A typical example is the analysis of the hydrocarbon fraction.
- *Thin-layer chromatography*: This is the method of choice for the complete fractionation of unsaponifiables. As in column chromatography, two different supports, alumina and silicagel, can be used. The former is particularly convenient in the study of unsaponifiables extracted from diethyl ether, even though it is not widely used; the latter is applicable to the fractionation of both petroleum and diethyl ether. Figure 6.11 shows unsaponifiables extracted from diethyl ether and

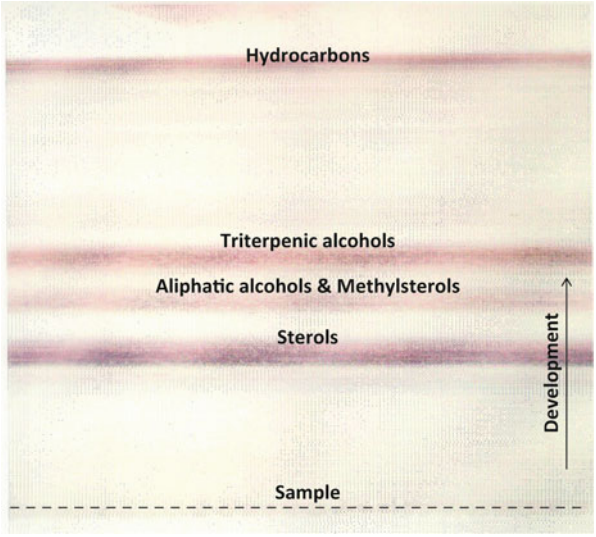


Fig. 6.11 Olive oil unsaponifiable fraction purified by thin-layer chromatography (*Source*: Authors)

purified in a silicagel thin layer. This method can be applied to unsaponifiables extracted from petroleum ether and containing FFAs, provided that great care is taken to utilize basic silicagel treated with diluted potassium hydroxide.

6.4.3 Hydrocarbons

In food matrices, most hydrocarbons have an odd number of carbon atoms, although there are also small amounts of ramified compounds with both *iso-* and *anteiso-structure*. In olive oils, there are small amounts of saturated, normal (C-15 to C-35), branching, terpenic, and even aromatic hydrocarbons; squalene ($C_{30}H_{50}$), a linear triterpenic polymer of isoprene, is the most important unsaturated hydrocarbon.

The dehydration of sterols during the refining process of olive oils also results in the formation of steroidal hydrocarbons (sterenes or steradienes) with two double bonds in the ring system (Lanzón 1990), although other steroidal hydrocarbons, whose structures have three double bonds in the ring system, have been detected in the sterene fractions of refined vegetable oils more recently (Bortolomeazzi et al. 2000). The quantification of stigmastadienes allows for the study of VOO genuineness (Table 16.6).

Low levels of pollutants (e.g., pesticides, volatile organic compounds, chlorinated hydrocarbons, aromatic hydrocarbons) can be found in unrefined oils. Thus, small amounts of methyl and ethyl benzene, which can derive from environmental pollution, have been quantified in VOOs using DHS-GC (Aparicio et al. 1997), while many other polycyclic aromatic hydrocarbons (PAHs) have been quantified by SPME-GC in Italian VOOs (Vichi et al. 2007). However, the most common current methods isolate PAHs from edible oils using SPE on silica cartridges, followed by HPLC with fluorometric detection that has proven to successfully separate PAHs from other hydrocarbons (PAHs coelute with other hydrocarbons in silica TLC) with a good quantitative response (Moret and Conte 1998).

6.4.3.1 Determination of Squalene

The determination of squalene in olive oil, which has a high content, can be carried out quickly. The method involves the preparation of a diluted solution of the oil in hexane with an added measuring standard, the most convenient being squalane, and elution in a silicagel column with mixtures of hexane/diethyl ether 95:5 (v/v). Squalene has an elution volume of approximately 85 ml; the eluted is concentrated and analyzed by GC with SE-54 or SE-52 columns (Fig. 6.12). The analysis is completed in less than 10 min, and the estimated response factor of squalene with respect to squalane is 0.963.

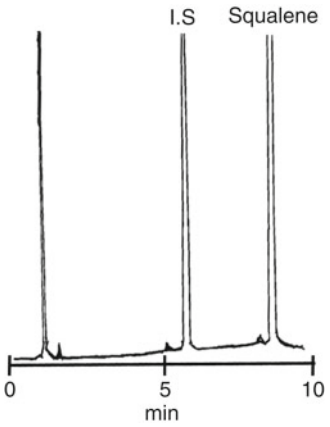


Fig. 6.12 Chromatogram showing virgin olive oil squalene eluted with SE-54 column (*Source*: Authors)

6.4.3.2 Determination of Stigmastadienes

Lanzón (1990) was the first to observe that some hydrocarbons are not naturally found in VOO, even though they show up during refining processes because of sterol dehydration; a typical case is the formation of stigmasta-3,5-diene from the dehydration of β-sitosterol. The consequence was the implementation of an analytical method particularly suited to detecting the presence of refined vegetable oils (e.g., olive, olive-pomace, sunflower, soybean, palm) in VOO, once it was established that refined oils contained stigmastadienes and virgin oils did not after analyzing thousands of samples inside the SEXIA project (Chap. 12). The method, based on the isolation of unsaponifiable matter, separation of steroidal hydrocarbon fractions by column chromatography on silicagel, and analysis by GC (capillary column of 25 m length coated with 5 % phenylmethylsilicone phase), has not been modified essentially in the official method (IOC 2001d). There are, however, proposals to improve it by a slight modification of the second separating step of the preparation of unsaponifiable matter. The use of less hexane and the elimination of the process of washing hexane extracts have been suggested, although no information has been reported about whether the changes improve the percentage of the relative standard deviation in reproducibility – $RSD_R > 6.2$ % – of the official method. Another proposal that uses a smaller amount of the sample (0.5 g of oil) is based on the purification of the sample on a silicagel column (15 g) with hexane (110 ml) and the quantification by GC (DB5 column of 25 m length) with an on-column injector and FID detector. RSD_R (%) is not, however, better than the official method.

6.4.3.3 Determination of n-alkanes, n-alkenes, and Terpenic Hydrocarbons

Hydrocarbons are not only used for detecting the presence of any refined edible oil in VOOs but also for VOO geographical traceability by means of the individual

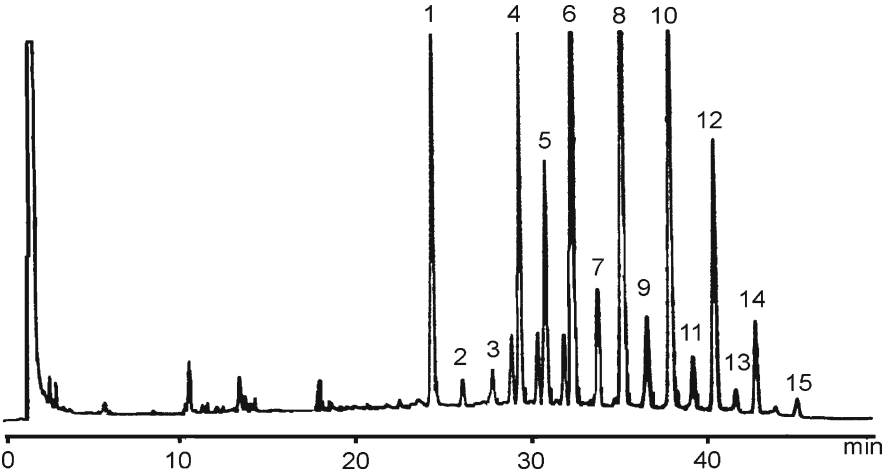


Fig. 6.13 Saturated linear hydrocarbon profile of olive oil obtained by gas–liquid chromatography. Notes: *1*: internal standard; *2*: C-21; *3*: C-22; *4*: C-23; *5*: C-24; *6*: C-25; *7*: C-26; *8*: C-27; *9*: C-28; *10*: C-29; *11*: C-30; *12*: C-31; *13*: C-32; *14*: C-33; *15*: C-35 (*Source*: Authors)

concentration of n-alkanes, n-alkenes, and terpenic hydrocarbons (Chap. 12). The first step in the analytical determination of these hydrocarbons (Aparicio and Alonso 1994) is the addition of 1 mL of n-iecosane to 20 g of olive oil before the saponification of the mixture with potassium hydroxide in ethanol (10 % in 75 mL added) for 90 min. The solution is passed to a 500 mL decanting funnel, and 100 mL of distilled water is then added, and the resulting mixture is extracted twice with 100 mL of hexane. The resulting solution is then evaporated to dryness in a rotary evaporator at 30 °C under reduced pressure. If an emulsion appears, then the solution should be left for a short time to allow it break up.

The compounds are separated from other compounds of the unsaponifiable matter by a chromatographic column of 1.5 cm i.d. and 50 cm long, filled with 15 g of silicagel slurried in hexane. The total unsaponifiable matter, dissolved hexane, is introduced onto the column head and eluted with hexane at a rate of 1 mL/min. Two fractions of 60 and 75 mL can be collected and concentrated to approximately 1 mL in a rotary evaporator at 30 °C under reduced pressure, but it is only the first fraction that is of interest for the mentioned hydrocarbons. A gas chromatograph equipped with a capillary column (30 m × 0.75 mm, 1 μm film thickness) coated with SPB-1 (chemically bonded methylsilicone) was used to determine the hydrocarbons under a programmed oven temperature that begins at 110 °C for 6 min and then rises at a rate of 5 °C/min to 300 °C. Figure 6.13 shows the chromatogram of the compounds identified and quantified in a VOO *var.* Picual.

6.4.3.4 Determination of Pesticides and Polycyclic Aromatic Hydrocarbons

The use of pesticides to increase crop yields, by the control of pests and diseases, is an almost inevitable part of modern agricultural practices, and its usage is subject to strict regulations, especially as regards the maximum residual limits (MRLs) in olives and olive oils. Furthermore, researchers have reported the presence of polycyclic aromatic hydrocarbons (PAHs) in edible oils and fats (van Stijn et al. 1996; Moret and Conte 2000), for example, benzo[a,e]pyrene, benzo[k]fluoranthene, benzo[g,h,i]perylene. The trade standards of the IOC (IOC 2005) have set the MRL in olive-pomace oil, as benzo(a)pyrene by wet weight, at 2 μg/kg.

These compounds are present in olive and olive-pomace oils at very low concentrations, which has prompted the need to implement simple methods with suitable sensitivity, selectivity, and accuracy for routine analysis. However, the complexity of the oily matrix makes the analysis difficult for the direct determination of multiresidues in oils, in addition to the low levels of the analytes and the variety of interferents potentially present. Thus, sample pretreatment is an essential part of the analytical process as the analyst must extract traces of analytes from the oil sample and avoid potential interferences by removing coextracted components from the matrix. Traditional sample preparation, however, relies on tedious, time-consuming procedures. It generally consists of an extraction step (e.g., saponification with alcoholic KOH and liquid–liquid partitioning) followed by one or more purification procedures (column chromatography, TLC, SPE), ending with the analytical determination by HRGC coupled to FID or MS, or by HPLC with spectrofluorometric detection. The laborious saponification step is avoided by analysts by liquid–liquid partitioning between dimethyl formamide and an organic solvent (e.g., n-hexane, cyclohexane), followed by cleanup on silicagel (Moret and Conte 2000). The pretreatment for pesticides, however, involves partitioning between hexane or petroleum ether and acetonitrile, followed by cleanup by adsorption on alumina or silicagel (Hiskia et al. 1998).

Recently, researchers have been interested in the simultaneous determination of pesticides and PAHs in olive and olive-pomace oils. Ballesteros et al. (2006) have proposed an expeditious and robust method based on GC–MS/MS technique to identify and quantify mixtures of pesticides and PAHs in these types of samples. The authors optimized gel permeation chromatography, widely used in multiresidue methods, for cleanup of the fat matrix using a mixture of 2 mL of *n*-hexane and 10 mL of acetonitrile and a shaking time of 30 min.

6.4.4 Tocopherols and Tocotrienols

Tocopherols and tocotrienols, collectively known as tocols, are heteroacid compounds, with high molecular weight, having vitamin E activity. They are lipid-soluble compounds easily oxidized in the presence of light, oxygen, alkaline pH, or

traces of transition metal ions. The term vitamin E is used to describe all tocol and tocotrienol derivatives that qualitatively exhibit the biological activity of α-tocopherol. Vitamin E is represented by a family of structurally related compounds (vitamers), eight of which are known to occur in nature, having been isolated from vegetable oils and other plant materials (Kamal-Eldin and Appelqvist 1996; Bramley et al. 2000). Vitamin E is the general term employed to designate tocopherols and tocotrienols, including α, β, γ, and δ species. The structure of tocotrienols differs from tocopherols by the presence of three *trans* double bonds in the hydrocarbon tail, but both series contain a polar chromanol ring linked to an isoprenoid-derived hydrocarbon chain. Thus, α, β, γ, and δ species of both tocopherols and tocotrienols differ with regard to the number and position of methyl groups on the chromanol ring (Eitenmiller and Lee 2004; Sayago et al. 2007). Tocotrienols have not been detected in VOO, while only the first three tocopherols have been quantified in percentages that vary from 52–87 % for α-tocopherol to 15–20 % and 7–23 % for β- and γ-tocopherols, respectively.

Tocopherols contribute to VOO antioxidant properties – they are the most effective group of lipophilic phenolic antioxidants – and give stability to VOOs. Their profile and composition is often a criterion of purity. There are verifiable evidence of their role in the quality and authenticity of VOO as well as health (Sayago et al. 2007; García-González et al. 2009).

The total content of tocopherols has usually been measured by the Emmerie and Engels method, which is based on a colored reaction between ferrous ion and α,α-bipyridilum. The determination, simple and quick, is colorimetric at 520 nm by extrapolating from a calibrating line of α-tocopherol or hydroquinone. Other methods are available, but this one is considered the best, although color development might be reduced. This effect can be attributed to the following causes: (a) substances other than tocopherol that reduce the ferric chloride; (b) substances that darken and obstruct color development; and (c) fat oxidation products. Among the different remedies proposed to eliminate these phenomena, the most widely used are (a) sample washing with sulfuric acid in petroleum ether, (b) saponification in the presence of pyrogallol or ascorbic acid, and (c) purification by chromatography.

Recently, luminescent methods have also been used for the determination of total tocopherols in VOO without prior separation (Escuderos et al. 2009).

GC procedures are also used for tocopherol analysis. The unsaponifiable matter, obtained in the presence of pyrogallol, must be extracted in advance with diethyl ether or cyclohexane. The unsaponifiable fraction is dissolved in hexane, deposited on a silicon-dioxide gel plate, and developed with hexane/ether (70:30, v/v). The tocopherol band is recovered with heptane/absolute alcohol (2:1, v/v) and analyzed in capillary columns after silanization (IUPAC 1987c) and once an internal standard is added. The elution order is δ-, γ-, and α-tocopherols (Slover et al. 1983). However, tocopherols coelute with epoxy squalenes in the preparative TLC fractionation of the unsaponifiable matter. These compounds derive from squalene oxidation and are precursors of steroid biosynthesis (i.e., cycloarthenol). The concentration of epoxy squalenes in olive oils depends on olive ripeness.

To eliminate the effects of tocopherol deterioration, due to the high temperatures used in GLC, HPLC methods have been developed. Tocopherols in oils can be determined either by direct normal-phase HPLC or by reversed-phase after saponification. HPLC is usually coupled with UV (usually 292 nm) or fluorescence detection at fixed wavelengths or electrochemical detection at a fixed oxidation/reduction potential (Cunha et al. 2006).

The method currently recommended by IUPAC (1987d) for the determination of tocopherols in VOO employs the direct analysis of the oil sample using normal-phase HPLC and fluorescence or UV detection. Tocopherols are directly analyzed through olive oil injection, dissolved in a hexane:isopropanol (99.8:0.2, v/v) mobile phase, in a Si-60 5 μm Lichrosorb column with a length of 250 mm and an internal diameter of 4 mm. The fluorescence detector characteristics are $\lambda_{ext} = 290$ nm and $\lambda_{em} = 330$ nm (IUPAC 1987d).

SPE has also been reported as an adequate procedure for sample preparation prior to HPLC analysis of tocopherols in VOO (Grigoriadou et al. 2007).

6.4.5 Sterols

The sterolic fraction is the most studied unsaponifiable matter. Sterols make up an extensive series of compounds with an analogous molecular structure; more than 200 have been reported in plants. They are made up of a tetracyclic cyclopenta[a] phenanthrene ring and a long flexible side chain at the C-17 carbon atom (Piironen et al. 2000). Based on the number of methyl groups at the C-4 position, sterols can be categorized into three groups, two (4,4-dimethyl), one (4-monomethyl), or none (4-desmethyl). 4,4-dimethylsterols and 4-monomethylsterols are metabolic intermediates in the biosynthetic pathway leading to end-product 4-desmethyl phytosterols, but they are usually present at low levels in most plant tissues (Moreau et al. 2002). 4-desmethylsterols, the most abundant class in olive oil, are commonly called phytosterols, while 4,4-dimethylsterols are called triterpenic alcohols, and 4-monomethylsterols are named methylsterols.

6.4.5.1 Phytosterols or 4-Desmethylsterols

The 4-desmethylsterols identified in olive oil (Azadmard-Damirchi and Dutta 2006) are cholesterol, 24-methylenecholesterol, campesterol, campestanol, stigmasterol, β-sitosterol, Δ^7-campesterol, $\Delta^{5,23}$-stigmastadienol, chlerosterol, sitostanol, Δ^5-avenasterol, Δ^7-avenasterol, $\Delta^{5,24}$-stigmastadienol, and Δ^7-stigmastenol; the normal levels of some of them are specified in various regulations (IOC 2010a; EC 2011). However, some of them have not been quantified in VOOs but appear in refined olive oils due to the refining process (e.g., $\Delta^{5,23}$-stigmastadienol), which makes it possible to distinguish ROOs from VOOs. Thus, $\Delta^{5,24}$-stigmastadienol increases its concentration during the oil-refining process, while the concentration of

Δ^5-avenasterol decreases (Amelotti 1985). Campesterol, stigmasterol, and β-sitosterol also suffer some degradation during refining (dehydration), although this cannot be directly observed in the chromatographic sterol profile since the amount of sterols that dehydrate is small in relation to the initial levels. In order to observe dehydration, it is necessary to analyze the hydrocarbon fraction searching for sterenes by the method described above.

Because the composition of 4-desmethylsterols is also typical of each oil or fat, it is very widely and reliably used to detect fraudulent mixtures of olive oil with other oils and fats (Chap. 16); for example, free and esterified sterols can detect the presence of refined hazelnut oil in olive oil (Mariani et al. 2006). When oils with a low sterol level (desterolized) are used to adulterate olive oil, even if determination of the sterols is difficult, confirmation can be obtained by analyzing the dehydration byproducts of sterols, which increase spectacularly with this practice (IOC 1997). The concentration of 4-desmethylsterols also varies with the cultivars, allowing for the varietal olive oil characterization (García-González et al. 2009).

The classic procedure to determine sterols consists of a gravimetric analysis, precipitating them as insoluble digitonids in an alcoholic solution of the fat and afterward determining the free sterols based on the fact that sterol esters do not combine with digitonin. Therefore, to determine the total sterols, it is necessary to free the combined sterols, which is achieved by saponification (Mehlenbacher 1960). Sterols that contain unsaturated bonds – such as cholesterol, ergosterol, and stigmasterol – can be determined by means of the Lieberman–Buchard colored reaction, color intensity being proportional to unsaturated sterol concentration. The reaction consists in adding acetic anhydride and sulfuric acid until a pink color appears.

At the present time, however, chromatographic methods are the most widely used for determining the composition of phytosterols. For the quantitative and qualitative analysis of sterols by GC or GC-MS it is necessary to separate and preconcentrate the different fractions of sterols. Such separation is necessary because some sterols of the various groups can overlap during GC analysis (Azadmard-Damirchi et al. 2005). Different methods are used to carry out the sample pretreatment; they are based on TLC, HPLC, and SPE.

Generally, the result of determination sterols determination comprising the sum of both possible forms (free and esterified) as their trimethylsilyl (TMS) ethers or sterol acetates, which improves their peak shape, volatility, and response factor (IOC 2001b; Cercaci et al. 2007). For their determination, it is necessary to isolate in advance the unsaponifiable fraction, preferably through the diethyl ether procedure, which guarantees total extraction of all sterols. Subsequently, the unsaponifiable material is purified by TLC on silicagel with different developing liquids, for example, hexane/ethyl acetate (85:15, v/v) or hexane/diethyl ether (60:40 or 70:30, v/v). The plate can be impregnated with alcoholic KOH for the purpose of retaining the FFAs in the unsaponifiable fraction. Then the sterols are recovered from the plate by scraping it and extracted with isopropyl ether, chloroform, or diethyl ether. Three large bands appear in the TLC plate. The first one contains triterpenic alcohols, some phytol, and aliphatic alcohols. The second is composed of the remaining

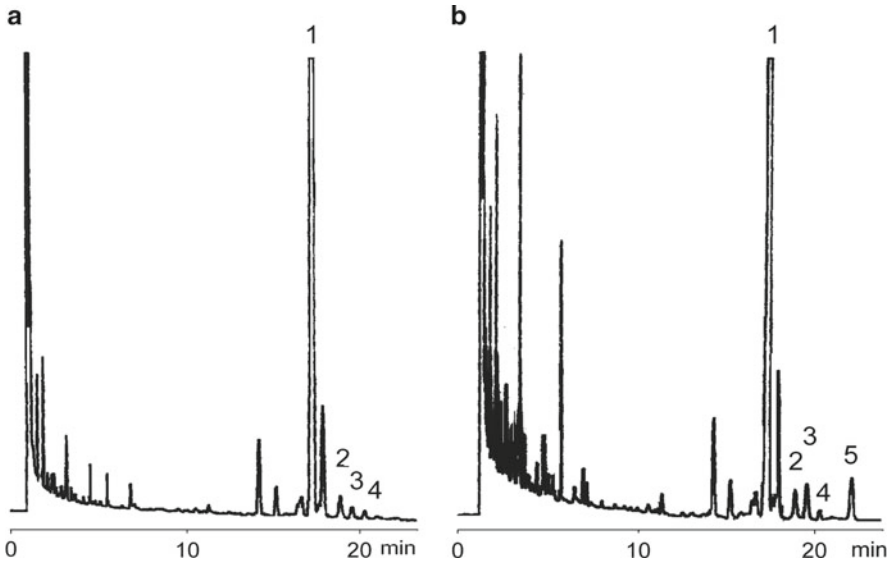


Fig. 6.14 Chromatogram of olive oil sterols analyzed by high-resolution gas chromatography. (**a**) using 200 μl of unsaponifiable; (**b**) using 300 μl of unsaponifiable matter. Note: *1*: β-sitosterol; *2*: $\Delta^{5,24}$-Stigmastadienol; *3*: Δ^{7}-stigmastenol; *4*: Δ^{7}-avenasterol; *5*: 24-methylene-cycloarthanol. (Camacho and Cert (1994), with permission of Grasas y Aceites)

phytol, aliphatic alcohols, and methyl sterols, and the third band contains sterols, erythrodiol, and uvaol.

However, the individual separation of sterols is not exempt from difficulties that can arise in determining components that appear at low concentrations. Such difficulties are due to the incomplete separation of the sterol fraction in TLC on silica-gel. The problem might be attributed to various causes resulting from an excess of the unsaponifiable matter deposited on the thin layer, an inadequate development of the plate, or defective scraping of the sterol band.

The optimum amount to deposit on the thin layer is 300 μL of a solution of the unsaponifiable fraction in chloroform. With such an amount, if the triterpenic alcohol content is high, an incomplete separation between these compounds and sterols usually takes place, causing small quantities of cycloarthenol and 24-methylene-cycloarthanol to remain at the same r_f as the phytosterols. As the gas-chromatographic analysis of the sterol fraction is being carried out, the cycloarthenol overlaps with the Δ^{7}-stigmastenol and the 24-methylene-cycloarthanol yields a peak at a retention time of 1.27 in relation to β-sitosterol (Fig. 6.14a). Therefore, when there is a peak at a relative retention time of 1.27 in the chromatographic register of a sterol fraction, it is likely that the value of the Δ^{7}-stigmasterol will be increased by the cycloarthenol, which can cause errors. Inasmuch as high-resolution GC is sufficiently sensitive in this case, it is wise to deposit only about 200 μL of the unsaponifiable solution on the thin layer. In this way, interference is prevented (Fig. 6.14b).

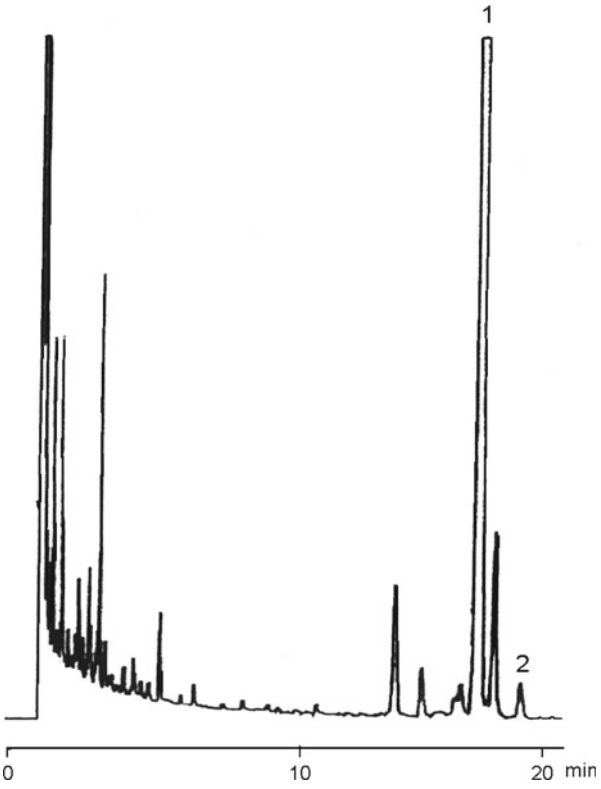


Fig. 6.15 Chromatogram of olive oil sterols showing loss of Δ^7-sterols due to an incomplete recovery from thin-layer chromatography (Camacho and Cert (1994), with permission of Grasas y Aceites)

As far as the possible mobile phases for TLC are concerned, hexane/diethyl ether (65:35, v/v) yield excellent results in that case, with just one development being applied in both cases. Experience shows, however, that the plate must undergo two developments in order to obtain a satisfactory separation.

It has also been proved that in TLC, Δ^7-sterols offer a slightly lower r_f than do the other sterols. As a result, if the sterol band is scraped too close to the lower edge, a significant amount of the former sterols can be lost (Fig. 6.15).

Because of the potential problems in using TLC to isolate the sterol fraction from the rest of the unsaponifiable material, as well as the high elution times needed, researchers have simplified the analysis of this and other fractions of unsaponifiable matter by means of an HPLC-GLC combination (Grob et al. 1990). The online coupling of an HPLC column with the stationary phase of silicagel with GC involves either the analysis of the underivatized sterol fraction or unsaponifiable derivatization before its injection onto the liquid chromatograph. An alternative method consists in using columns of reversed-phase C-18 liquid to separate the free sterol fraction from an oil sample. The problem of the transfer of great amounts of water or similar solvents that are usually used in this kind of phase is solved by using GC

Table 6.7 Comparison of results from analysis of sterolic fraction of different samples purified by HPLC and TLC

	Sample 1		Sample 2		Sample 3		Sample 4	
Sterols	HPLC	TLC	HPLC	TLC	HPLC	TLC	HPLC	TLC
24-methylenecholesterol	–	–	0.13	–	–	–	0.17	–
Campesterol	4.12	3.4	3.28	3.2	3.44	3.29	3.15	3.05
Campestanol	0.24	–	0.33	0.33	0.31	0.33	0.32	0.31
Stigmasterol	1.66	1.57	1.18	1.21	1.4	1.36	1.27	1.24
Δ^7-campesterol	0.31	0.38	0.19	0.16	–	–	–	–
$\Delta^{5,24}$-stigmastadienol	–	–	1.37	1.31	–	–	–	1.46
Clerosterol	0.93	0.84	1.71	1.49	1.27	1.00	1.76	1.54
B-sitosterol	83.74	84.56	82.73	83.59	83.45	83.78	84.41	83.89
Sitostanol	1.24	1.3	1.06	0.87	0.92	0.88	0.89	0.81
Δ^5-avenasterol	5.83	6.1	4.42	4.2	7.25	7.45	4.25	4.03
$\Delta^{5,23}$-stigmastadienol	0.55	0.41	2.46	2.36	0.69	0.53	2.42	2.3
Δ^7-stigmastenol	0.77	0.71	0.44	0.5	0.53	0.47	0.58	0.54
Δ^7-avenasterol	0.44	0.51	0.58	0.63	0.56	0.59	0.65	0.69
Total sterols (mg/kg)	3,642	3,792	1,901	1,875	1,719	1,683	1,816	1,891

Note: Sample 1: olive-pomace oil spiked with sunflower oil; Samples 2–4: olive oil. Values in percentage except for total sterols

with a programmed temperature vaporizer (PTV) injector instead of on-column injection and solvent-elimination precolumns (Señoráns et al. 1995, 1996).

The most reliable and widely used technique is the off-line HPLC-GLC coupling (i.e., separation of the fractions of sterols or other unsaponifiable components by HPLC with a silicagel column, collection of the fraction, elimination of the solvent, further derivation, collection of the fraction, elimination of the solvent, further derivation, and injection onto GC). This technique is identical to the traditional method except for the substitution of TLC purification for HPLC, which involves considerable savings in both time and solvent. For example, for correct separation of the sterol fraction from total unsaponifiables on a 20×20 cm thin layer with a 0.24 mm phase width, it is necessary to use approximately 100 mL of eluting mixture, with the developing time being approximately 1 h or more. When the separation is performed in a 250 mm long HPLC silicagel column, with an internal diameter of 4 mm and 5 μm particle size, it takes only 14 min, with the solvent consumption being approximately 15 mL. Table 6.7 shows data on the sterol content of various samples analyzed at the Instituto de la Grasa through purification using TLC and HPLC.

It can be observed that Δ^7-sterols give a higher value when sterols are purified by means of HPLC, which might point to their loss following TLC because of scraping the sterol band too narrowly.

Sterol injection can be performed either directly or after derivation with a silanizing reactant. The sterols are then separated by GC in nonpolar capillary columns, such as SE-52 or SE-54 (Lercker et al. 1981). A common column phase used for

general phytosterol analysis is composed of 95 % dimethyl-5 % diphenyl-polysiloxane (DB-5, HP-5, PTE-5, RTX-5, SPB-5), but sitosterol and Δ^5-avenasterol overlap to some extent. Mid-polarity columns achieve good resolution of Δ^5-sterols from Δ^7-sterols. Thus, for example, DB-1701 column (14 % cyanopropyl-phenyl-methylpolysiloxane) has been suggested by Dutta and Normén (1998), while thermostable GC columns with a stationary phase of 65 % phenyl-35 % dimethylpolysiloxane have been proven to separate efficiently the TMS derivatives of compounds that have a higher boiling point than β-sitosterol (Rodriguez-Estrada et al. 2002).

Sterol quantification is carried out by adding an internal standard, α-cholestanol (EC 1991), which presents a relative retention time to β-sitosterol similar to that of cholesterol. This method can present a disadvantage. As the chromatographic column ages and loses resolving potential (TPN), both sterols might coincide in retention time.

Some methodologies have been suggested the independent analysis of free sterols by using liquid chromatography in columns with nonpolar absorbents and even HPLC systems attached to mass spectrometry (Amelotti 1985). The prevalent methodologies used for their isolation and determination were based on gas chromatography (Bortolomeazzi et al. 1990; Biedermann et al. 1993; Cercaci and Lercker 2000) although attention should be paid to the percentages of recovery that can be lower than 60–70 % because of the methodology used (Cercaci and Lercker 2000). However, it is possible an independent and adequate quantification of the single fractions of free and esterified sterols by a preparative method that involves the separation of the nonpolar fraction (containing esterified sterols) from the polar fraction (containing free sterols) by silicagel column chromatography (Mariani et al. 2006). The proposed method has three steps: (i) the preparation of the silicagel chromatography column, (ii) the separation of the free and esterified sterols, and (iii) the independent quantification of free and esterified sterols by GLC (IOC 2001b).

Prior to the separation of the nonpolar–polar fractions, the silicagel chromatography column, containing 25 g of silicagel and 80 ml of n-hexane, should be washed with n-hexane, opening the stopcock, and the solvent should be allowed to flow away until it reaches approximately 2 mm above the silicagel. Concerning the olive oil sample, 5 gm – to which 1 mL of an internal standard solution (100 mg of α-cholestanol and 50 mg cholesteryl stearate in 100 mL of *n*-hexane) was added – is dissolved with 20 mL of n-hexane. The resulting sample is passed through the chromatography column until the sample reaches about 2 mm above the silica surface. Then 150 mL of an *n*-hexane/diethyl ether mixture (87:13 v/v) is passed (flow rate: 2 ml/min). The eluate contains the nonpolar fraction (esterified fraction), which is evaporated completely by means of a rotary evaporator. Immediately 150 mL of diethyl ether is passed through the column and the new eluate, which contains the polar fraction (free sterols), is also evaporated completely. The nonpolar fraction is saponified with an ethanolic potassium hydroxide solution (IOC 2001b) and the unsaponifiable matter is extracted with diethyl ether. The sterol fractions are separated by TLC and analyzed by GC with a FID and a capillary column (25 m × 0.25 mm × 0.15 μm) coated with SE-52.

6.4.5.2 Triterpenic Alcohols or 4,4-Dimethylsterols

Triterpenic alcohols (4,4-dimethyl sterols), which are not present in many other edible oils, have an extra OH or COOH group in comparison with triterpenic dialcohols, such as erytrodiol and uvaol.

Triterpenic alcohols, as the total fraction of aliphatic alcohols, are analyzed by saponifying olive oil with potassium hydroxide after the addition of an internal standard like C-20 or C-21 and by extracting the unsaponifiable matter with diethyl ether and later purifying the unsaponifiable material on a plate. Triterpenic alcohols are more polar than tocopherols, which often leads to an insufficient separation in silica TLC. However, TLC isolation of linear and triterpenic alcohols is not complicated, and their separation from each other can be achieved on a 20×40 silicagel plate with a thickness of 0.25 mm and elution with hexane/ethyl acetate (80:15, v/v), where the r_f are phytosterols = 0.24, linear alcohols = 0.38, triterpenic alcohols 6 = 0.52. An SPE method was recently developed with stepwise elution by increasing the polarity of solvent mixture (*n*-hexane and diethyl ether) in order to separate olive oil sterol fractions (Azadmard-Damirchi and Dutta 2006) without the potential TLC problems. The SPE method is simpler and less time consuming than TLC and results in higher recoveries of 4,4-dimethylsterols.

No analytical problems, however, have been observed for their GC determination in a nonpolar column (100 % dimethylpolysiloxane or a 5 % phenyl-95 % dimethylpolysiloxane). Concerning authentication, isomerization of triterpenic alcohols in the refining steps (Ntsourankoua et al. 1994) might evince illegal admixtures of VOOs with refined ones.

6.4.5.3 Methyl Sterols or 4-Monomethylsterols

Methyl sterols are intermediates of the biosynthesis of phytosterols from squalene and are present at relatively low levels in olive oils compared with other unsaponifiable components. Olive oil methyl sterols are separated by TLC with a mobile phase of hexane/ethyl acetate (85:15, v/v) (Aparicio and Alonso 1994).

GC analysis of methyl sterols can be carried out under the same conditions as for phytosterols (EC 1991). These compounds can be quantified together with linear alcohols in the same chromatogram (Aparicio and Alonso 1994).

6.4.6 Sterol Oxidation Products

Phytosterols are unsaturated molecules that are prone to the formation of sterol oxidation products (SOP) as hydroxy, keto, epoxy, and triol derivatives; for example, oxides of β-sitosterol, campesterol, and stigmasterol can be produced by

thermo-oxidation (7-hydroxy-, 7-keto-, and epoxy-derivatives) and chemical synthesis (triol derivatives).

The first step for determining SOPs is a cold saponification to diminish the formation of artifacts and the decomposition of keto and epoxy derivatives. The internal standard should be added to the sample beforehand to allow quantification. 19-hydroxycholesterol is the most common internal standard, although a double standard (7α-hydroxycholesterol and 7-ketocholesterol) is suggested for accurate quantification of hydroxyl and keto derivatives of phytosterols in olive oils (Bortolomeazzi et al. 2003).

In a second step, sterol oxides are isolated and enriched by silica or NH_2 SPE. The SPE method, however, is not able to completely remove sterols from the sterol oxide fraction, so the remaining sterols can overlap with some SOPs during GC analysis. GC-FID or GC-MS are used for SOP quantification (Johnsson and Dutta 2003; Apprich and Ulberth 2004). GC-MS is recommended for identification purposes due to overlaps, though LC-MS allows for the determination of SOPs without sample pretreatment and derivatization (Kemmo et al. 2007, 2008). Nonpolar columns have been used for SOP separation (100 % dimethylpolysiloxane or a 5 % phenyl-95 % dimethylpolysiloxane), though thermostable polar columns also provide efficient separations (Rodriguez-Estrada et al. 2002). A better determination has been achieved by combining two capillary columns of different polarities (midpolar and a nonpolar columns) (Johnsson and Dutta 2003).

6.4.7 Linear Alcohols

Linear alcohols can be either of an even chain of carbon atoms, which is the case with olive oil alcohols, or of an odd chain, as in some seed oils; this criterion is used to identify mixtures of these seed oils with olive oil (Oborn and Ullman 1986). Olive oil linear alcohols are composed of a homolog series of primary fatty alcohols having 20–32 carbon atoms; however, some of them, such as phytol, are artifacts generated by saponification (Fig. 6.16). Phytol, which is present in VOOs at low concentrations, is generated from chlorophyll decomposition by alkaline treatment during saponification.

As with phytosterols, the free and esterified alcohols in olive oil can be quantified. For the determination of free alcohols, the oil sample dissolved in hexane is deposited on a silicagel plate or in a column of the same material and eluted with hexane:diethyl ether (60:40 or 70:30, v/v). They can be directly chromatographed, while silanized derivatives can be used with an SE-30 or SE-54 or SE-52 nonpolar column (EC 1991). After acetylation, they can also be chromatographed on polar columns (diethylene glycol succinate). The use of capillary columns makes the analysis much easier because of their separating power. Initially, the cold-on-column injection procedure came into use because split injection produced discrimination of the sample in relation to the alcohol chain length. At present, however, split injectors no longer present this handicap and can be used in the

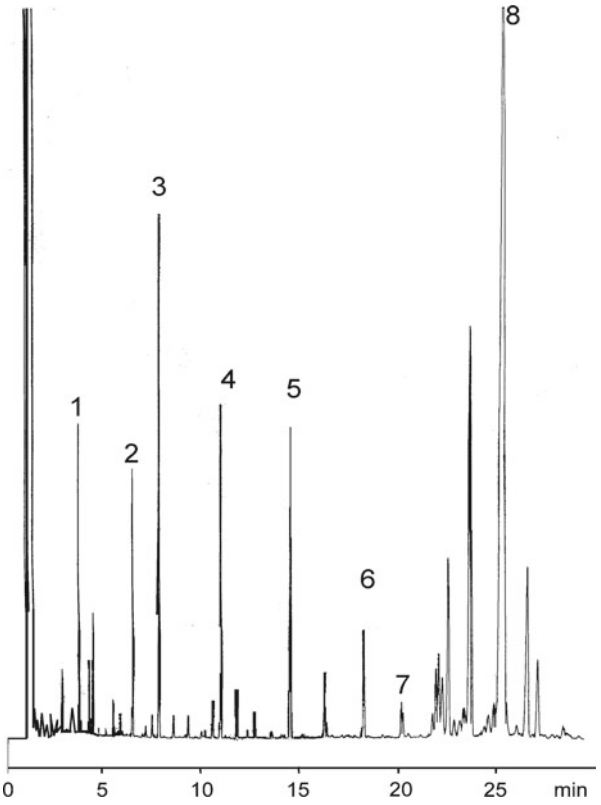


Fig. 6.16 Chromatogram showing olive oil aliphatic alcohols and 4,4-methyl sterols using a capillary column with split injection. Notes: *1*: phytol; *2*: geranyl-geraniol; *3*: internal standard (C-21); *4*: C-22; *5*: C-24; *6*: C-26; *7*: C-28; *8*: methylene-cholesterol (*Source*: Authors)

analysis of this type of compound, which means a noticeable improvement over cold-on-column injection.

6.4.8 Triterpenic Dialcohols

The presence of two pentacyclic triterpenic alcohols, erythrodiol and uvaol, has been observed in the unsaponifiable fraction of both olive-pomace oil and olive oil, their chemical structure having been revealed by GC-MS. Analytical determination of these two diols is put into practice, together with that of the sterol fraction (Fig. 6.17). Until now, their quantification has been accomplished by referring them to the whole fraction of sterols plus triterpenic dialcohols (EC 1991). The resolution of these diols has improved a great deal with the use of capillary columns, and, although not habitually practiced, they can be quantified using betulin as an internal standard. Betulin is used because it is an isomer of erythrodiol and, hence, its

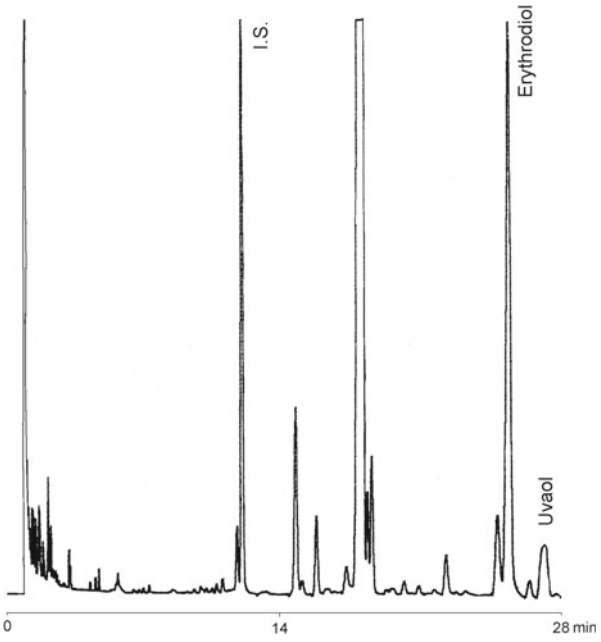


Fig. 6.17 Chromatogram of olive-pomace oil showing triterpenic alcohols: erythrodiol and uvaol (*Source*: Authors)

response factor is 1. In olive oil, this determination is carried out in order to elucidate the presence of olive-pomace oil in olive oil (Mariani et al. 1987), although it must be assessed together with the wax or aliphatic alcohol content to ascertain the presence of olive-pomace oil because the content of erythrodiol, like that other compounds, also depends on olive variety.

6.5 Challenges and Perspectives

The panoply of analytical methods described in this chapter for quantifying chemical compounds present in olive oils and olive-pomace oils are not only due to the interest of researchers in a better knowledge of this edible oil but also because olive oil is one of the most strictly regulated food products.

Instrumental advances have led to great success in the identification and quantification of chemical compounds with validated methodologies; most of them have been internationally accepted for trade standards. However, and despite the arsenal of analytical techniques available at the moment, a considerable investment in perfecting techniques and developing new ones is still required to solve new authenticity and traceability issues. Today, there is a clamor, from all actors involved in the olive oil world, for a rapid and universal method that can be used for all those issues, although today this vision remains a utopia.

Meanwhile, certain objectives have been achieved that are related to the methods' validation (Chap. 16) and the building of a large database that makes it possible to know the reproducibility and robustness with fine certainty factors. Furthermore, official methods should be open to techniques other than chromatography since they can offer rapid, robust, and precise analyses of many of the series of chemical compounds analyzed in this chapter. Finally, although the perfecting of analytical methods is the main objective of the chemical analyst, this objective should not be limited exclusively to the improvement of columns, solvents, and so on but also used for the analysis of results obtained with chemometric tools.

References

Amelotti G (1985) Problematiche inerenti la valutazione della frazione sterolica. Riv Ital Sost Grasse 62:337–344

Aparicio R (2004) Final report project G6RD-CT2000-00440 (MEDEO). Commission of the European Communities (Growth Program), Brussels (Belgium)

Aparicio R, Alonso V (1994) Characterization of virgin olive oils by SEXIA expert system. Prog Lipid Res 33:29–38

Aparicio R, McIntyre P (1998) Fats and oils authentication. In: Lees M (ed) Food authenticity: issues and methodologies. Eurofins Scientific, Nantes

Aparicio R, Morales MT, Alonso V (1997) Autentication of European virgin olive oils. J Agric Food Chem 45:1076–1083

Apprich S, Ulberth F (2004) Gas chromatographic properties of common cholesterol and phytosterol oxidation products. J Chromatogr A 1055:169–176

Aragón A, Toledano RM, Cortes JM, Villén J, Vázquez A (2011) Wax ester composition of monovarietal olive oils from designation of origin (DO) "Campos de Hellin". Food Chem 129:71–76

Azadmard-Damirchi S, Dutta PC (2006) Novel solid-phase extraction method to separate 4-desmethyl-, 4-monomethyl-, and 4,4'-dimethylsterols in vegetable oils. J Chromatogr A 1108:183–187

Azadmard-Damirchi S, Savage GP, Dutta PC (2005) Sterol fractions in hazelnut and virgin olive oils and 4,4'-dimethylsterols as possible markers for detection of adulteration of virgin olive oil. J Am Oil Chem Soc 82:717–725

Ballesteros E, García Sánchez A, Ramos Martos N (2006) Simultaneous multidetermination of residues of pesticides and polycyclic aromatic hydrocarbons in olive and olive-pomace oils by gas chromatography/tandem mass spectrometry. J Chromatogr A 1111:89–96

Biedermann M, Grob K, Mariani C (1993) Transesterification and on-line LC-GC for determining the sum of free and esterified sterols in edible oils and fats. Fat Sci Technol 95:127–133

Biedermann M, Haase-Aschoff P, Grob K (2008) Wax ester fraction of edible oils: analysis by on-line LC-GC-MS and GC×GC-FID. Eur J Lipid Sci Technol 110:1084–1094

Bortolomeazzi R, Frega N, Lercker G (1990) Rapid determination of free and esterified sterols using prepacked silica columns. Ital J Food Sci 4:231–234

Bortolomeazzi R, De Zan M, Pizzale L, Conte LS (2000) Identification of new steroidal hydrocarbons in refined oils and the role of hydroxy sterols as possible precursors. J Agric Food Chem 48:1101–1105

Bortolomeazzi R, Cordaro F, Pizzale L, Conte LS (2003) Presence of phytosterol oxides in crude vegetable oils and their fate during refining. J Agric Food Chem 51:2394–2401

Boukhchina K, Sebai A, Cherif H, Kallel P, Mayer M (2004) Identification of glycerolphospholipids in rapessed, olive oil, almond, and sunflower oils by LC-MS and LC-MS-MS. Can J Chem 82:1210–1215
Bramley PM, Elmadfa I, Kafatos A, Kelly FJ, Roxborough HE, Schuch W, Sheehy PJA, Wagner KH (2000) Vitamin E. J Sci Food Agric 80:913–938
Calvano CD, Palmisano F, Zambonin CG (2005) Laser desorption/ionization time-of-flight mass spectrometry of triacylglycerols in oils. Rapid Commun Mass Spectrom 19:1315
Carelli A, Cert A (1993) Comparative study of the determination of triacylglycerol in vegetable oils using chromatographic techniques. J Chromatogr 630:213–222
Cercaci L, Lercker G (2000) Proposta di un metodo di determinazione degli steroli liberi e degli steroli totali nelle sostanze grasse. Boll Chim Igien 51:177–183
Cercaci L, Passalacqua G, Poerio A, Rodriguez-Estrada MT, Lercker G (2007) Composition of total sterols (4-desmethyl-sterols) in extra virgin olive oils obtained with different extraction technologies and their influence on the oil oxidative stability. Food Chem 102:66–76
Cert A, Di Giacinto L, Faberi A, Mariani C, Lacoste F et al (2011) New methods proposed to IOC. Internal communication
Christie WW (1992) Detectors for high-performance liquid chromatography of lipids with special reference to evaporative light-scattering detection. In: Christie WW (ed) Advances in lipid methodology – one. Oily Press, Ayr, pp 239–271
Christie WW (2011a) Preparation of ester derivatives of fatty acids for chromatographic analysis. In: Christie WW (ed) The AOCS lipid library. AOCS Press. Available from http://lipidlibrary.aocs.org/topics/ester_93/index.htm
Christie WW (2011b) What is a lipid? In: Christie WWW (ed) The AOCS lipid library. AOCS Press. Available from http://lipidlibrary.aocs.org/lipids/whatlip/index.htm
Cozzolino R, De Giulio B (2011) Application of ESI and MALDI-TOF MS for triacylglycerols analysis in edible oils. Eur J Lipid Sci Technol 113:160–167
Cuhna S, Oliveira MBPP (2006) Discrimination of vegetable oils by triacylglycerols evaluation profile using HPLC/ELSD. Food Chem 95:518–524
Cunha SC, Amaral JS, Fernandes JO, Oliveira MB (2006) Quantification of tocopherols and tocotrienols in portuguese olive oils using HPLC with three different detection systems. J Agric Food Chem 54:3351–3356
Dobarganes CM, Márquez-Ruiz G (2007) Formation and analysis of oxidized monomeric, dimeric and higher oligomeric triglycerides. In: Erickson MD (ed) Frying: chemistry, nutrition and practical applications, 2nd edn. AOCS Press, Champaign, pp 87–110
Dutta PC, Normén L (1998) Capillary column gas–liquid chromatographic separation of Δ5-unsaturated and saturated phytosterols. J Chromatogr A 816:177–184
Eitenmiller RR, Lee J (2004) Vitamin E, food chemistry, composition and analysis. Marcel Dekker, New York, p 2
Escuderos ME, Sayago A, Morales MT, Aparicio R (2009) Evaluation of α-tocopherol in virgin olive oil by a luminiscent method. Grasas Aceites 60:336–342
European Communities (EC) (1991) Official journal of the commission of the European communities. Regulation No. 2568/91, L248, 5 Sept 1991
European Communities (EC) (1992a) Official journal of the commission of the European communities. Regulation No. 1429/92, L150, 2 June 1992
European Communities (EC) (1992b) Official journal of the commission of the European communities. Regulation No. 1996/92, L199, 18 July 1992
European Communities (EC) (1993) Official journal of the commission of the European communities. Regulation No. 183/93, L 22, 30 Jan 1993
European Communities (EC) (2011) Official journal of the commission of the European communities.. Regulation No. 61/2011, L 23, 27 Jan 2011
Fasciotti M, Netto ADP (2010) Optimization and application of methods of triacylglycerol evaluation for characterization of olive oil adulteration by soybean oil with HPLC-APCIMS-MS. Talanta 81:1116–1125

Frega N, Bocci F, Lercker G (1993) High-resolution gas-chromatographic determination of diacylglycerols in common vegetable oils. J Am Oil Chem Soc 70:175–177
Garcés R, Mancha M (1993) One-step lipid extraction and fatty acid methyl esters preparation from fresh plant tissues. Anal Biochem 211:139–143
García-González DL, Viera-Macías M, Aparicio-Ruiz R, Morales MT, Aparicio R (2007) Validation of a method based on triglycerides for the detection of low percentages of hazelnut oil in olive oil by column liquid chromatography. J AOAC Int 90:1346–1353
García-González DL, Aparicio-Ruiz R, Aparicio R (2009) Olive oil. In: Kamal-Eldin A, Moreau R (eds) Gourmet and health-promoting speciality oils. AOCS Press, Urbana, pp 33–72
Geeraert E, Sandra P (1985) Capillary GC of triglycerides in fats and oils using a high temperature phenylmethylsilicone stationary phase, Part I. J High Resolut Chromatogr Commun 8:415–422
Geeraert E, Sandra P (1987) Capillary GC of triglycerides in fats and oils using a high temperature phenylmethylsilicone stationary phase. Part II. J Am Oil Chem Soc 64:100–105
Geeraert E, Schepper D (1982) Structure elucidation of triglycerides by chromatographic techniques. Part 1: capillary GC of ozonized triglycerides. J High Resolut Chromatogr Commun 5:80–84
Geeraert E et al (1987) Polar capillary GLC of intact natural diacyl and triacylglycerols. In: Kuksis A (ed) Chromatography of lipids in biomedical and clinical diagnosis. Journal of chromatography library, vol 37. Elsevier Science, Amsterdam, pp 48–75
Gertz C, Fiebig H-J (2006) Isomeric diacylglycerols – determination of 1,2- and 1,3 diacylglycerols in virgin olive oil. Eur J Lipid Sci Technol 108:1066–1069
Grandgirard A, Sebedio JL, Fleury J (1984) Geometrical isomerization of linolenic acid during heat treatment of vegetable oils. J Am Oil Chem Soc 61:1563–1568
Grigoriadou D, Androulaki A, Psomiadou E, Tsimidou MZ (2007) Solid phase extraction in the analysis of squalene and tocopherols in olive oil. Food Chem 105:675–680
Grob K, Lanfranchi M, Mariani C (1990) Evaluation of olive oils through the fatty alcohols, the sterols and their esters by coupled LC-GC. J Am Oil Chem Soc 67:626–634
Heo SH, Hou CT, Kim BS (2009) Production of oxygenated fatty acids from vegetable oils by *Flavobacterium* sp. strain DS5. N Biotechnol 26:105–108
Hiskia AE, Atmajidou ME, Tsipi DF (1998) Determination of organophosphorus pesticide residues in Greek virgin olive oil by capillary gas chromatography. J Agric Food Chem 46:570–574
Holčapek M, Jandera P, Zderadička P, Hrubá L (2003) Characterization of triacylglycerol and diacylglycerol composition of plant oils using high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. J Chromatogr A 1010:195–215
International Olive Council (IOC) (1997) COI/T.15/Doc. No 2/Rev. 6. Trade standard applying to olive oils and olive-pomace oils. Madrid
International Olive Council (IOC) (2001a) COI/T.20/Doc. No 24. Preparation of the fatty acids methyl esters from olive oil and olive-pomace oil. Madrid
International Olive Council (IOC) (2001b) COI/T.20/Doc. No 10/Rev. 1. Determination of the composition and content of sterols by capillary-column gas chromatography. Madrid
International Olive Council (IOC) (2001c) COI/T.20/Doc. No 17/Rev. 1. Determination of trans unsaturated fatty acids by capillary column gas chromatography. Madrid
International Olive Council (IOC) (2001d) COI/T.20/Doc. no. 11/Rev. 2. Determination of stigmastadienes in vegetable oils. Madrid
International Olive Council (IOC) (2005) RES-1/93-IV/05. Detection of polycyclic aromatic hydrocarbons in edible olive oils and olive-pomace oils. Madrid
International Olive Council (IOC) (2007) COI/T.20/doc. No. 18/Rev.2. Determination of wax content by capillary column gas chromatography. Madrid
International Olive Council (IOC) (2010a) COI/T.15/NC No 3/Rev.5. Trade standard applying to olive oils and olive-pomace oils. Madrid
International Olive Council (IOC) (2010b) COI/T.20/Doc. No 20/Rev. 3. Determination of the difference between actual and theoretical content of triacyglycerols with ECN 42. Madrid

International Olive Council (IOC) (2010c) COI/T.20/Doc. No 28/Rev. 1. Determination of the content of waxes, fatty acid methyl esters and fatty acid ethyl esters by capillary gas chromatography. Madrid

International Olive Council (IOC) (2011) Determination of composition and content of diacylglycerols by capillary gas chromatography, in vegetable oils. Internal report, Madrid

International Union of Pure and Applied Chemistry (IUPAC) (1987a) Method 2.301. Preparation of the fatty acid methyl esters. In: Paquot C, Haufenne A (eds) Standard methods for the analysis of oils, fats and derivatives, 7th edn. Blackwell Scientific Publications, Oxford, UK, pp 123–129

International Union of Pure and Applied Chemistry (IUPAC) (1987b) Method 2.507. Determination of polar compounds in frying fats. In: Paquot C, Haufenne A (eds) Standard methods for the analysis of oils, fats and derivatives, 7th edn. Blackwell Scientific Publications, Oxford, UK, pp 216–219

International Union of Pure and Applied Chemistry (IUPAC) (1987c) Method 2.411, identification and determination of tocopherols. In: Paquot C, Haufenne A (eds) Standard methods for the analysis of oils, fats and derivatives, 7th edn. Blackwell Scientific Publications, Oxford, UK, pp 174–182

International Union of Pure and Applied Chemistry (IUPAC) (1987d) Method 2.432, determination of tocopherols and tocotrienols in vegetable oils and fats by HPLC. In: Paquot C, Haufenne A (eds) Standard methods for the analysis of oils, fats and derivatives, 7th edn. Blackwell Scientific Publications, Oxford, UK, pp 2432/1–2432/7

Jakab A, Héberger K, Fornács E (2002) Comparative analysis of different plant oils by high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. J Chromatogr A 976:255–263

Johnsson L, Dutta PC (2003) Characterization of side-chain oxidation products of sitosterol and campesterol by chromatographic and spectroscopic methods. J Am Oil Chem Soc 80:767–776

Kalo PJ, Kemppinen A (2012) Regiospecific analysis of TAGs using chromatography, MS, and chromatography-MS. Eur J Lipid Sci Technol 114:399–411

Kamal-Eldin A, Appelqvist L (1996) The chemistry and antioxidant properties of tocopherols and tocotrienols. Lipids 31:671–701

Kemmo S, Ollilainen V, Lampi AM, Piironen V (2007) Determination of stigmasterol and cholesterol oxides using atmospheric pressure chemical ionization liquid chromatography/mass spectrometry. Food Chem 101:1438–1445

Kemmo S, Ollilainen V, Lampi AM, Piironen V (2008) Liquid chromatography mass spectrometry for plant sterol oxide determination in complex mixtures. Eur Food Res Technol 226:1325–1334

Koidis A, Boskou D (2006) The contents of proteins and phospholipids in cloudy (veiled) virgin olive oils. Eur J Lipid Sci Technol 108:323–328

Kuksis A, McCarthy MJ (1962) Gas–liquid chromatographic fractionation of natural triglyceride mixtures by carbon number. Can J Med Sci 40:679–686

Lanzón A (1990) Changes produced in the components of the unsaponifiable of olive oil as consequence of the refining process. Ph.D. thesis, University of Seville, Spain

León-Camacho M (1997) Isomerization of *cis-trans* fatty acids during the deodorization of edible oils. Ph.D. thesis, Faculty of Chemistry, University of Seville, Spain

Lercker G, Frega N, Conte LS, Capella P (1981) La gascromatografia su colonne capillari (HRGC) nello studio dell'insaponificabile degli oli vegetali. Riv Ital Sostanze Grasse 63:324–330

Mariani C, Venturini M (1996) Sull'aumento delle cere durante la conservazione degli oli di oliva. Riv Ital Sost Grasse 73:489–498

Mariani C, Fedeli E, Morchio G (1987) Sul contenuto di eritrodiolo assoluto quale possibilità di individuare il sansa nell´olio di oliva. Riv Ital Sost Grasse 64:359–363

Mariani C, Bellan G, Lestini E, Aparicio R (2006) The detection of the presence of hazelnut oil in olive oil by free and esterified sterols. Eur Food Res Technol 223:655–661

Márquez-Ruiz G, Dobarganes MC (2006) High-performance size-exclusion chromatography for lipid analysis in organic media. In: Mossoba MM, Kramer JKG, Brenna JT, McDonald RE

(eds) Lipid analysis and lipidomics: new techniques and applications. AOCS Press, Champaign, pp 205–238
Mehlenbacher VC (1960) The analysis of fats and oils. Garrard Press, Champaign
Moreau RA, Whitaker BD, Hicks KB (2002) Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. Prog Lipid Res 41:457–500
Moret S, Conte L (1998) Off-line LC-LC determination of PAHs in edible oils and lipidic extracts. J High Resolut Chromatogr 21:253–257
Moret S, Conte L (2000) Polycyclic aromatic hydrocarbons in edible fats and oils: occurrence and analytical methods. J Chromatogr A 882:245–253
Nimal Ratnayake WM, Hansen SL, Kennedy MP (2006) Evaluation of the CP-Sil 88 and SP-2560 GC columns used in the recently approved AOCS official method Ce 1h-05: Determination of *cis*-, *trans*-, saturated, monounsaturated, and polyunsaturated fatty acids in vegetable or non-ruminant animal oils and fats by capillary GLC method. J Am Oil Chem Soc 83:475–488
Nota G, Naviglio D, Romano R, Sabia V, Spagna-Musso S et al (1999) Determination of the wax ester content in olive oils. Improvement in the method proposed by EEC regulation 183/93. J Agric Food Chem 47:202–205
Ntsourankoua H, Artaud J, Guerere M (1994) Etude des alcools triterpeniques d'une huile d'olive vierge et d'une huile de grignons raffinée. Ann Fals Exp Chim 926:91–107
Oborn RE, Ullman AH (1986) A capillary gas chromatographic method for the characterization of linear fatty alcohols. J Am Oil Chem Soc 63:95–97
Pérez-Camino MC, Moreda W, Cert A (1996) Determination of diacylglycerol isomers in vegetable oils by solid-phase extraction followed by gas chromatography on a polar phase. J Chromatogr A 721:305–314
Pérez-Camino MC, Moreda W, Cert A (2001) Effects of olive fruit quality and oil storage practices on the diacylglycerol content of virgin olive oils. J Agric Food Chem 49:699–704
Pérez-Camino MC, Cert A, Romero-Segura A, Cert-Trujillo R, Moreda W (2008) Alkyl esters of fatty acids a useful tool to detect soft deodorized olive oils. J Agric Food Chem 56:6740–6744
Piironen V, Lindsay DG, Miettinen TA, Toivo J, Lampi AM (2000) Plant sterols: biosynthesis, biological function and their importance to human nutrition. J Sci Food Agric 80:939–966
Rodriguez-Estrada MT, Frega N, Lercker G (2002) Applications on thermostable polar capillary columns. Grasas Aceites 53:76–83
Ross KL, Hansen SL, Tu T (2011) Reversed-phase analysis of triacylglycerols by ultra-performance liquid chromatography-evaporative light scattering detection (UPLC-ELSD). Lipid Technol 23:14–16
Sayago A, Marín MI, Aparicio R, Morales MT (2007) Vitamin E and vegetable oils. Grasas Aceites 58:74–86
Señoráns FJ, Herraiz M, Tabera J (1995) On-line reversed-phase liquid chromatography-capillary gas chromatography using a programmed temperature vaporizer as interfase. J High Resolut Chromatogr Commun 18:433–438
Señoráns FJ, Tabera J, Herraiz M (1996) Rapid separation of free sterols in edible oils by on-line coupled reversed phase liquid chromatography-gas chromatography. J Agric Food Chem 44:3189–3192

Chapter 7
Chromatographic Methodologies: Compounds for Olive Oil Color Issues

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties*,
DOI 10.1007/978-1-4614-7777-8_7, © Springer Science+Business Media New York 2013

7.1 Introduction

Color and appearance constitute the first contact we have with food. They determine our preferences and influence our choices. In the set of feelings that food gives us, their influence is such that they can even subjectively modify other sensations such as taste and odor. Experience shows that when we do not see color, we have problems identifying the flavor.

Concerning olive fruit, its color changes from deep green to yellow-green and then to purple and black as it grows, ripens, and matures. The green color of the tissue is caused by chlorophyllic and carotenoid pigments, and their concentrations fall progressively during ripening, giving way to the synthesis of anthocyanin compounds, which appear first as small reddish spots on the skin (turning color or mottled fruit) that cover more and more of the fruit's surface in purple until it becomes black at full maturation. Finally, anthocyanin synthesis invades the interior of the pulp, pigmenting the whole fruit. Only chlorophylls and carotenoids, which are fat-soluble, are transferred to the virgin olive oil, giving it its highly valued natural color.

Virgin olive oil (VOO), as a natural food, has its own color, which can vary from deep green to gold, depending on the cultivar and olive ripeness. In terms of the total pigment content of the oil, the extraction process entails a loss of pigment, which affects the chlorophyll pigments (80%) more than the carotenoids (50%). Many factors, both agronomical and technological, can affect the pigment profile in VOO. Poor processing or storage conditions of olive oil can lead to a deterioration or destruction of its natural coloring compounds. In this case, artificial colorings are fraudulently added to the olive oil to retrieve the color expected by consumers. Although chlorophylls and carotenoids in food are mainly valued for this chromatic function, recent research has demonstrated that these compounds are more than just color in food technology. Besides their biological activity, their incidental transformations associated with the ripening or postharvest process, or those derived from the specific system of processing the food, make them quality indicators for the end product.

The aim of this chapter is to provide a basic understanding of the general aspects of these pigments such as distribution, location, structures, functions, biological activities, and, in particular, the spectroscopic and chemical properties that are necessary to know to carry out their identification. We propose to provide a critical description of the available information on methodologies used for chromatographic analysis, including both those allowing the direct injection of diluted oil and those that require an enrichment step using liquid-phase extraction (LPE) or solid-phase extraction (SPE). We also summarize information on the presence of chlorophyll and carotenoid pigments in fruit and olive oil and their changes in profile and concentration associated with metabolism, processing, and storage conditions. Finally, recent researches related to the kinetics of pigments, prediction models of their evolution over time, and future trends in methods of pigment analysis are presented.

7.2 General Aspects of Chlorophyll Pigments

7.2.1 Distribution, Location, and Structure

Chlorophyll pigments are widespread in the natural environment, the chlorophylls *a* and *b* being the most abundant and best known. Chlorophyll *a* is found in all photosynthetic organisms, except some groups of bacteria. Chlorophyll *b* is found in all vascular plants and in algae belonging to the Chlorophyta and Euglenophyta divisions of green algae. Other types of chlorophylls (*c* and *d*) are found in algae, and bacteriochlorophyll only in photosynthesizing bacteria. Recently, a new chlorophyll was discovered, chlorophyll *f* (Chen et al. 2010), with a maximum absorption displaced to the red region of the spectrum, 706 nm, as compared to chlorophyll *a*. Only chlorophylls *a* and *b* are found in vascular plants, with a dry weight content of 0.6–1.2%. The ratio between both varies between 3 and 1, depending on genetic and environmental factors, mainly time and intensity of exposure to the sun.

In vascular plants, chlorophylls are found in a specific type of plastid. These are eukaryotic organelles responsible for the production and storage of important chemical compounds and known as chloroplasts because of their photosynthetic capacity. This organelle has an internal medium, the stroma, and a lipid double-membrane system in the form of sacs known as thylakoids (Strasburger et al. 2004).

Chemically, chlorophylls are classified in the porphyrin group. They have a basic common structure, the *porphin* (Fig. 7.1), with four units of pyrrole linked at the

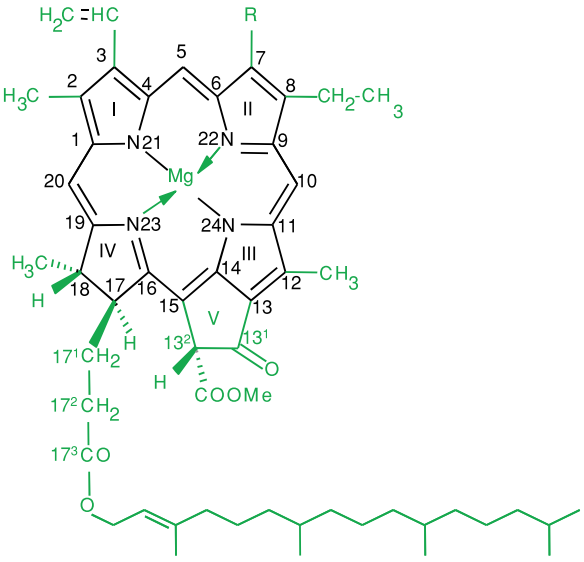

Fig. 7.1 Structures of porphin (*in black*) and chlorophylls (*in green*). R=CH_3 for chlorophyll *a* and R=CHO for chlorophyll *b* (*Source*: Authors)

alpha positions by methylene bridges, in a planar aromatic system, which is highly stable and amenable to the formation of chelates with metal ions. In this basic structure, 11 conjugated double bonds form the chromophore group able to absorb radiation in the visible spectrum. The color of a porphyrin compound depends both on the nature of the central atom and the porphyrin ring substituents. For the chlorophylls, the metal ion that forms the complex is Mg^{2+} and belongs to the most common group of reduced porphyrins, the dihydroporphyrins, in which the saturated carbon atoms are found on one of the pyrrolic rings. As additional substituents, it also contains a chain of modified propionic acid in the form of cyclic beta-ketoester (V- or isocyclic ring) and in the C-17, a chain of esterified propionic acid with a diterpenic alcohol of 20 carbon atoms, the phytol, which (Fig. 7.1) favors its insertion in the lipid bilayer of the thylakoids. The difference between chlorophyll *a* and *b* lies in the substituent of the C-7; chlorophyll *a* has a methyl group while chlorophyll *b* has a formyl group.

7.2.2 Functions and Biological Activities

Chlorophylls are the most abundant pigments in nature, possibly thanks to the key role they play in photosynthesis. They are able to absorb radiant energy and transfer it directly to the photosynthetic reaction center for transformation into chemical energy. All photosynthetic cells also contain other accessory pigments such as carotenoid, phycocyanin, or phycoerythrin, which act as supplementary light receptors in parts of the visible spectrum not completely covered by chlorophyll. The photosynthetic apparatus is organized into two large supramolecular complexes, with noncovalent pigment-protein bonds. These are called photosystems (PSs) and are embedded in the structure of the lipid bilayer of the thylakoid. The PSII is responsible for the photooxidation of water to oxygen and the supply of electrons, while the PSI catalyzes the energy transfer to the ferredoxin-NADP complex, thus producing intracellular energy for the assimilation of carbon dioxide (Salisbury and Ross 1992).

In vegetable foods, the primary feature of chlorophylls derived from their chromophoric capacity, which is responsible for the highly valued green color that the consumer associates with something's being fresh or freshly prepared. Furthermore, these compounds exhibit a series of biological activities of interest to the scientific community with respect to improving health through eating habits and the development of functional foods. In both in vitro tests and in vivo tests using animal models, chlorophyllic compounds have demonstrated antioxidant (Ferruzzi et al. 2002) and antimutagenic activities (Dashwood et al. 1998), modulating action of the xenobiotic enzymes (Fahey et al. 2005), and induction of apoptotic events in cancer cell lines (Chan et al. 2006), all of them related to the prevention of degenerative diseases. However, although chlorophylls are found widely distributed in vegetables, very little is known about their bioavailability. Until recently, they were assumed to

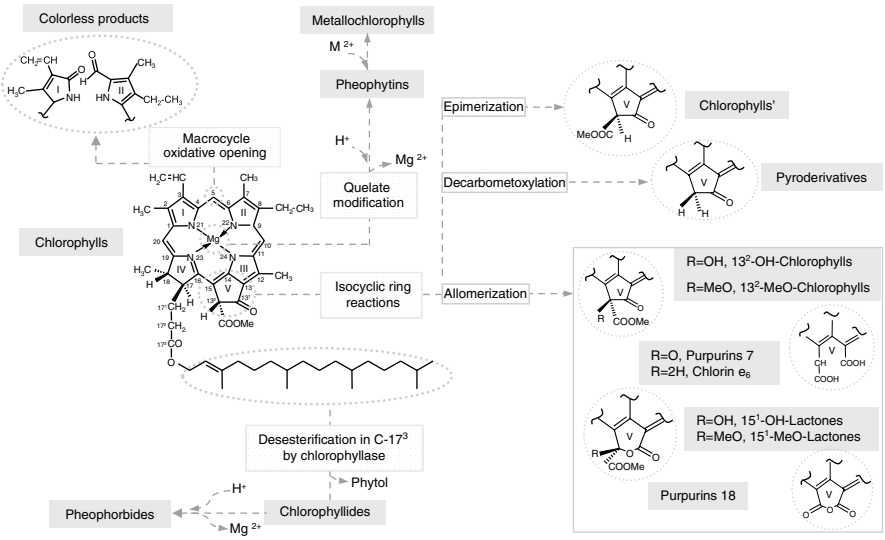

Fig. 7.2 Changes in chlorophyll structure (*Source*: Authors)

have a physiological role exclusively limited to the interception of mutagenic compounds in the gastrointestinal tract because it was assumed that they could not be absorbed by humans (Dashwood 1997). However, recent research has demonstrated their effective absorption in Caco-2 epithelial cell growths (Ferruzzi and Blakeslee 2007; Gallardo-Guerrero et al. 2008; Gandul-Rojas et al. 2009) and by humans (Egner et al. 2000), opening up areas of research in this field.

7.2.3 Chemical Properties

The principal structural transformations that may occur to a chlorophyll molecule mainly affect the chelate, the phytol ester bond (C-17^3), the isocyclic ring (C-13^2), and the basic porphyrin structure (Fig. 7.2). Mg^{2+} is rapidly replaced in acid conditions by two *hydrogens* protons, generating pheophytins and modifying the properties of the chromophore. Chlorophyll *a*, a green-blue color, forms a gray-colored pheophytin *a*, and in the case of chlorophyll *b*, the green-yellow color turns brown and is called pheophytin *b*. The reinsertion of Mg^{2+} into the tetrapyrrolic ring is a difficult reaction; however, other divalent metals such as Zn and Cu are inserted more easily, recovering the green color and obtaining greater stability (Inoue et al. 1994). The deesterification in the C-17^3 is a specific reaction catalyzed by chlorophyllase and resulting in chlorophyllides and pheophorbides (Holden 1961). Loss of the phytol leads to a significant

increase in polarity but does not modify the properties of the chromophore. The non-specific deesterification of the phytol occurs in highly acid or alkaline conditions, together with other collateral oxidative reactions (Hynninen 1991).

The isocyclic ring undergoes reactions of a very different nature. The mildest one is the epimerization of the C-13^2, which only involves a slight change in the polarity of the molecule. The oxidative substitution in the C-13^2 of the carbometoxy group ($COOCH_3$) by hydrogen generates the pyroderivatives, with identical coloring and spectroscopic properties of its forerunners but with a lower polarity.

The oxidation of the isocyclic ring with triplet molecular oxygen $^3O_2$ is generically referred to as allomerization and occurs due to a free-radical mechanism with the enolate anion as intermediate (Hynninen and Hyvärinen 2002). In an aqueous medium, the hydrogen of the C-13^2 is replaced by an OH-group, while in an alcohol solution, e.g., methanol, the substitution is a metoxyl group (MeO-) (Kuronen et al. 1993). These modifications only involve a slight increase in polarity.

Reactions in a slightly alkaline medium and an inert atmosphere produce the solvolysis of the isocyclic ring, and the resulting compounds are of the chlorin e_6 and rhodin g_7 types, according to whether they come from the *a* or *b* series, respectively (Hynninen 1991), and if in addition C-13^2 oxidizes, the isocyclic ring results in purpurin 7 (Mínguez-Mosquera et al. 1996). A cyclic ester (lactone ring) may also be formed, leading to OH-lactone chlorophyll, and in more severe oxidation conditions, the formation of a cyclic anhydride occurs, resulting in a purpurin 18. In all these reactions, although the basic structure of the macrocycle remains, the spectrum of electronic absorption is modified significantly, as are the adsorption properties, because these are far more polar compounds (Kuronen et al. 1993).

Lastly, the most disruptive transformation of the chlorophyll molecule involves the opening of the macrocycle, in which the chlorophyll molecule loses its original chromophore properties, which results in colorless products. In physiological conditions, the oxygenolitic breakdown starts between carbons four and five and is catalyzed by the pheophorbide *a* oxygenase enzyme (PaO), a monoxygenase located in the membrane of the chloroplasts. Another type of oxidation process that is developed via radicalary reactions also leads to the discoloration of the chlorophyllic compounds (Matile et al. 1999).

7.2.4 Spectroscopic Properties

7.2.4.1 Ultraviolet-Visible Electron Absorption Spectrum

The conjugated double-bond system that constitutes the basic structure of porphyrin is responsible for the electronic absorption spectrum of chlorophyllic compounds. These are very characteristic spectra formed by a number of relatively pronounced bands, in the yellow, red, and near-infrared regions, and a band of high absorption in the violet or near-violet region, called the Soret band. The presence of the latter indicates that the basic porphyrin structure has not been broken (Hoff and Amesz 1991).

Although the spectra of all chlorophyllic derivatives have common characteristics, there are differences in the location of the maxima and in the relation between the Soret band and the secondary absorption bands, which permit the use of the ultraviolet-visible (UV-vis) electronic absorption spectroscopy as a valuable tool for the preliminary identification of these compounds.

Structural modifications affecting the chelate are those that lead to the most striking changes in the absorption spectrum because they alter the properties of the chromophore. Figure 7.3a, b compares chlorophyll *a*, pheophytin *a*, and Cu-pheophytin *a* spectra. The deesterification of the phytol does not affect the structure of the chromophore, and the electronic absorption spectrum remains unchanged. This also occurs with the epimerization or decarbomethoxylation in the C-13[2] and the primary allomerization reactions that lead to hydroxy- or methoxy- derivatives. On the other hand, the other oxidative reactions in the isocyclic ring that involve its opening (Fig. 7.3c), the formation of a lactone ring, or a cyclic anhydride as phytol-purpurin 18 *a* (Fig. 7.3d) do result in significant modifications to the electronic absorption spectrum. In chlorophyllic derivatives from the *b* series, the structural changes produce similar changes in the corresponding electronic absorption spectra (Fig. 7.3e, f).

7.2.4.2 Fluorescence Spectrum

The conjugated structure of the tetrapyrrolic macrocycle is responsible for the fluorescent nature of the chlorophyllic pigments. The fluorescence spectra are very similar to the light absorption spectra in the visible part, although chlorophyll *a* shows a greater intensity of fluorescence in comparison with chlorophyll *b*. Similarly, the pheophytin *a* and pheophytin *b* spectra are similar to those for the corresponding chlorophylls (Loftus and Carpente 1971). The fluorimetric methods, although far more selective and sensitive than the spectrophotometric methods, are not as widely used. It is possible to detect these compounds in quantities of picomoles using this technique (Hornero-Méndez et al. 2005). The majority of chlorophyllic compounds show a characteristic pink fluorescence when illuminated by ultraviolet light, permitting the use, since a long time ago, of this property for its detection in thin-layer chromatography (TLC) (Zonneveld et al. 1984). The exceptions include certain derivatives such as the metallochlorophylls of Cu and the purpurins 18 (Mínguez-Mosquera et al. 1996), which do not have this property, and in this case it becomes a very useful test for a preliminary identification.

7.2.4.3 Other Spectroscopic Properties

Nuclear magnetic resonance (NMR), infrared spectrometry (IR), and Raman resonance have been valid tools for the structural elucidation of chlorophylls and derivatives since their development (Abraham and Rowan 1991; Schick and Bocian 1987).

The application of mass spectrometry to the characterization and identification of chlorophylls and derivatives has only been possible since the development of desorption and ionization methods suitable for nonvolatile and thermolabile compounds.

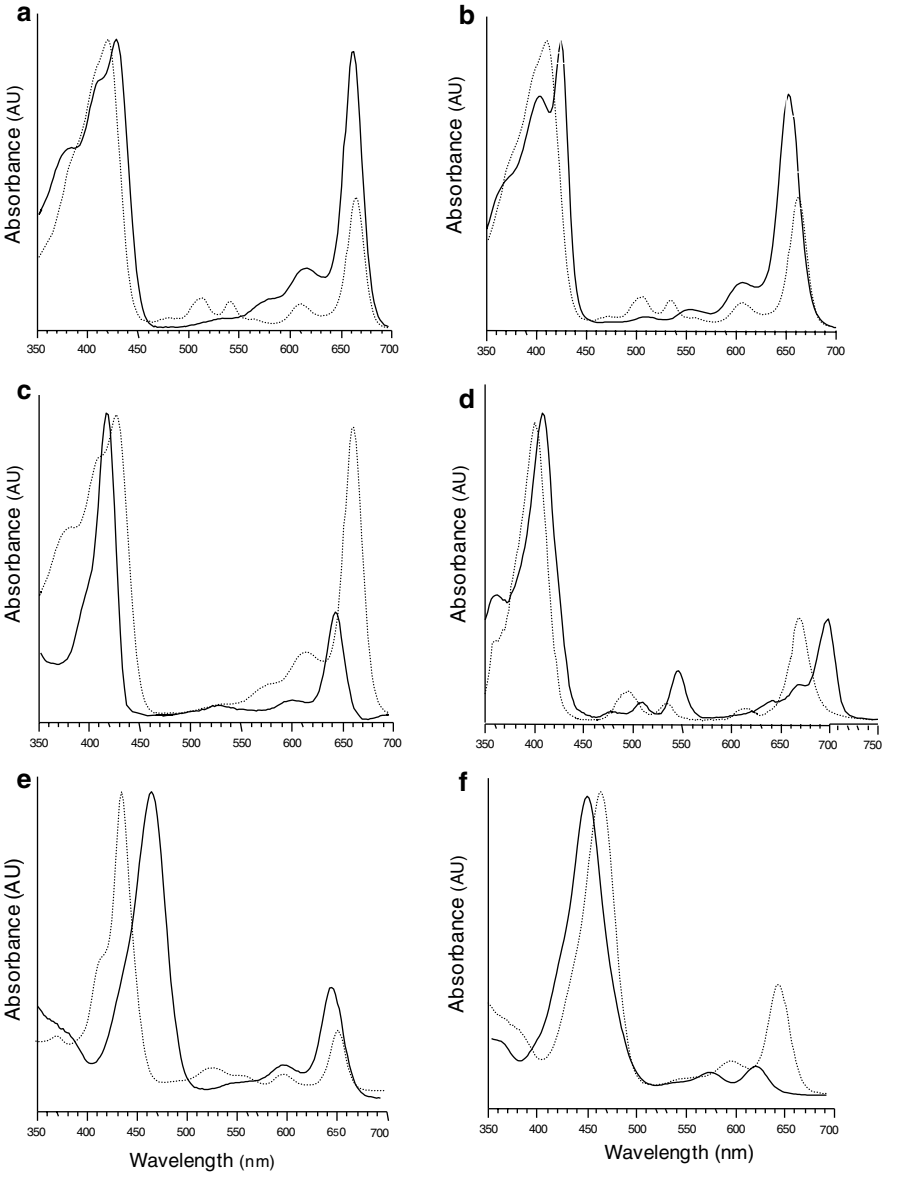

Fig. 7.3 Comparison of electronic absorption spectra of chlorophyll compounds. (**a**) chlorophyll *a* (—) and pheophytin *a* (····); (**b**) pheophytin *a* (····) and Cu-pheophytin *a* (—); (**c**) chlorophyll *a* (····) and Mg-phytol-chlorin e_6 (—); (**d**) 15^1-OH-lactone pheophytin *a* (····) and phytol-purpurin *a* 18 (—); (**e**) chlorophyll *b* (—) and pheophytin *b* (····); (**f**) chlorophyll *b* (····) and Mg-phytol-rhodin g_7 (—) (*Source*: Authors)

The combination of desorption techniques and tandem mass spectrometry (MS/MS) has also been applied to the characterization of chlorophylls and derivatives (Hunt and Michalski 1991; van Breemen et al. 1991). The latest applications in this field combine high-performance liquid chromatography (HPLC) with MS for the analysis of chlorophyllic compounds using either fast-atom bombardment as a source of ionization (Kostiainen et al. 1995) or atmospheric pressure chemical ionization (APCI) (Hyvärinen and Hynninen 1999; Verzegnassi et al. 1999; Zissis et al. 1999).

7.2.5 Stability During Food Processing

The release of acids after the loss of cellular integrity is a common occurrence in vegetables in which the internal medium has a pH that may be as low as 5.5. The reaction of pheophytinization is therefore the most generalized transformation in the postharvesting and processing of vegetables, occurring with different reaction rates depending on the degree of acidity of the food. The loss of cellular integrity thus permits the contact of endogenous enzymatic systems with their substrates, giving rise to hydrolytic or oxidation reactions in the chlorophylls. In the case of materials with chlorophyllase enzyme activity, the chlorophylls partially convert into their dephytylated derivatives, chlorophyllides and pheophorbides.

During food conservation, chlorophyll degradation may continue until oxidation to colorless products, a process that may involve different oxidative enzymatic systems (Gross 1991; Holden 1965; Mínguez-Mosquera et al. 1990). The free radicals formed as intermediate products in the catalysis are directly responsible for the cooxidation of chlorophylls. In light conditions, the photooxidation of chlorophyll takes place. Given the capacity of chlorophylls to absorb light energy and transfer it to oxygen, converting it into the singlet oxygen reactive species, a series of radicalary reactions are unleashed that also lead to the destruction of the chlorophyll (Gutierrez-Rosales et al. 1992).

In vegetable oils, pheophytinization is again the most common reaction due to the general release of acids produced with the mechanical breakup of the plant tissue. Pheophytin *a* is the main pigment in such oils, and its amount varies depending on whether the extraction of the oil is carried out by pressing, as is the case with olive oil (Mínguez-Mosquera et al. 1990, 1992; Rahmani and Csallany 1991), or by solvent extraction (Ward et al. 1994). Furthermore, the conditions of the process enhance the action of the chlorophyllase enzyme, and dephytylated chlorophyllic derivatives may be found in the oil, principally pheophorbide *a* (Gandul-Rojas and Mínguez-Mosquera 1996a, b). Oxidation reactions take place also while oil is stored, and pyropheophytins and allomerized chlorophyllic derivatives have been found in stored oil (Ward et al. 1994; Gandul-Rojas et al. 1999b; Roca et al. 2003). Concerning olive oil refining processes, pyropheophytinization is the most general reaction in a physical refining process with a deodorization step involving nitrogen

(Gandul-Rojas et al. 1999b), while in a chemical refining process, almost all the chlorophyllic compounds are eliminated through the adsorption process with bleaching earth or a deodorization step at high temperature (Didi et al. 2009; Manjula and Subramanian 2006).

7.3 General Aspects of Carotenoid Pigments

7.3.1 Distribution, Location, and Structure

Carotenoid pigments are widely found among living beings, although higher concentrations and greater variety are found within the plant kingdom. In plants, carotenoids are found and accumulate in specialized subcellular organules known as plastids, specifically in chloroplasts and chromoplasts. Chloroplasts are present in photosynthetically active tissues, where almost all the carotenoids are found in the form of chlorophyll-carotenoid-protein complexes (photosystems PSI and PSII) located in the thylakoids. However, the organules specializing in the massive accumulation of carotenoids are the chromoplasts present in flowers, some ripe fruit, and some roots and tubers.

Carotenoids are chemically classified as terpenoids and the majority has a central skeleton of 40 carbon atoms, formed of eight isoprenoid units (C_5). The biosynthesis of this skeleton takes place from two molecules of an intermediate C_{20} (geranylgeranyl diphosphate), which produces phytoene, the generic precursor to the carotenoids. The phytoene undergoes a series of successive desaturations, up to four, increasing the conjugation and modifying the chromophore characteristic of these pigments. Subsequent modifications of the structure, such as cyclization to one or both ends of the chain, hydroxylation, or the introduction of other oxygenated functions, results in the huge variety of carotenoids found in nature. At present, more than 700 different carotenoids have been identified (Britton et al. 2004).

Carotenoids are divided into two main groups: the carotenes, which, strictly speaking, are hydrocarbons, and the xanthophylls, derived from the former and which contain oxygenated functions. Xanthophylls can be found esterified with fatty acids. Carotenoids may have an acyclic structure, or contain a ring of five or six carbons on one or both ends. Possible end groups that may be present in a carotenoid molecule are identified using the Greek letters β, ε, γ, κ, ϕ, χ, ψ. Figure 7.4 shows the seven end groups that have been found in natural carotenoids, together with the structures and numbering system of the carotenoids, using lycopene and β-carotene as examples of acyclic and bicyclic carotenoids, respectively. The numbering considers the carotenoid structure as a dimer. First, the left side is numbered in a linear manner, starting with the carbon that is linked to two methyls and ending with the carbon 15 (the opposite end of the first monomer). Next, the methyls are numbered from left to right up to 20. The right half is numbered in an equivalent manner, from right to left with prime numbering. Figure 7.5 shows the structure of the xanthophylls that may be found in olive oil.

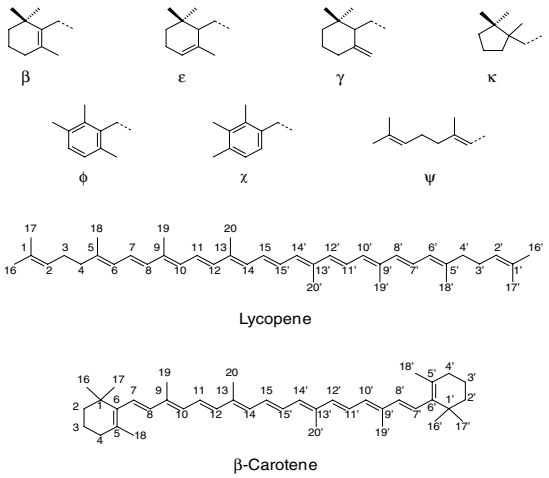

Fig. 7.4 Structures for seven end groups found in natural carotenoids and the C_{40} scheme and numbering system of carotenoids, illustrated by lycopene and β-carotene as examples of acyclic and bicyclic carotenoids, respectively (*Source*: Authors)

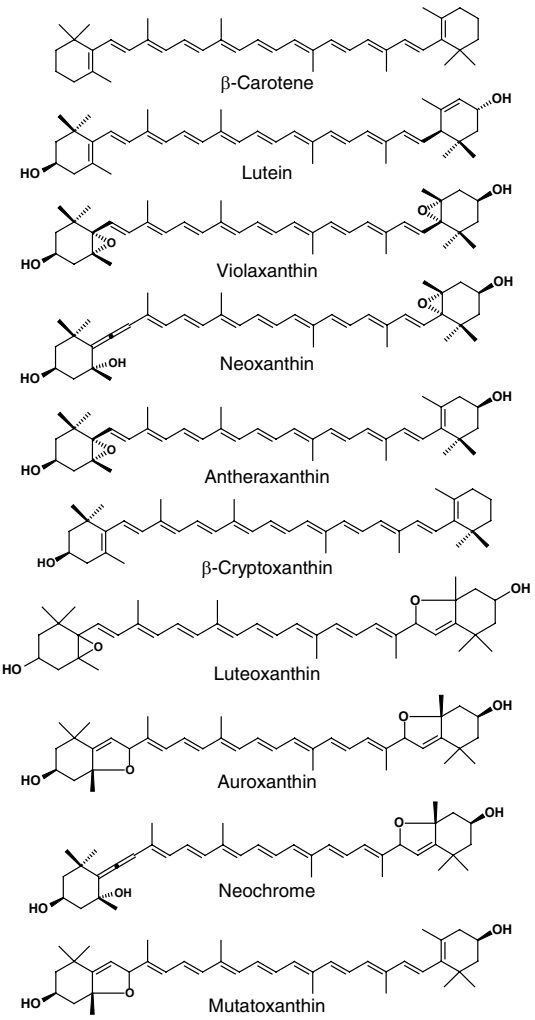

Fig. 7.5 Structures of xanthophylls found in olive oils (*Source*: Authors)

7.3.2 Biological Functions

The main function of carotenoids in plants is to serve as accessory pigments to collect light energy that is then transferred to the chlorophylls, which transduce it during photosynthesis. In addition, carotenoids play a major role in protecting against oxidation damage, in particular the photoprotective effect on the chlorophylls and photosynthetic appliance as it cancels (quenching effect) highly reactive forms of triplet chlorophylls (^{3}Chl) and singlet oxygen ($^1O_2$) formed during the process of capturing light energy. In addition, carotenoids have an antioxidant capacity with which they protect other systems sensitive to oxidizing damage from oxidant-free radicals. Furthermore, carotenoids have a principal function as precursors of vitamin A and occupy at present an important place among food components of interest with respect to human health (Britton et al. 2008, 2009).

7.3.3 General Properties

Carotenoids are lipophilic compounds that are generally insoluble in water unless they contain a highly polar group. When they are isolated, whether dry or in solution, they are highly sensitive to light and heat, which can lead to *cis-trans* isomerization. Although the carotenoid structure is relatively resistant to extreme pH values, i.e., acids and alkalis, these agents may provoke certain transformations. In acidic conditions, isomerization of the xanthophylls with 5,6-epoxide groups takes place, and these are converted into 5,8-furanoids. Some xanthophylls, such as fucoxanthin and astaxanthin, are exceptionally unstable under alkaline conditions, which cause autooxidation of the 3-hydroxy-4-oxo terminal groups. The conjugated double-bond system makes the carotenoids particularly susceptible to oxidative discoloration caused by oxygen in the air.

Interactions with other molecules in their microenvironment could modify the chemical and physical properties of carotenoids in vivo, and these would be very different from those displayed by free carotenoids in organic solutions. These interactions with molecules, especially proteins, may be critical for the operation or actions of carotenoid molecules in vivo, for example, in membranes (Britton et al. 1995a).

7.3.4 Spectroscopic Properties

Carotenoid pigments have a series of spectroscopic properties that permit the application of different methods for the analysis and elucidation of their structure. This section summarizes the most general characteristics of the UV-vis light absorption spectroscopy, one of the most basic analytic methods that is widely used

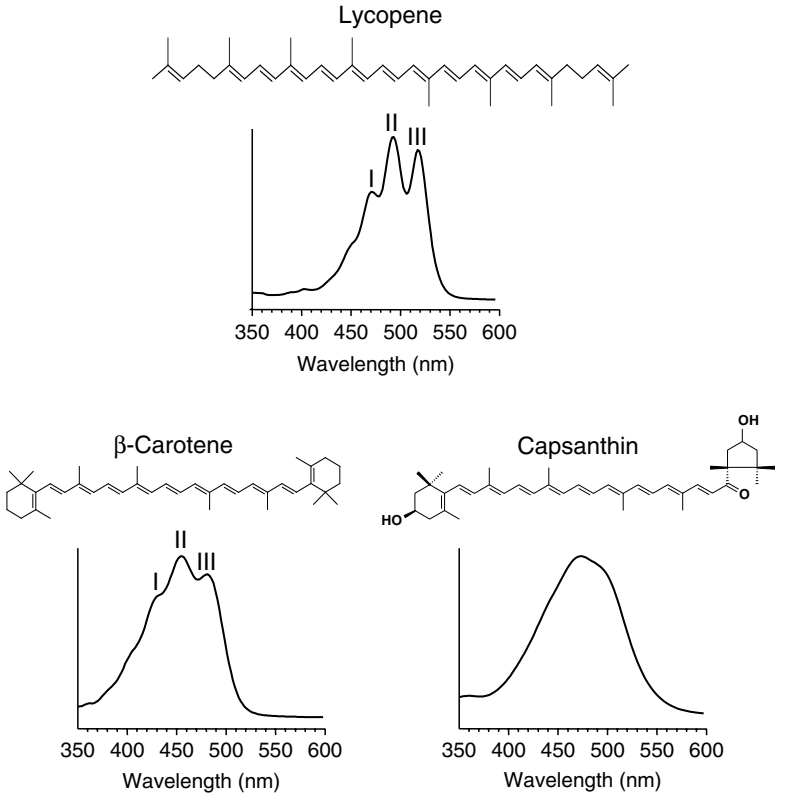

Fig. 7.6 Structures and UV-visible spectra of lycopene, β-carotene, and capsanthin (*Source*: Authors)

in carotenoid research. Other physical techniques are generally more specialized (circular dichroism, IR spectroscopy, Raman resonance, NMR spectroscopy, mass spectroscopy, X-ray crystallography) and require a thorough knowledge of and experience in these techniques (Britton et al. 1995b).

Carotenoid pigments have a characteristic visible light absorption spectrum due to the conjugated double-bond system of their hydrocarbonate chain (polyene). For a given carotenoid the positions of the maximum light absorption bands (λmax) depend on the number of conjugated double bonds of the molecule (Fig. 7.6). Roman numerals (I, II, III) are preferred when referring to the absorption maxima. The fine structure of the spectrum of acyclic carotenoids usually is more defined than the corresponding to cyclic (monocyclic and bicyclic) carotenoids. This is related to the noncoplanarity of the terminal rings with the central polyenic chain. The first of the absorption maxima in carotenoids with two β-type rings, for example β-carotene, appears as a shoulder. In the majority of ketocarotenoids, such

as capsanthin, where the carbonyl group is conjugated with the polyene chain, the fine structure is lost, and only a principal broad band is observed with very weak inflections on one or the other side. Figure 7.6 compares the fine structure of the UV-vis spectra for various carotenoids. It is important to compare not only the positions of the absorption maxima but also the shape and fine structure (defined by % III/II).

7.3.5 Stability During Food Processing

Carotenoids are very stable in living tissue thanks to their association with proteins and membranes or their aggregation in microcrystals. However, when tissues break, for example in olive milling, the loss of cellular compartmentalization brings them into contact with substances that may structurally modify them or even cause their destruction. Furthermore, gentle heat treatments in the absence of air induce the *trans-cis* isomerization of certain double bonds and, as the number of *cis* double bonds increases, the strength of the color and the nutritional value of these components decrease. As mentioned in Sect. 7.3.3, the presence of acids may cause the isomerization of xanthophylls with 5,6-epoxide groups to 5,8-furanoids, which will also affect the strength of the color. The main cause of deterioration in carotenoids is oxidation, a reaction catalyzed by light, lipoxidases, and lipid peroxides, which may result in their discoloration.

7.4 Analysis of Chlorophylls and Carotenoids in Olive Oil

Thin-layer chromatography (TLC) on silica gel provided the first study with quantitative results of chlorophyllic pigments and carotenoids in olive oil (Mínguez-Mosquera et al. 1990). Chlorophylls *a* and *b*, and their corresponding derivatives free of magnesium-pheophytins *a* and *b*- were identified in the chlorophyllic fraction. In the yellow pigment fraction, lutein and β-carotene were identified as the major carotenoids, with an epoxidated xanthophyll series that included neoxanthin, violaxanthin, antheraxanthin, and their respective furanoid isomers, neochrome, luteoxanthin, auroxanthin, and mutatoxanthin. At present, HPLC methodologies have replaced TLC in the analysis of these compounds, although the last named continue to be of great use in the isolation and purification of standards (Mínguez-Mosquera et al. 2007).

Methods that use normal phase chromatographic columns offer an advantageous reduction in the time needed to analyze these pigments in vegetable oils (Seppanen et al. 2003) as they permit a direct injection of the oil sample, after dilution to 5–8 % in the mobile phase, thereby avoiding any prior purification treatment of the sample. However, the oil dilution implies working with low sensitivity and do not permit the

determination of minor pigments such as oxidized chlorophyll derivatives or epoxidated xanthophylls, which are compounds included in a proposal of new quality parameters and VOO varietal discrimination (Aparicio-Ruiz et al. 2009; Roca et al. 2003; Gandul-Rojas et al. 2000).

In order to develop a more sensitive pigment analysis, different enrichment techniques have been put forth to purify samples and remove the lipid compounds that interfere in the chromatographic separation of pigments. Among them, the usual method for extracting an unsaponifiable fraction cannot be generally implemented since the alkaline conditions of the saponification reaction provoke total chlorophyll transformation. Thus, less aggressive and specific purification methodologies have been worked out for the simultaneous analysis of chlorophyllic and carotenoid pigments in olive oil. The first of these methods uses cellulose columns (Fraser and Frankl 1985) or so-called freezing-out techniques (Aitzetmuller 1989), but recoveries of only 60–70% were obtained. At present, both SPE systems and LPE methodologies are used to obtain recoveries of up to 94% (Cichelli and Pertesana 2004; Mateos and García-Mesa 2006; Mínguez-Mosquera et al. 1992).

7.4.1 Extraction and Purification Methodologies

7.4.1.1 Liquid-Phase Extraction

Liquid-liquid extraction methods permit the separation of compounds from a complex matrix on the basis of their different solubilities in two immiscible solvents. The solvents N,N-dimethylformamide (DMF) and hexane have been successfully used for olive oil (Mínguez-Mosquera et al. 1992). Chlorophylls, chlorophyllic derivatives, and xanthophylls are retained in DMF because of their polarity, while interfering lipids, carotenes, and esterified xanthophylls are extracted in the hexane phase. The procedure involves dissolving the oil sample (10–15 g) directly in 100 mL DMF, saturated with $MgCO_3$, and extracting five times with a volume of 50 mL hexane in a separation funnel. Pigments must be recovered from this polar phase by transferring them to an apolar solvent due to the high boiling point of DMF. For this purpose, the DMF phase is added to 400 mL of a 10% NaCl solution previously cooled to 4°C; it is shaken gently and then extracted with 100 mL of ethyl ether; a cool temperature is necessary because it is an exothermic reaction. After separation, the bottom aqueous phase is again extracted with ethyl ether (50 mL) to ensure quantitative recovery of pigments, and lastly it is rejected to eliminate polyphenols and other soluble compounds. The ethyl ether phases are combined and then filtered through a desiccant such as Na_2SO_4. The water-free solution is evaporated to dryness at temperatures below 30°C. The final residue is dissolved in an appropriate volume of acetone (1.5 mL) and stored in darkness at −20°C until the chromatographic analysis. Prior to the analysis, the solution should be centrifuged at 13,000 × g or filtered through 0.45 μm.

The five successive hexane extractions are combined, desiccated with Na_2SO_4, and concentrated by evaporation to a final volume of 25 mL in a volumetric flask. The carotene concentration is determinated by spectrophotometric measurement at 454 nm ($\varepsilon_{454} = 139.2$).

Based on this method, Maia et al. (2008) validated a micro method that reduces the size of the sample tenfold (1 g oil) as well as the solvent volumes. All the extraction procedures are performed in microvials, the separation of phases is carried out using centrifugation, and the evaporation of solvents is carried out with nitrogen stream. The procedure has only been applied to the determination of lutein and β-carotene in olive oil.

7.4.1.2 Solid-Phase Extraction

SPE is a simple, versatile, and low-cost technique. The bibliography on olive oil describes this purification technique with different absorbents such as C_{18}, diol, and silica gel (Hornero-Méndez et al. 2005; Gertz and Fiebig 2006; Mateos and García-Mesa 2006) (Table 7.1). In general terms, and with respect to carotenoids, the retention levels of lutein and β-carotene are high enough with C_{18} and C_{30} phases, while the retention level of lutein is fine with diol and silica gel phases (Shen et al. 2009). With respect to chlorophyllic derivatives, the C_{18} phase does not permit total retention of pheophytin *a*.

The general procedure for olive oil starts with the activation of the cartridge with methanol, followed by its conditioning with hexane. Next, the oil sample (1 g) is dissolved in hexane (4–6 mL) and applied to the column. The solvent is collected in a 10 mL volumetric flask. The interfering lipid compounds are removed by washing the cartridge with hexane (3–5 mL) and collected in the same flask. Next, the analytes are eluted with acetone (3–5 mL) and the solvent evaporated to dryness at temperatures below 30°C. The final residue is dissolved in an appropriate volume of acetone (0.2–0.3 mL) and stored in darkness at −20°C until chromatographic analysis. Prior to the analysis, the solution is centrifuged at 13,000 × g or filtered through 0.45 μm.

β-carotene and some quantities of pheophytin *a* and pyropheophytin *a* are not retained in cartridges with C_{18} phase after the washing step with hexane. Quantitative estimation of these analytes can be made using spectrophotometric measurements, after bring to 10 mL in the volumetric flask the hexane phase. Under these conditions, β-carotene is determined at 454 nm ($\varepsilon_{454} = 139.2$), and the sum of pheophytin *a* and pyropheophytin *a* is measured at 660 nm ($\varepsilon_{660} = 53.3$). Two C_{18} cartridges in tandem avoid the losses since the percentage of recoveries of chlorophyllic derivatives is higher than 94%. Cartridges with a diol phase also permit a satisfactory retention of xanthophylls and chlorophyllic compounds with recoveries > 98%. Gertz and Fiebig (2006) proposed to use a silica gel phase and a petroleum ether (40–60°C)/ethyl ether (9:1) mixture as a purification solvent of lipid compounds, although no data are given about the retention percentages of the analytes.

Table 7.1 Solid-phase extraction methods for pigments analyses in olive oils

Sorbent	Procedure				Analyte elution	Retention grade/ recovery[a,b]	References
	Activation	Conditioning	Sample application	Purification			
Octadecyl C_{18}	Methanol	Hexane	1g + hexane (4 mL)	Hexane (3 mL)	Acetone (5 mL)	β-carotene: NR/100 % Phy *a* and pyrophy *a*: PR/101 % Xanthophylls: R/104 %	1
LC-Si (300 mg)			0.3g + hexano (1 mL)	Hexane (10 mL)	Acetone (6 mL)	No data	2
Octadecyl C_{18} /18% (× 2 in series)		Petroleum ether (65–95 °C)	0.5 mL	Petroleum ether (65–95 °C) (12 mL)	Acetone (3 mL)	β-carotene: no data Chlorophylls: R/>94% Xanthophylls: no data	3
Diol-phase (500 mg/3 mL)	Methanol	Hexane	1g + hexane (4 mL)	Hexane (5 mL)	Acetone (3 mL)	β-carotene: NR Chlorophylls: R/>98 % Xanthophylls: R/>98 %	4
Silica gel (1,000 mg/6 mL)			0.3 g + hexane (1 mL) + petroleum ether (40–60 °C) (2 × 1 mL)	Petroleum ether (40–60 °C)/ethyl ether (9:1) (2 × 5 mL)	Acetone (5 mL)	No data	5

[a]Analyte retention grade in absorbent phase; *NR* not retained, *PR* partially retained, *R* retained

[b]Analytes eluted during purification stage (not retained) are quantitatively estimated by spectrophotometric measurements at 454 nm (β-carotene) and 660 nm (pheophytin *a* + pyropheophytin *a*)

References: 1, Mínguez et al. (1992); 2, Serani and Piacenti (2001); 3, Hornero et al. (2005); 4, Mateos and García-Mesa (2006); 5, Gertz and Fiebig (2006)

7.4.1.3 Saponification for Carotenoids

Saponification is a generalized and widely used technique, which in addition to removing lipids and chlorophyllic compounds results in the release of esterified xanthophylls. In fact, some carotenoids have been described as alkali-sensitive, and significant losses of xanthophylls, especially of epoxy carotenoids, have been described in the saponification process (Khachik et al. 1986). Therefore, the percentages of losses or transformation of carotenoids are directly related to the process harshness. Processes carried out at warm temperatures for short periods of time (5–10 min) cause significant transformations of carotenes and xanthophylls into oxidation products, while soft conditions (12–16 h at room temperature) minimize the loss of carotenes, though the degradation of the xanthophylls cannot be avoided. These transformations are greatly minimized if gentle saponification is carried out inside a nitrogen atmosphere or in the presence of an antioxidant (Kimura et al. 1990).

Stancher et al. (1987) used a standard gentle saponification for the determination of lutein and β-carotene in olive oils. Briefly, the procedure involves adding oil sample (10 g) to 100 mL of a mixture of ethanol/50% KOH aqueous solution (1:1) that contains 0.5% ascorbic acid. The reaction is left stirring overnight at room temperature. The saponified mixture is extracted with 200 mL ethyl ether and the ether phase is then washed twice with 100 mL phosphate buffer solution (pH 7.4) and once with water. Finally, it is desiccated with Na_2SO_4 anhydride and the solvent evaporated.

An alternative saponification method has been used for the identification of carotenes and esterified xanthophylls in Spanish varietial VOOs (Gandul-Rojas et al. 1999c). In this case, the oil sample is dissolved in ethyl ether (100 mL) and mixed with the same volume of KOH solution at 20% in methanol. After 1 h of reaction, the soaps are separated with the addition of water, and the carotenoids move to the higher ether phase. The washing of the ether phase is repeated until reaching a neutral pH of 7, then it is desiccated by filtering through Na_2SO_4 anhydride, and finally the solvent is evaporated.

7.4.2 Methodologies by HPLC Analysis

The chromatographic techniques currently used for the analysis of pigments in olive oil can be classified into two groups, those that use systems in a normal stationary phase and those that use reverse-stationary-phase systems (Table 7.2).

Rahmani and Csallany (1985, 1991) were the first to apply an HPLC methodology in normal phase for the determination of chlorophylls, pheophytins, and β-carotene in olive oil using hexane/2-propanol (98.5:1.5 v/v) in isocratic conditions as the mobile phase. However, these conditions are not sufficiently polar to allow elution of lutein, a major carotenoid in VOO. Psomiadou and Tsimidou (1998) proposed a gradient elution system between n-Hexane/2-propanol (99:1, v/v) (A) and 5% 2-propanol (B) to resolve this problem, thereby allowing the simultaneous determination of α-tocopherol, β-carotene, chlorophylls *a* and *b*, pheophytins *a* and *b*, and lutein within a reasonable time length (20 min). The separation was achieved on

Table 7.2 High-performance liquid chromatography methods for pigment analysis in olive oils

Stationary phase	Mobile phases[a]		Elution system	F.R.[b]	Injection[c]	Detection	A.T[d]	Analyzed compounds[e]	Comments	References[f]
Normal phase										
µ-Porasil 10 µm; 3.69 mm×30 cm	I	*n*-Hexane/2-propanol (98.5:1.5 v/v)	Isocratic	0.8	D- (5 %)	Absorbance 409 nm, 430 nm 432 nm, 452 nm	20	Chls, Phys, β-car	No lutein No minority pigments	1
LiChrospher-Si 60; 5 µm; 4.0 mm×25 cm	II	A: *n*-Hexane/2-propanol (99:1 v/v) B: 2-propanol	Isocratic: 0 % B (10′); Lineal: 0–5 % B (4′); Isocratic: 5 % B (6′); Lineal: 5–0 % B (4′); Isocratic: 0 % B (6′)	1.2	D- (8 %) in A	Absorbance 410 nm, 430 nm 453 nm, 294 nm	20	Chls, Phys, β-car, Lutein, Tocopherols	No minority pigments	2
LiChrobsorb Si-60; 5 µm; 4.6 mm×25 cm	I	*n*-Hexane/2-propanol (98.5:1.5 v/v)	Isocratic	1.0	D- (5 %)	Absorbance 409 nm, 430 nm 433 nm, 452 nm Fluorescence λ_{ex} = 295 nm λ_{em} = 330 nm	20	Chls, Phys, β-car, Tocopherols, Tocotrienols	No lutein No minority pigments	3
Reverse phase										
Zorbax ODS; 6 µm; 4.6 mm×25 cm	III	Acetone/methanol (75:25)	Isocratic	0.5	C-cellulose columns	Absorbance 665 nm	30	Chls, Phys, Pyrophys	Recoveries only 60–70 % Specific for chl. pigments	4
LiChrobsorb RP 18; 5 µm; 5.0 mm×25 cm	IV	A: methanol B: acetone/water (95:5)	Isocratic: 5 % B (6′); Lineal: 5–50 % B (1′); Isocratic: 50 % B (19′); Lineal: 50–5 % B (1′); Isocratic: 5 % B (3′)	2.0	C-freezing-out	Fluorescence λ_{ex} = 409 nm λ_{em} = 667 nm	30	Chls, Phys, Pyrophys	Recoveries only 60–70 % Specific for chl. pigments	5

(continued)

Table 7.2 (continued)

Stationary phase	Mobile phases[a]		Elution system	F.R.[b]	Injection[c]	Detection	A.T[d]	Analyzed compounds[e]	Comments	References[f]
Spherisorb ODS-2; 5 µm; 4.0 mm×25 cm	V	A: water/IPR/methanol (1:1:8 v/v/v) B: acetone/methanol (1:1 v/v)	Lineal: 25–75 % B (8′); Isocratic: 75 % B (2′); Convex-4: 75–90 % B (8′); Concave-10: 90–100 % B (5′); Concave-10: 100–25 % B (7′)	2.0	C-SPE or LPE	Absorbance 410 nm, 430 nm	23	Chls, Phys, Carots		6
LC-18-DB; 3 µm; 4.6 mm×15 cm	VI	Water/methanol/acetone (4/36/60)	Isocratic	1.0	C-SPE	Absorbance 410 nm		Chls, Phys. Pyrophys	Specific for chlorophyll pigments	7
YMC^{TC} C_{30}; 5 µm; 4.6 mm×25 cm	VII	A: methanol/MTBE/AA/water (88:5:5:2) B: methanol/MTBE/AA (20:78:2)	Isocratic: 0 % B (5′); Linear: 0–85 % B (40′); Linear: 85–100 % B (5′)		D-(33 %) in MTBE/methanol (1:1)	Electro chemical 200–620 mV	50	Chls, Phys, Carots, Tocopherols	No minority pigments	8
Spherisorb ODS-2; 5 µm; 4.6 mm×25 cm	V	A: water/IPR/methanol (1:1:8 v/v/v) B: acetone/methanol (1:1 v/v)	Linear: 25–75 % B (7′); Isocratic: 75 % B (3′); Convex-5: 75–90 % B (10′); Concave-5: 90–100 % B (4′); Concave-5: 100–25 % B (6′)	1.5	C-SPE or LPE	Absorbance 430 nm Fluorescence λ_{ex}=440 nm λ_{em}=660 nm	30	Chls, Phys, Carots	No data for β-carotene	9
Spherisorb ODS-2; 5 µm; 4.6 mm×25 cm	VIII	Acetone/methanol (1:1)	Isocratic	2.0	C-SPE	Absorbance 410 nm Fluorescence λ_{ex}=410 nm λ_{em}=672 nm	7	Phys, Pyrophys	Rapid method for a specific determination of phy *a* and pyrophy *a*	10

Partisil ODS; 3.5 µm; 4.6 mm × 25 cm	VI	Water/methanol/acetone (4:36:60)	Isocratic	1.0	C-SPE	Absorbance 410 nm	10	Phys, Pyrophys	Rapid method for a specific determination of phy *a* and pyrophy *a*	11
YMC^{TC} C_{30}; 5 µm; 4.6 mm × 25 cm	X	A: methanol/MTBE/ water (90:7:3 v/v/v) B: methanol/MTEB/ water (7:90:3 v/v/v)	Linear: 5–35 % B (30′); Linear: 35–40 % B (10′); Linear: 40–95 % B (10′);	0.8	C-LPE	Absorbance 410 nm, 430 nm 444 nm, 465 nm 665 nm	50	Chls, Phys, Carots, Pyrophys		12
Spherisorb ODS-2; 3 µm; 4.6 mm × 20 cm	V	A: water/IPR/methanol (1:1:8 v/v/v) B: acetone/methanol (1:1 v/v)	Lineal: 25–75 % B (8′); Isocratic: 75 % B (2′); Linear: 75–90 % B (8′); Linear: 90–100 % B (5′); Isocratic: 100 % B (7′)	1.25	C-LPE	Absorbance 400 nm, 410 nm 430 nm, 450 nm	30	Chls, Phys Carots, Pyrophys		13
Mediterranean Sea; 3 µm; 4.6 mm × 20 cm	V	A : water/IPR/methanol (1:1:8 v/v/v) B: acetone/methanol (1:1 v/v)	Lineal: 25–75 % B (8′); Isocratic: 75 % B (2′); Linear: 75–90 % B (8′); Linear 90–100 % B (5′); Isocratic: 100 % B (15′)	1.25	C-LPE	Absorbance 430 nm, 654 nm 633 nm	43	Chls, Phys, Pyrophys, Carots, Cu-chls	To detect adulteration with E-141i	14

[a] *MTBE* methyl-*tert*-butyl ether, *AA* ammonium acetate, *IPR* ion-pair reagent (0.05 M tetrabutylammonium and 1M ammonium acetate aqueous solution)

[b] *F.R.* flow rate (mL/min)

[c] D (n %): direct injection of oil sample after dilution at n % in mobile phase. C: concentrated extract of pigment obtained by liquid-phase extraction (LPE) or solid-phase extraction (SPE)

[d] *A.T.* analysis time (min)

[e] *Chls* chlorophylls, *Phys* Pheophytins, *Pyphys* pyropheophytins, *Carot* Carotenoids, *β-car* β-carotene, *Cu-chl* copper chlorophyll derivatives

[f] Ref.: They are sorted first according to HPLC system in normal or reverse phase and second by chronological order

References: 1, Rahmani and Csallany (1985; 1991); 2, Psomiadou and Tsimidou (1998; 2001); 3, Seppanen et al. (2003); 4, Fraser and Frankl (1985); 5, Aitzetmuller (1989); 6, Mínguez-Mosquera et al. (1992); 7, Serani and Piacenti (2001); 8, Puspitasari-Nienaber et al. (2002); 9, Cichelli and Pertesana (2004); 10, Hornero-Méndez et al. (2005); 11, Gertz and Fiebig (2006); 12, Giuffrida et al. (2007); 13, Aparicio-Ruiz et al. (2010; 2011); 14, Roca et al. (2010)

a 250×4 mm i.d. LiChrospher-Si 60, 5 μm column at a 1.2 mL/min flow rate. The authors suggested an injection volume of 20 μL.

The first quantitative method for simultaneous reverse-phase HPLC analysis of chlorophylls and carotenoids in olive oils was developed by Mínguez-Mosquera et al. (1992). Once a concentrated extract of olive oil pigments has been obtained, by LPE or SPE, they are chromatographically separated using C_{18} stationary-phase columns (e.g., Spherisorb ODS-2 and Mediterranean Sea), protected by precolumns (1 cm×0.4 cm i.d.) packed with the same material. The flow rate varies depending on the size of the stationary-phase particle (2 mL/min for 5 μm particle size or 1.25 mL/min for 3 μm particle size). The suggested injection volume is 20 μL, and the elution system is a gradient between two mobile phases: A: water/ion pair reagents/methanol (1:1:8, v/v) and B: acetone-methanol (1:1, v/v). The ion pair reagent is a solution of tetrabutylammonium acetate (0.05 M) and ammonium acetate (1 M) in water. This reagent can be replaced with water when the analysis of deesterified chlorophyllic derivatives is not required. After use, the column should be stored in acetone/methanol (1:1, v/v). The basic gradient system starts with 25% B and changes to 100% B along different gradient steps (Table 7.2). These elution conditions permit the separation of 19 compounds in 23 min (Gandul-Rojas and Minguez-Mosquera 1996a). Figure 7.7 shows typical chromatograms for VOO pigment extracts from Arbequina and Hojiblanca cultivars.

The final gradient stage can be adjusted slightly to detect a fraudulent addition of colorant E-141i (copper compounds of chlorophylls) in olive oil (Roca et al. 2010). These compounds, with a lower polarity than the chlorophyllic derivatives of olive oil, are satisfactorily resolved by maintaining the final conditions at 100% B for 15 min before returning to the initial conditions.

New chromatographic methodologies – based on the use of reverse-phase YMC C_{30} columns (25 cm×0.4 cm i.d., 5 μm) with a binary gradient system with mixtures of methanol, methyl-*tert*-butyl ether (MTBE), water, and/or ammonium acetate – have been suggested recently for an effective separation of lutein, β-carotene, and pheophytin *a* with direct injection of the oil sample (10–20 mg) diluted at 33% in MTBE/methanol (1:1) (Puspitasari-Nienaber et al. 2002). Giuffrida et al. (2007) optimized a chromatographic separation system C_{30}-RP-HPLC that was applied to concentrated extracts of olive oil pigments obtained by LPE. The method allows a good resolution of 19 pigments in 53 min. The use of the C_{30} stationary phase significantly increases the chromatographic analysis time in comparison with the C_{18} phase, although it permits a more efficient separation of *cis-trans* isomers, lutein, α-carotene, β-carotene, and lycopene. The method is useful in the analysis of highly pigmented vegetable oils such as palm oil (Puspitasari-Nienaber et al. 2002) but is not recommended for the analysis of less pigmented oils such as VOO.

The most generalized form of determining chlorophylls and carotenoids is based on the measurement of their absorbance with a photodiode detector. A wavelength of 430 nm is adequate for their simultaneous detection since it is between the maximum absorbance of pheophytin *a* (410 nm) and that of chlorophyll *b* (466 nm); the absorbance maxima of the other pigments are in between. The pigments are identified by cochromatography with their respective standards and from the characteristics of the UV-vis spectrum registered online from 350 to 800 nm with

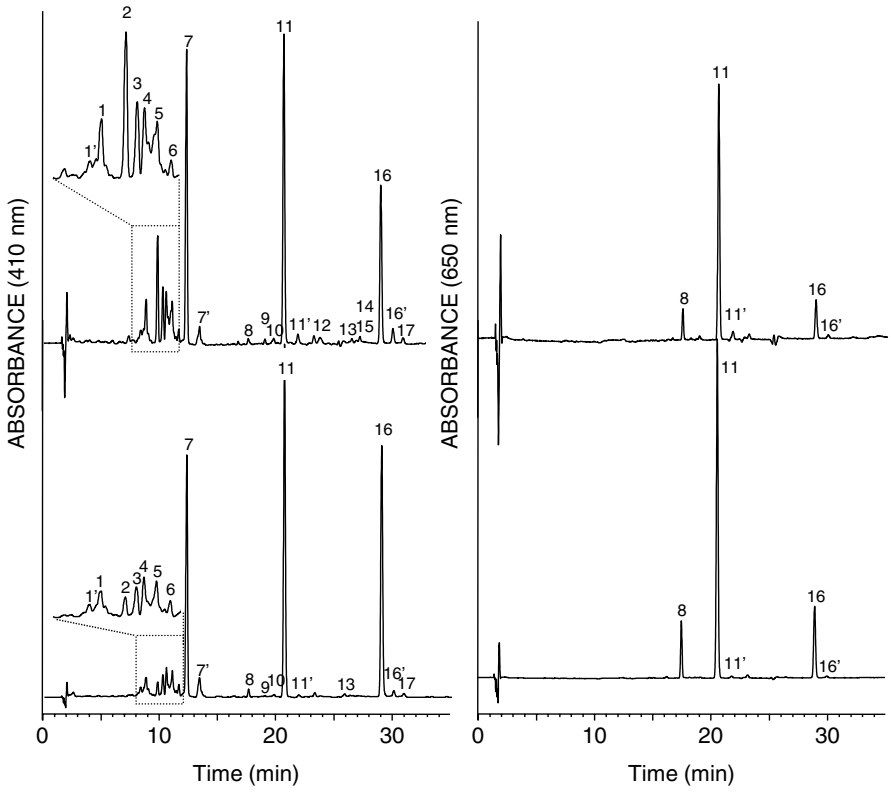

Fig. 7.7 HPLC chromatograms of pigment fraction from virgin olive oil of Arbequina variety (*upper*) and Hojiblanca variety (*lower*). Detection at 410 and 650 nm. Peaks: (*1*) neoxanthin, (*1′*) neoxanthin isomer, (*2*) violaxanthin, (*3*) luteoxanthin, (*4*) auroxanthin, (*5*) antheraxanthin, (*6*) mutatoxanthin, (*7*) lutein, (*7′*) lutein isomers, (*8*) chlorophyll *b,* (*9*) 13²-OH-chlorophyll *a,* (*10*) β-cryptoxanthin, (*11*) chlorophyll *a,* (*11′*) chlorophyll *a′*, (*12*) violaxanthin monoesterified, (*13*) pheophytin *b,* (*14*) antheraxanthin esterified, (*15*) neoxanthin esterified, (*16*) pheophytin *a,* (*16′*) pheophytin *a′*, (*17*) β-carotene (*Source*: Authors)

a photodiode detector. Table 7.3 lists the spectroscopic and chromatographic characteristics that enable their identification. The numbered compounds correspond to peaks identified in recently obtained VOO as shown in Fig. 7.7, and the unnumbered compounds correspond to peaks identified in VOOs after storage or adulteration whose characteristics are also included to facilitate their identification.

At the same time, chromatograms can be obtained at other wavelengths (410 nm, 450 nm), thereby allowing lower detection limits (LOD) of certain pigments (Table 7.3). Other wavelengths such as 650 nm (Fig. 7.7) or 633 nm, at which carotenoid compounds do not absorb, are highly suited to detect the fraudulent addition of the already mentioned colorant E-141i (copper compounds of chlorophylls) in olive oil.

The calibration curves for quantification should be obtained from pure pigment standards. Duplicate injections should be made of five different volumes of each standard solution and the calibration equation obtained using linear regression

Table 7.3 Chromatographic and spectroscopic characteristics of pigments from virgin olive oils separated RP-HPLC

					Spectral data in HPLC eluent: λ_{max}[d]												Detection[e]	
					I		II		III		IV		V		VI			
Peak[a]	Pigment	k_c[b]	$r_{i,st}$[c]	Soret	M	R	M	R	M	R	M	R	M	R	M	R	λ	LOD
	Carotenoids																	
1	Neoxanthin	3.35	0.34		414		438		466	90							430	0.12
1′	Neoxanthin isomer	3.59	0.37		414		438		466	90							430	0.12
	Neochrome	3.83	0.39		398		422		448	78							430	0.60
2	Violaxanthin	4.11	0.42		417		440		471	94							450	0.09
3	Luteoxanthin	4.34	0.44		399		422		449	107								
4	Auroxanthin	4.49	0.46		380		400		424	103							430	0.33
5	Antheraxanthin	4.76	0.49		(425)		446		474	22								
6	Mutatoxanthin	5.06	0.52		(408)		428		455	39								
7	Lutein	5.42	0.55		424		446		474	60							450	0.08
7′	Lutein isomer	6.01	0.61		418		440		478	42							450	0.08
10	β-cryptoxanthin	9.35	0.95		(431)		452		479	25							450	0.08
12	Violaxanthin monoesterified	11.44	1.17		417		440		471	94								
14	Antheraxanthin esterified	13.07	1.33		(425)		446		474	22								
15	Neoxanthin esterified	13.24	1.35		414		438		466	90								
17	β-carotene	15.19	1.55		(432)		454		481	26							450	0.19
	Chlorophyll compounds																	
8	Chlorophyll *b*	8.20	0.84	466	350	3.15					(570)	16.80	600	8.69	650	2.80	450	0.33
9	13^2-OH-chlorophyll *a*	8.95	0.91	432	(394)	1.49	(420)	1.10	(534)	25.10	(590)	8.37	616	4.48	666	1.27	430	0.31
	15^1-OH-lactone-pheophytin *b*	8.45	0.92	427			(416)	1.89	522	15.38	(560)	17.57	(600)	20.50	651	4.47		

11	Chlorophyll *a*	9.81	1.00	432	(394)	1.49	(420)	1.10	(534)	25.10	(590)	8.37	616	4.48	666	1.27	430	0.30
11′	Chlorophyll *a*′	10.45	1.06	432	(394)	1.49	(420)	1.10	(534)	25.10	(590)	8.37	616	4.48	666	1.27	430	0.30
	15^1-OH-lactone-pheophytin *a*	10.37	1.13	399					499	10.97	531	18.00	614	22.91	670	2.57		
	Cu-pheophytin *b*	11.11	1.21	443							540	15.53	590	6.47	633	2.38		
13	Pheophytin *b*	12.93	1.32	435			412	2.27	524	13.62	(558)	20.42	598	16.33	654	3.83	430	0.27
16	Pheophytin *a*	14.18	1.45	409	(400)	1.07	(376)	1.31	505	8.78	537	9.62	609	10.63	666	1.85	410	0.34
16′	Pheophytin *a*′	14.73	1.50	409	(400)	1.07	(376)	1.31	505	8.78	537	9.62	609	10.63	666	1.85	410	0.34
	Pyro-pheophytin *b*	14.81	1.51	435			412	2.27	524	13.62	(558)	20.42	598	16.33	654	3.83	430	0.25
	Cu-pheophytin *a*	15.11	1.54	424	(384)	1.65	400	1.02	506	20.11	548	12.07	606	3.29	654	1.00	410	0.88
	Cu-pyro-pheophytin *b*	15.40	1.57	443							540	15.53	590	6.47	633	2.38		
	Pyro-pheophytin *a*	17.17	1.75	409	(400)	1.07	(376)	1.31	505	8.78	537	9.62	609	10.63	666	1.85	410	0.32
	Cu-pyro-pheophytin *a*	18.93	1.93	424	(384)	1.65	400	1.02	506	20.11	548	12.07	606	3.29	654	1.00	410	0.77

Source: Authors

The values in parenthesis indicate inflection points in the absorption spectrum

[a]Numbered peaks according to Fig. 7.7. Peaks not numbered correspond to compounds from stored or adulterated virgin olive oil

[b]Retention factor, $k_c = (t_r - t_m)/t_m$, where t_r = retention time of pigment peak and t_m = retention time of an unretained component

[c]Relative retention, $r_{i,st} = k_c/k_{st}$; *st* = chlorophyll *a*

[d]M = position maximum (nm) and R = absorbance ratio of Soret band to absorbance at wavelength indicated for chlorophyll compounds and 100III/II for carotenoids

[e]*LOD* limit of detection (ng) considering a signal-to-noise ratio of about 3 under UV-visible detection at λ (nm)

analysis (see Chap. 12 for more information). LODs can be calculated considering a signal-to-noise ratio of approximately 3. Usually, under analytical conditions in which 10 g VOO are concentrated by LPE into 1.5 mL acetone, 20 μL are injected into the chromatograph and analyzed under UV-visible detection, it permits LODs < 6.6 ppb.

The fluorescence detectors are more selective and have lower LODs concerning chlorophyllic compounds (Cichelli and Pertesana 2004; Hornero-Méndez et al. 2005). The most suitable excitation and emission wavelengths for the simultaneous detection of all the chlorophyllic derivatives (Cichelli and Pertesana 2004) are $\lambda_{ex} = 440$ nm and $\lambda_{em} = 660$ nm. The specific determination of pheophytin *a* and pyropheophytin *a* can be improved by using acetone/methanol mixture (1:1) in isocratic conditions that permits a fine separation in less than 9 min (Table 7.2) and by adjusting λ_{ex} to 410 nm and $\lambda_{em} = 672$ nm (Hornero-Méndez et al. 2005). The high levels of sensitivity and selectivity offered by electrochemical detectors (coulometric electrochemical array detector) have also been applied to vegetable oils (Puspitasari-Nienaber et al. 2002) for the simultaneous detection of tocopherols and pigments, with detection limits improved 1,000-, 25-, and 5-fold for carotenoids, tocopherols, and chlorophyllic derivatives, respectively.

7.5 Chlorophylls and Carotenoids in Virgin Olive Oils

7.5.1 Metabolism in Olive Fruits: Intervariety Differences

As mentioned earlier, chlorophylls and carotenoids play a central role in photosynthesis, and it has been reported that the photosynthesis that takes place in the olive fruit contributes to the biosynthesis of olive oil (Sánchez and Harwood 2002; Conde et al. 2008). Therefore, due to the essential and universal role of chlorophylls and carotenoids, the pigment profile in vascular plant chloroplasts is almost uniform. Based on this premise, the total pigment content in the olive fruit permits differentiation between varieties, where Hojiblanca and Picual stand out as varieties with high pigmentation, compared to others, such as Arbequina, Blanqueta, and Cornicabra, included in the low pigmentation varieties. Figure 7.8 shows the evolution in chlorophyll and carotenoid content in two extreme varieties (Hojiblanca and Arbequina) during the growth and ripening of their olives; the synthesis and stabilization of chlorophylls can be observed in the olive growth phase, while the catabolic phase takes place during the ripening phase. In general terms, the evolution profile of chlorophylls *a* and *b* is relatively similar, with chlorophyll *a* representing approximately 80% and chlorophyll *b* 20% of the chlorophyllic fraction (Roca and Mínguez-Mosquera 2001a). But in addition to these pigments, clearly the majority of the chromatographic profile shows a differential buildup of chlorophyllic catabolites between varieties. Thus, whereas Hojiblanca and Picual basically have chlorophylls *a* and *b* and traces of 13^2-OH-chlorophyll *a*, Arbequina also accumulates dephytylated

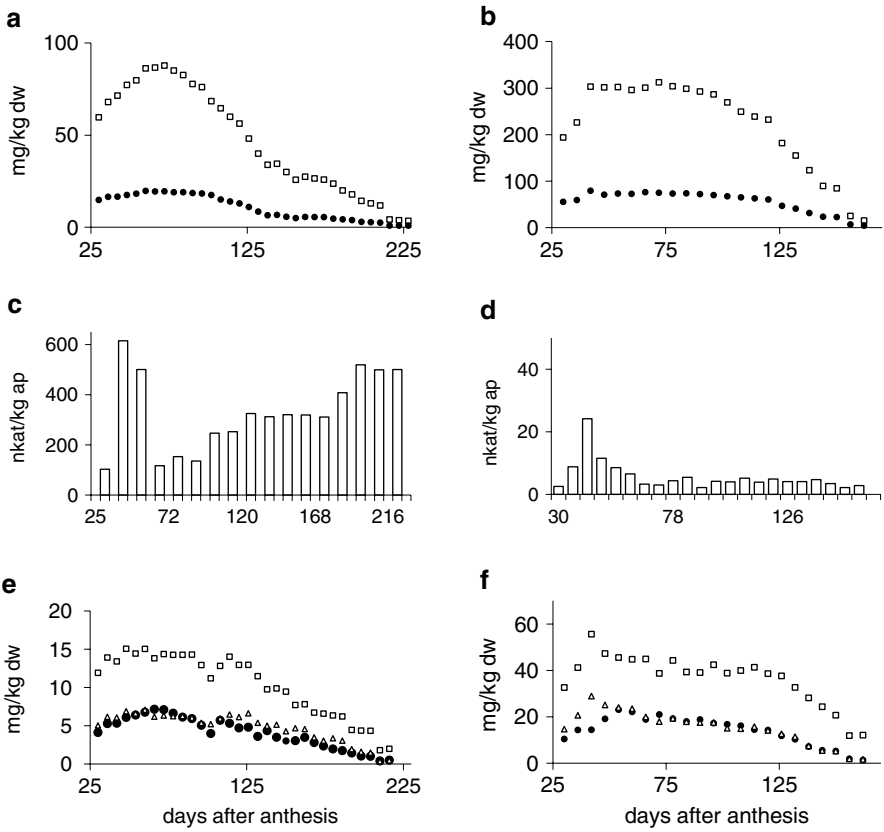

Fig. 7.8 Pigment content (mg/kg dry weight) and chlorophyllase activity (nkat/kg acetone powder) during fruit growth phase and ripening in Arbequina (**a**, **c, e**) and Hojiblanca varieties (**b**, **d, f**). (**a**, **b**) Chlorophyll content: (□) chlorophyll *a*, (●) chlorophyll *b*. (**c**, **d**) chlorophyllase activity. (**e**, **f**) carotenoid content: (□) lutein, (●) ß-carotene and (▲) violaxanthin + antheraxanthin + neoxanthin (*Source*: Authors)

chlorophylls: 13^2-OH-chlorophyllide *a*, chlorophyllide *a*, pyrochlorophyllide *a*, and pheophorbide *a* (Fig. 7.2) (Roca and Mínguez-Mosquera 2003a).

This differential accumulation of dephytylated chlorophylls in some varieties of olives is due to the high levels of the chlorophyllase enzyme, the first enzyme involved in the degradation of chlorophylls. As an example, Fig. 7.8 shows the inter-variety differences of chlorophyllase activity measured between varieties (Roca and Mínguez-Mosquera 2003a). It can be seen how the levels may be 30–100 times higher in Arbequina than in Hojiblanca. But in addition to the dephytylated chlorophyllic derivatives, oxidized chlorophylls have also been detected in different amounts in the pigment profile of different varieties of olive oil. The formation of these oxidized catabolites has been attributed to the peroxidase enzyme, as the

presence of this enzyme activity in the thylakoid of the olive fruit in the presence of H_2O_2 and phenols has been demonstrated together with its role in the formation of 13^2-OH-chlorophyll *a* (Gandul-Rojas et al. 2004; Roca et al. 2007).

In general, almost all olive fruits are green when unripe (like the majority of fruits), and the photosynthetic activity decreases and the chlorophylls disappear as the fruit ripens. Alternatively, the concentration of carotenoids decreases (as is usual in fruits in which ripening is associated with the synthesis of anthocyanins). Consequently, in varieties such as Gordal, Manzanilla, Picual, Hojiblanca, Cornicabra, or Blanqueta, the qualitative pattern of chloroplasts does not change (Mínguez-Mosquera and Gallardo-Guerrero 1995; Roca and Mínguez-Mosquera 2001a). In these cases, regardless of the high oil content of the ripe olive, the xanthophylls remain unesterified, which is a sign that chloroplasts remain intact during the ripening process. However, studies of fruit from the Arbequina variety have demonstrated a completely different metabolism. Thus, in addition to the carotenoid derivatives present in other olives, the Arbequina variety has exclusive pigments, such as esterified violaxanthin, neoxanthin, and antheraxanthin, as well as α-carotene, ζ-carotene, and phytofluene, which may be significant from a chemotaxonomic point of view when differentiating this variety (Roca and Mínguez-Mosquera 2001c; Criado et al. 2007a). To conclude, in this fruit, the chloroplasts do not remain intact during the ripening process but are transformed into chromoplasts.

The only carotenoid representative from the ß, ε-series, and present in the olive fruit is lutein, which is also the main pigment of the chloroplast. Its concentration increases during the growth of the fruit, it remains constant until the ripening process begins, and then its concentration gradually decreases. With respect to the carotenoids from the β, β-series (β-carotene, neoxanthin, violaxanthin, antheraxanthin, and ß-cryptoxanthin), their accumulation during growth depends on the availability of the corresponding carotenoid precursor. During the ripening process, catabolism is accelerated (Roca and Mínguez-Mosquera 2003b).

7.5.2 Changes in Profile and Concentration of Pigments

The color of virgin olive oil is exclusively due to the solubilization in the oil of the chloroplastic pigments present in the fresh fruit, together with transformations that these pigments might experience during the process of virgin olive oil extraction. In fact, the pigment concentration in virgin olive oils depends on the cultivar (Roca and Mínguez-Mosquera 2001b; Criado et al. 2007a), olive ripeness (Mínguez-Mosquera et al. 1990; Gallardo-Guerrero et al. 2002; Criado et al. 2006), agricultural practices such as irrigation (Criado et al. 2007b), and oil extraction process. In general, as the temperature of the malaxation process increases in a range between 20 and 30°C, the concentration of the pigments in the oil increases (Ranalli et al. 2001, 2005; Stefanoudaki et al. 2011). The heat treatments of olive fruit prior to olive extraction also increase the content of both chlorophyll and carotenoid

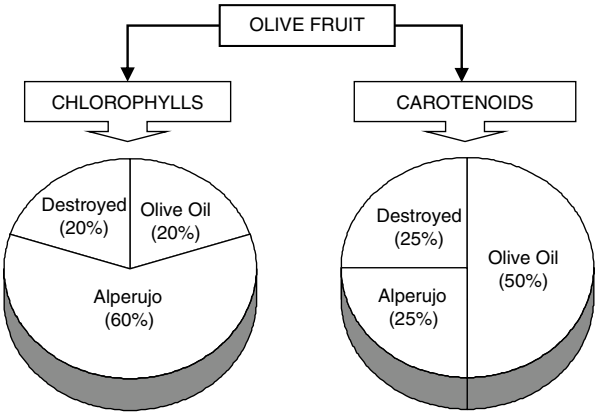

Fig. 7.9 Distribution (%) of chlorophylls and carotenoids present in olive fruit when processed for virgin olive oil (*Source*: Authors)

compounds (Luaces et al. 2005). Furthermore, the addition of coadjuvants (enzymes, microtalc, or NaCl) during the oil extraction process generally increases the pigment content of the resultant oils (Ranalli et al. 2005; Criado et al. 2007b; Pérez et al. 2008). The pigments detected in virgin olive oil are chlorophylls *a* and *b*, lutein, β-carotene, violaxanthin, neoxanthin, antheraxanthin, and β-cryptoxanthin, all of them coming from the fresh fruit, together with those formed during the extraction process, pheophytins *a* and *b*, luteoxanthin, auroxanthin, neochrome, and mutatoxanthin (Mínguez-Mosquera et al. 1990, 1992; Gandul-Rojas and Mínguez-Mosquera 1996a). The formation of these pigments is due to the fact that during the milling process a series of acids is released from the tissues of the fruit, which in the malaxation and centrifugation period favors the pheophytinization of chlorophylls and the isomerization of the carotenoids with 5,6-epoxide groups in their structure to the 5,8-furanoid derivatives.

On average, only 20% of the chlorophylls and 50% of the carotenoids present in the olive fruit are transferred to VOO. Recent studies have pointed out that most pigments (60% for chlorophylls and 25% for carotenoids) are occluded in the byproduct *alperujo* (Gallardo-Guerrero et al. 2002) (Fig. 7.9).

The presence of exclusive pigments – such as deesterified chlorophyllic derivatives or esterified xanthophylls – can be used as markers of varietal VOO. On the other hand, three ratios have been proposed (Gandul-Rojas et al. 2000; Roca et al. 2003) for determining the genuineness and correct processing of Spanish VOOs:

Ratio of total chlorophylls to total carotenoids of between 0.5 and 1.4.
Ratio of minor carotenoids to lutein of between 0.2 and 1.2.
Ratio of lutein to β-carotene of greater than 1.

The color of VOO may range from dark green to pale yellow depending on the composition of the chlorophyllic and carotenoid pigments of the source fruit. The

average consumer identifies deeply colored oil with VOO, whereas little pigmented olive oils tend to remind the consumer of refined seed oils. As a result, some producers reinforce the color of the olive oil by adding colorants, even though this practice is fraudulent according to European Regulations (EC 1994). Therefore, the detection of pigments not found in the olive fruit, or the alteration of quantitative ratios among certain pigments, indicates adulteration of the color of the marketed oil (Roca et al. 2010).

The green fat-soluble colorant E-141i, known as a cupric derivative of chlorophyll, is the most widely used colorant in the adulteration of the color of olive oil. It is obtained from different edible vegetable sources by extracting the pigments with solvent and then adding copper to the resultant solution. Although its chlorophyllic composition might therefore vary, depending on the raw material and the process used to obtain it, analyses of various commercial samples of the colorant E-141i (Roca et al. 2010) have shown that 99.6% of chlorophyllic pigments present in the colorant E-141i are not from olive oil; the main pigment in that colorant is Cu-pyropheophytin *a*. Therefore, the simple detection of one of these compounds in an unknown olive oil would permit detection of adulteration (Roca et al. 2010).

The fraudulent use of other colorants that do not contain Cu has also been confirmed. These include E-140i (so-called chlorophyll or natural green) and carotenoid compounds such as E-160 (carotenes) and E-161 (lutein). Consequently, another useful marker of adulteration is the lutein/β-carotene ratio or the structural form of the lutein. There are cases of olive oil in which β-carotene is found exclusively or in a percentage of more than 90%. This ratio is not found in olive oil and indicates the addition of the colorant E-160. In other cases, lutein is found in esterified form (less polar), which is not present in the olive fruit despite the high fatty acid content. Therefore, the presence of this compound indicates adulteration with the colorant E-161.

The genuine qualitative pigment profile of the olive oil is affected by the small degree of oil degradation that occurs during storage even under appropriate conditions of darkness and controlled temperature (Gallardo-Guerrero et al. 2005). There is a general advance of the reactions started during the extraction process. Basically, pheophytinization is completed and a certain degree of allomerization continues. Likewise, this begins a new reaction, so-called pyropheophytinization, that is significantly affected by temperature (Fig. 7.2) (Aparicio-Ruiz et al. 2010). Therefore, the percentage of pyropheophytins has been suggested as a chemical marker for monitoring degradation of VOO (Gandul-Rojas et al. 1999b; Gallardo-Guerrero et al. 2005; Anniva et al. 2006).

The presence of pyropheophytins in food, on the other hand, has always been associated with thermal treatment (Schwartz et al. 1981), and this explains why its presence in VOOs had been connected to their spiking with “deodorato” olive oils. The deodorization under vacuum conditions and at low temperature (<100°C) with a nitrogen stream allows for the removal of volatile compounds responsible for VOO sensory defects, which would not allow classifying a VOO as extra virgin but as ordinary or lampante. This process does not modify the basic chemical compounds of the VOO with the exception of pyropheophytins whose concentration

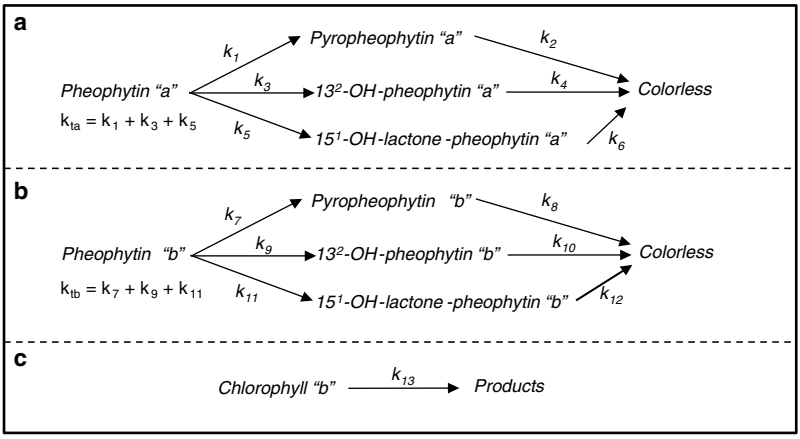

Fig. 7.10 Kinetic mechanisms of thermodegradation pathway of chlorophylls in virgin olive oil: (**a**) kinetic mechanism of pheophytin *a* thermodegradation; (**b**) kinetic mechanism of pheophytin *b* thermodegradation; (**c**) kinetic mechanism of total thermodegradation of chlorophyll *b* (*Source*: Authors)

increases (Gandul-Rojas et al. 1999c; Serani and Piacenti 2001). Therefore, the percentage of pyropheophytins has also been suggested as a chemical marker of VOO thermal treatments (Gertz and Fiebig 2006).

These results have prompted researchers to study the kinetic behavior of chlorophyll pigments to establish prediction models of their evolution over time as a function of temperature.

7.5.3 Thermal Degradation Kinetics and Prediction Models

Kinetic models are becoming increasingly useful in the study of changes in the chemical composition of foods and in predicting their shelf life. The formulation of the chemical reactions in the degradation of pigments is immediately amenable to the design of kinetic models that make it possible to predict these changes. Experimental data are, therefore, necessary for estimating the parameters of the mathematical model and, with the help of statistical algorithms, can be used to determine which parameters are relevant to the prediction.

Thermodegradation of chlorophylls and carotenoids in VOOs, heated at different temperatures, in the absence of light and oxygen, has enabled the determination of different first-order degradation kinetic mechanisms of relevant chlorophyllic and carotenoid pigments (Aparicio-Ruiz et al. 2010, 2011) (Figs. 7.10 and 7.11). In the group of chlorophyllic pigments, pheophytin *a* does not directly degrade to colorless products but through a complex kinetic mechanism of parallel and consecutive reactions (Fig. 7.10a) in pyropheophytin *a* (due to the loss of the carbomethoxide

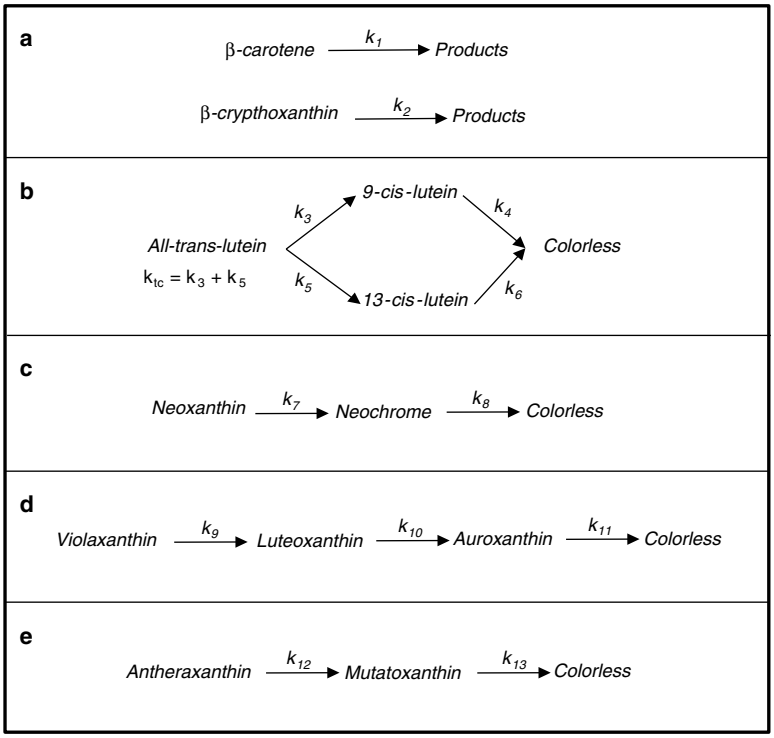

Fig. 7.11 Kinetic mechanisms of thermodegradation pathway of carotenoids in virgin olive oil: (**a**) kinetic mechanism of total thermodegradation of ß-carotene and ß-cryptoxanthin; (**b**) kinetic mechanism of all-trans-lutein thermodegradation; (**c**) kinetic mechanism of neoxanthin thermodegradation; (**d**) kinetic mechanism of violaxanthin thermodegradation; (**e**) kinetic mechanism of antheraxanthin thermodegradation (*Source*: Authors)

group from the pheophytin), in 13^2-OH-pheophytin *a* (due to the allomerization of the C-13^2 of the isocyclic ring), and in 15^1-OH-lactone-pheophytin *a* (due to the formation of a lactone ring) (Fig. 7.2). In turn, these compounds degrade to colorless products through a subsequent reaction due to the breakup of the porphyrin ring (Aparicio-Ruiz et al. 2010).

The kinetic constant of the reaction leading to the formation of pyropheophytin *a* (k_1) (Fig. 7.10a) has much higher values than those resulting in OH-pheophytin *a* (k_3) and lactone-pheophytin *a* (k_5). This is because in oil heated in the absence of light and oxygen, the reaction to form pyropheophytin *a* (Fig. 7.12), which is more dependent on temperature, is favored to the detriment of the other two reactions, which take place by a mechanism via free radicals in which oxygen is a critical factor. On the other hand, reactions via free radicals could be affected by the reaction medium since triacylglycerols and some minor compounds with antioxidant activity (e.g., polyphenols, tocopherols, and carotenoids) could modulate the reaction.

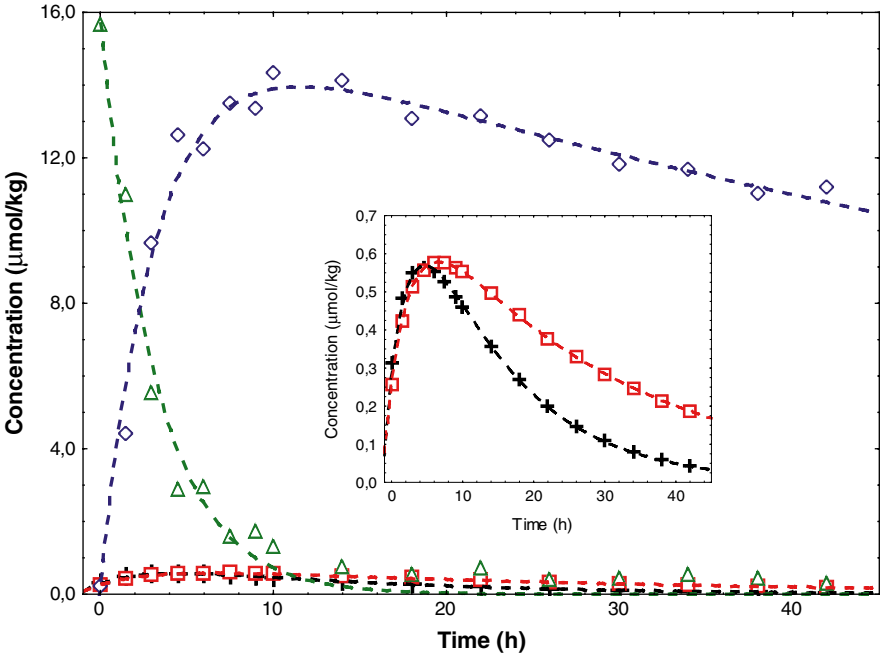

Fig. 7.12 Evolution of concentration time of pheophytin *a* (Δ), pyropheophytin *a* (◇), 13^2-OH-pheophytin *a* (□), and 15^1-lactone-pheophytin *a* (+) in VOO for 42 h at 120°C and their corresponding nonlinear regression fits (- -) (*Source*: Authors)

Although a recently extracted VOO contains chlorophylls *a* and *b*, the total thermodegradation to colorless products, including any possible intermediate product (pheophytin *b*, 13^2-OH-chlorophyll *b*, 15^1-OH-lactone-chlorophyll *b*) has only been determined for chlorophyll *b* (Fig. 7.10b, c). Comparatively, the kinetic constant for chlorophyll *b* (k_{13}) is approximately equal to that corresponding to pheophytin *b* (k_{tb}) and greater than that for pheophytin *a* (k_{ta}), demonstrating the greatest stability of pheophytin *a* against thermodegradation (Aparicio-Ruiz 2008).

Similar mechanisms of cascade reactions are also involved in the degradation of carotenoid pigments; Fig. 7.11a shows the total degradation mechanisms for β-carotene and β-cryptoxanthin to colorless products. Figure 7.11b, on the other hand, shows the degradation mechanism of all-trans-lutein that occurs through parallel and consecutive reactions. The first reactions result in structural isomerizations where the formation of 13-*cis*-lutein is favored over 9-*cis*-lutein. The second reactions generate colorless products due to the breakdown of the polyenic chain (Aparicio-Ruiz et al. 2011).

The degradation mechanisms of neoxanthin, violaxanthin, and mutatoxanthin (Fig. 7.11c–e) occur through consecutive reactions that involve the reorganization of the 5,6-epoxide group to 5,8-furanoide and its subsequent degradation to colorless products due to the rupture of the polyenic chain (Aparicio-Ruiz and Gandul-Rojas 2012).

These studies indicate that the carotenoids of VOO are gradually destroyed, due to thermal degradation, in a well-established order; violaxanthin is the most vulnerable to thermal degradation and, therefore, the first to degrade. The following sequence orders the compounds according to fastest (violaxanthin) to slowest (9-cis-lutein) degradation rates: violaxanthin, luteoxanthin, auroxanthin, β-carotene, antheraxanthin, mutatoxanthin, neochrome, β-cryptoxanthin, neoxanthin, lutein, 13-cis-lutein, and 9-cis-lutein. Contrary to what might be expected, the total degradation constant of carotenoids is on average 3.6 times higher than that of chlorophylls, indicating a higher thermal stability of the latter (Aparicio-Ruiz 2008; Aparicio-Ruiz et al. 2011; Aparicio-Ruiz and Gandul-Rojas 2012).

7.5.4 Prediction Models

Kinetic studies of the thermodegradation of pigments in VOOs have therefore enabled the design and validation of mathematical prediction models.

This is the case with pyropheophytin *a*, a compound proposed as a chemical marker for VOO subjected to thermal treatments (Gandul-Rojas et al. 1999c; Serani and Piacenti 2001; Gertz and Fiebig 2006) and for VOO freshness (Gandul-Rojas et al. 1999a; Gallardo-Guerrero et al. 2005; Anniva et al. 2006).

In 2001, Serani and Piacenti established an empirical correlation between pheophytin and pyropheophytin contents, analyzing VOOs of diverse geographical origins and with different shelf lives, and proposed an index (cold index) derived from that correlation that sought to distinguish between the genuine and "deodorato" VOOs. Later, Gallardo-Guerrero et al. (2005) demonstrated that pyropheophytin content is a highly variable parameter that depends not only on the operating variables (time and temperature) but also on the initial pheophytin content in the VOO, and this led the researchers to propose instead the relative content of pyropheophytin *a*, which is expresed as

$$\%\ \text{Pyphy}\ a = \left[\frac{\text{Pyropheophytin}\ a}{\text{Pyropheophytin}\ a + \text{Pheophytin}\ a}\right] \times 100$$

This new chemical parameter is independent of the absolute amounts and can be directly calculated from the area ratio of the corresponding peaks in the HPLC chromatogram, eliminating the calibration process. By substituting the kinetic parameters established experimentally in the foregoing equation (Aparicio-Ruiz et al. 2012), we obtain the prediction model for pyropheophytin *a* as a function of temperature and time:

$$\%\text{Pyphy}\,a(t) = \left[\frac{\dfrac{k_1[\text{Phy}\ a]_0}{k_2 - k_{ta}}\left[e^{-k_{ta}\cdot t} - e^{-k_2\cdot t}\right] + [\text{Pyphy}\ a]_0 e^{-k_2\cdot t}}{\dfrac{k_1[\text{Phy}\ a]_0}{k_2 - k_{ta}}\left[e^{-k_{ta}\cdot t} - e^{-k_2\cdot t}\right] + [\text{Pyphy}\ a]_0 e^{-k_2\cdot t} + [\text{Phy}\ a]_0 e^{-k_{ta}\cdot t}}\right] \times 100,$$

where [Phy a]$_0$ is the initial concentration of pheophytin a, and k_1, k_2, and k_{ta} are the kinetic constants involved in the formation/degradation of pyropheophytin a (Fig. 7.10a). In a recently extracted VOO, the initial concentration of pyropheophytin a [Pyphy a]$_0$ should be zero, and therefore the equation can be simplified.

The successful validation of the prediction model, with oils analyzed by other analysts, led to the conclusion that the proposed model is useful to producers and wholesalers for an a priori estimation of the shelf life of a VOO, based on the percentage of pyropheophytin a, as a function of the oil storage temperature that can be expressed as a function of real time to daily, monthly, or yearly average. Analyses of the most general and unfavorable cases of the model show that VOOs should be stored at a controlled temperature (or annual average) of no more than 22°C to prevent the percentage of pyropheophytins from reaching 15% before the usual shelf life (Aparicio-Ruiz et al. 2012).

7.6 Trends and Perspectives

Single-column (one-dimensional) chromatography analysis has been used for many years as a standard separation tool for analyzing pigments. Multidimensional chromatography has emerged as an interesting alternative for analyzing complex samples. Thus, peak capacity enhancement achievable by multidimensional chromatography is far higher than that obtained after improving, by any means, one-dimensional chromatography (Herrero et al. 2009). Comprehensive two-dimensional liquid chromatography (LC×LC) is a powerful separation technique that couples two independent separations with orthogonal selectivity. The use of this technique entails analyzing the whole sample in the two dimensions independently using a switching valve as a transfer system between them. The use of two identical sample loops installed in this switching valve allows for collecting and injecting fractions eluting from the first dimension into the second dimension continuously. This technique has been applied successfully in the determination of esterified carotenoides (Dugo et al. 2009), and its use is increasing.

A different approach is the use of conventional C_{30} LC columns coupled in series in a complex matrix. It has been demonstrated that the use of this coupling can enhance both the resolution and separation of such complex samples, increasing the peak capacity by as much as 25% (Dugo et al. 2008).

HPLC employing various detecting techniques, like diode array detector (DAD) and mass spectrometry (MS), is currently the method of choice for carotenoid analysis. Mass spectrometric analyses, which provide molecular weight and characteristic fragmentation patterns, may then provide final confirmation of individual carotenoid identities when used in conjunction with retention and spectral characteristics. In this sense, many carotenoids possess very similar or even identical UV-vis spectra, and MS is of great help as it allows for the differentiation of compounds with diverse molecular masses. However, there are also carotenoids that have the same molecular weight. In these cases, DAD analysis allows for

identification. Many recent works dealing with this subject have been published, coupling UV-vis and MS detection using both APCI and ESI (Crupi et al. 2010).

The development of techniques based on photoacoustic and photothermal phenomena has progressed significantly in recent years. The methods, which essentially rely on the conversion of absorbed energy into heat, permit the optical as well as thermal characterization of samples in practically any aggregate condition. These nondestructive methods offer several advantages over the conventional approaches: a substantial simplification of sample preparation, the ability to perform studies on opaque and intractable samples, and the possibility for subsurface profiling of the optical and thermal properties of the layers in inhomogeneus and layered samples. These techniques have been extensively used with carotenoids (Bicanic 2011).

Although, at the moment, the profile of chlorophyllic and carotenoid pigments present in an olive oil is not included in the regulated quality standards (IOC 2009), a recent regulation has included the color of oils as one of the obligatory quality parameters (USDA 2010). Color makes a significant impression on the consumer and has a major effect on the acceptance of the oil due to the current association of green color with high-quality oils and pale yellow color with refined oils. As mentioned earlier, this has led certain producers to strengthen the color in very different ways, usually by adding colorants such as E-141i (copper chlorophylls), E-160 (β-carotene), and E-161 (lutein), although these are not the only ones. Chromatographic methods and control indexes have been established for all these methods to facilitate the detection of adulteration. However, to date, none of these is official.

Finally, the latest agronomic trends involve the development of new cultivars with the primary goal of producing large quantities of very high-quality olive oil in an efficient and economical way (Chap. 2). The chemical characterization of their fruits and oils includes information on the metabolism of chlorophylls and carotenoids. Perhaps the development of new cultivars with certain characteristics requested by consumers is the path of the future.

Those are points to be developed in the future. The chlorophyllic and carotenoid compositions of VOO are parameters not only of authenticity but also of quality.

References

Abraham RJ, Rowan AE (1991) Nuclear magnetic resonance spectroscopy of chlorophyll. In: Scheer H (ed) Chlorophylls. CRC Press, Boca Raton, pp 797–834

Aitzetmuller K (1989) Chlorophyll degradation products in vegetable-oils. Fett Wiss Technol 91:99–105

Anniva C, Grigoriadou D, Psomiadou E, Tsimidou MZ (2006) Pheophytin α degradation products as useful indices in the quality control of virgin olive oil. J Am Oil Chem Soc 83:371–375

Aparicio-Ruiz R (2008) Characterization of thermodegradation reactions of carotenoid and chlorophyllic compounds of virgin olive oil. Ph.D. thesis, University of Sevilla (Spain)

Aparicio-Ruiz R, Gandul-Rojas B, Roca M (2009) Pigment profile in non-spanish olive varieties (*Olea europaea* L. var. coratina, frantoio, and koroneiki). J Agric Food Chem 57:10831–10836

Aparicio-Ruiz R, Minguez-Mosquera MI, Gandul-Rojas B (2010) Thermal degradation kinetics of chlorophyll pigments in virgin olive oils. 1. Compounds of series a. J Agric Food Chem 58:6200–6208

Aparicio-Ruiz R, Minguez-Mosquera MI, Gandul-Rojas B (2011) Thermal degradation kinetics of lutein, β-carotene and β-cryptoxanthin pigments in virgin olive oils. J Food Compos Anal 24:811–820

Aparicio-Ruiz R, Gandul-Rojas B (2012) Thermal Degradation Kinetics of Neoxanthin, Violaxanthin, and Antheraxanthin in Virgin Olive Oils. J Agric Food Chem 60:5180–5195

Aparicio-Ruiz R, Roca M, Gandul-Rojas B (2012) Mathematical model to predict formation of pyroheophytin a in virgin olive oil during the storage. J Agric Food Chem 60:7040–7049

Bicanic DD (2011) On the photoacoustic, photothermal and colorimetric quantification of carotenoids and other phytonutrients in some foods: a review. J Mol Struct 993:9–14

Britton G, Liaaen-Jensen S, Pfander H (1995a) Carotenoids. Volume 1A: isolation and analysis. Birkhäuser Verlag, Basel

Britton G, Liaaen-Jensen S, Pfander H (1995b) Carotenoids. Volume 1B: spectroscopy. Birkhäuser Verlag, Basel

Britton G, Liaaen-Jensen S, Pfander H (2004) Carotenoids. Handbook. Birkhäuser Verlag, Basel

Britton G, Liaaen-Jensen S, Pfander H (2008) Carotenoids. Volume 4: natural functions. Birkhäuser Verlag, Basel

Britton G, Liaaen-Jensen S, Pfander H (2009) Carotenoids. Volume 5: nutrition and health. Birkhäuser Verlag, Basel

Chan JY, Tang PM, Hon PM, Au SW, Tsui SK, Waye MM, Kong SK, Mak TC, Fung KP (2006) Pheophorbide *a*, a major antitumor component purified from *Scutellaria barbata*, induces apoptosis in human hepatocellular carcinoma cells. Planta Med 72:28–33

Chen M, Schliep M, Willows RD, Cai ZL, Neilan BA, Scheer H (2010) A red-shifted chlorophyll. Science 329:318–1319

Cichelli A, Pertesana GP (2004) High-performance liquid chromatographic analysis of chlorophylls, pheophytins and carotenoids in virgin olive oils: chemometric approach to variety classification. J Chromatogr 1046:141–146

Conde C, Serge D, Gerós H (2008) Physiological, biochemical and molecular changes occurring during olive development and ripening. J Plant Physiol 165:1545–1562

Criado MN, Motilva MJ, Ramo T, Romero MP (2006) Chlorophyll and carotenoid profile and enzymatic activities (chlorophyllase and lipoxygenase) in olive drupes from the fruit setting period to harvest time. J Am Soc Hortic Sci 131:593–600

Criado MN, Motilva MJ, Goñi M, Romero MP (2007a) Comparative study of the effect of the maturation process of the olive fruit on the chlorophyll and carotenoid fractions of drupes and virgin oils from Arbequina and Farga cultivars. Food Chem 100:748–755

Criado MN, Romero MP, Motilva MJ (2007b) Effect of the technological and agronomical factors on pigment transfer during olive oil extraction. J Agric Food Chem 55:5681–5688

Crupi P, Milella RA, Antonacci D (2010) Simultaneous HPLC-DAD-MS (ESI+) determination of structural and geometrical isomers of carotenoids in mature grapes. J Mass Spectrom 45:971–980

Dashwood RH (1997) Chlorophylls as anticarcinogens. Int J Oncol 10:721–727

Dashwood RH, Negishi T, Hayatsu H, Breinholt V, Hendricks J, Bailey G (1998) Chemopreventive properties of chlorophylls towards aflatoxin B1: a review of the antimutagenicity and anticarcinogenicity data in rainbow trout. Mutat Res 399:245–253

Didi MA, Makhoukhi B, Azzouz A, Villemin D (2009) Colza oil bleaching through optimized acid activation of bentonite. A comparative study. Appl Clay Sci 42:336–344

Dugo P, Herrero M, Giuffrida D, Ragonese C, Giacomo D, Mondello L (2008) Analysis of native carotenoid composition in orange juice using C_{30} columns in tandem. J Sep Sci 31:2151–2160

Dugo P, Giuffrida D, Herrero M, Donato P, Mondello L (2009) Epoxycarotenoids esters analysis in intact orange juices using two-dimensional comprehensive liquid chromatography. J Sep Sci 32:973–980

Egner PA, Stansbury KH, Snyder EP, Rogers ME, Hintz PA, Kensler TW (2000) Identification and characterization of chlorin e_4-ethyl ester in sera of individuals participating in the chlorophyllin chemoprevention trial. Chem Res Toxicol 13:900–906

Fahey JW, Stephenson KK, Dinkova-Kostova AT, Egner PA, Kensler TW, Talalay P (2005) Chlorophyll, chlorophyllin and related tetrapyrroles are significant inducers of mammalian phase 2 cytoprotective genes. Carcinogenesis 26:1247–1255

Ferruzzi MG, Blakeslee J (2007) Digestion, absorption, and cancer preventive activity of dietary chlorophyll derivatives. Nutr Res 27:1–12

Ferruzzi MG, Böhm V, Courtney PD, Schwartz SJ (2002) Antioxidant and antimutagenic activity of dietary chlorophyll derivatives determined by radical scavenging and bacterial reverse mutagenesis assays. J Food Sci 67:2589–2595

Fraser MS, Frankl G (1985) Detection of chlorophyll derivatives in soybean oil by HPLC. J Am Oil Chem Soc 62:113–121

Gallardo-Guerrero L, Roca M, Mínguez-Mosquera MI (2002) Distribution of chlorophylls and carotenoids in ripening olives and between oil and alperujo when processed using a two-phase extraction system. J Am Oil Chem Soc 79:105–109

Gallardo-Guerrero L, Gandul-Rojas B, Roca M, Mínguez-Mosquera MI (2005) Effect of storage on the original pigment profile of Spanish virgin olive oil. J Am Soc Chem Soc 82(1):33–39

Gallardo-Guerrero L, Gandul-Rojas B, Mínguez-Mosquera MI (2008) Digestive stability, micellarization, and uptake by Caco-2 human intestinal cell of chlorophyll derivatives from different preparations of pea (*Pisum sativum* L.). J Agric Food Chem 56:8379–8386

Gandul-Rojas B, Mínguez-Mosquera MI (1996a) Chlorophyll and carotenoid composition in virgin olive oils from various Spanish olive varieties. J Sci Food Agric 72:31–39

Gandul-Rojas B, Mínguez-Mosquera MI (1996b) Chlorophyllase activity in olive fruits and its relationship with the loss of chlrorophyll pigments in the fruits and oils. J Sci Food Agric 72:291–294

Gandul-Rojas B, Roca-López-Cepero M, Carmona-Ramón C, Mínguez-Mosquera MI (1999a) Stability of chlorophyll pigments during storage of virgin olive oil. In Mínguez-Mosquera MI, Jarén-Galán M, Hornero-Méndez D (eds*)* Proceeding of 1st international congress on pigments in food technology, Sevilla, pp 271–276

Gandul-Rojas B, Roca M, Minguez-Mosquera MI (1999b) Chlorophyll and carotenoid pattern in virgin olive oil. Adulteration control. In: Minguez-Mosquera MI, Jaren-Galan M, Hornero-Mendez H (eds) Proceeding of the 1st international congress on pigments in food technology (PFI), Sevilla, PFT Press, pp 381–386

Gandul-Rojas B, Roca-López-Cepero M, Mínguez-Mosquera MI (1999c) Chlorophyll and carotenoid patterns in olive fruits, *Olea europaea* Cv. Arbequina. J Agric Food Chem 47:2207–2212

Gandul-Rojas B, Cepero MRL, Mínguez-Mosquera MI (2000) Use of chlorophyll and carotenoid pigment composition to determine authenticity of virgin olive oil. J Am Oil Chem Soc 77:853–858

Gandul-Rojas B, Roca M, Mínguez-Mosquera MI (2004) Chlorophyll and carotenoid degradation mediated by thylakoid-associated peroxidative activity in olives (Olea europaea) cv. Hojiblanca. J Plant Physiol 161:499–507

Gandul-Rojas B, Gallardo-Guerrero L, Mínguez-Mosquera MI (2009) Influence of the chlorophyll pigment structure on its transfer from an oily food matrix to intestinal epithelium cells. J Agric Food Chem 57:5306–5314

Gertz C, Fiebig HJ (2006) Pyropheophytin a – determination of thermal degradation products of chlorophyll a in virgin olive oil. Eur J Lipid Sci Technol 108:1062–1065

Giuffrida D, Salvo F, Salvo A, La Pera L, Dugo G (2007) Pigments composition in monovarietal virgin olive oils from various sicilian olive varieties. Food Chem 101:833–837

Gross J (1991) Pigments in vegetables. Chlorophylls and carotenoids. Van Nostrand Reinhold, New York, pp 3–74

Gutierrez-Rosales F, Garrido-Fernández J, Gallardo-Guerrero L, Gandul-Rojas B, Mínguez-Mosquera MI (1992) Action of chlorophylls on the stability of virgin olive oil. J Am Oil Chem Soc 69:866–871

Herrero M, Ibáñez E, Cifuentes A, Bernal J (2009) Multidimensional chromatography in food analysis. J Chromatogr A 1216:7110–7129

Hoff AJ, Amesz J (1991) Visible absorption spectroscopy of chlorophylls. In: Scheer H (ed) Chlorophylls. CRC Press, Boca raton, pp 723–738

Holden M (1961) The breakdown of chlorophyll by chlorophyllase. Biochem J 78:359–364

Holden M (1965) Chlorophyll bleaching by legume seeds. J Sci Food Agric 16:312–325

Hornero-Méndez D, Gandul-Rojas B, Mínguez-Mosquera MI (2005) Routine and sensitive SPE-HPLC method for quantitative determination of pheophytin a and pyropheophytin a in olive oils. Food Res Int 38:1067–1072

Hunt JE, Michalski TJ (1991) Desorption-ionization mass spectrometry of chlorophyll. In: Scheer H (ed) Chlorophylls. CRC Press, Boca raton, pp 835–854

Hynninen PH (1991) Chemistry of chlorophylls: modifications. In: Scheer H (ed) Chlorophylls. CRC Press, Boca raton, pp 145–210

Hynninen PH, Hyvärinen K (2002) Tracing the allomerization pathways of chlorophylls by ^{18}O-labelling and mass spectrometry. J Org Chem 67:4055–4061

Hyvärinen K, Hynninen PH (1999) Liquid chromatographic separation and mass spectrometric identification of chlorophyll b allomers. J Chromatogr A 837:107–116

Inoue H, Yamashita H, Furuya K, Nonomura Y, Yoshioka N, Li S (1994) Determination of copper (II) chlorophyllin by reversed-phase high-performance liquid chromatography. J Chromatogr 679:99–104

International Olive Council (IOC) (2009) COI/T.15/NC no 3/Rev. 4. Trade standard applying to olive oils and olive-pomace oils. Madrid

Khachik F, Beecher GR, Whittaker NF (1986) Separation, identification, and quantification of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by liquid-chromatography. J Agric Food Chem 34:603–616

Kimura M, Rodriguez-Amaya DB, Godoy HT (1990) Assessment of the saponification step in the quantitative-determination of carotenoids and provitamins-A. Food Chem 35:187–195

Kostiainen R, Hyvärinen K, Hynninen PH (1995) Fast-atom bombardment mass spectra of the 13^2-epimers of 13^2-chlorophyll a. Rapid Commun Mass Spectrom 9:555–558

Kuronen P, Hyvärinen K, Hynninen PH, Kilpelainen I (1993) High-performance liquid chromatographic separation and isolation of the methanolic allomerization products of chlorophyll *a*. J Chromatogr 654:93–104

Loftus ME, Carpente JH (1971) Fluorometric method for determining chlorophylls *a*, *b*, and *c*. J Mar Res 29:319–323

Luaces P, Pérez AG, García JM, Sanz C (2005) Effects of heat-treatments of olive fruit on pigment composition of virgin olive oil. Food Chem 90:169–174

Maia L, Casal S, Oliveira MBPP (2008) Validation of a micromethod for quantification of lutein and beta-carotene in olive oil. J Liquid Chromatogr Rel Technol 31:733–742

Manjula S, Subramanian R (2006) Membrane technology in degumming, dewaxing, deacidifying, and decolorizing edible oils. Crit Rev Food Sci Nut 46:569–592

Mateos R, García-Mesa JA (2006) Rapid and quantitative extraction method for the determination of chlorophylls and carotenoids in olive oil by high-performance liquid chromatography. Anal Bioanal Chem 385:1247–1254

Matile P, Hörtensteiner S, Thomas H (1999) Chlorophyll degradation. Ann Rev Plant Physiol Plant Mol Biol 50:67–95

Mínguez-Mosquera MI, Gallardo-Guerrero L (1995) Disappearance of chlorophylls and carotenoids during the ripening of the olive. J Sci Food Agric 69:1–6

Mínguez-Mosquera MI, Gandul-Rojas B, Garrido-Fernández J, Gallardo-Guerrero L (1990) Pigment present in virgin olive oil. J Am Oil Chem Soc 67:192–196

Mínguez-Mosquera MI, Gandul-Rojas B, Gallardo-Guerrero ML (1992) Rapid method of quantification of chlorophylls and carotenoids in virgin olive oil by high-performance liquid chromatography. J Agric Food Chem 40:60–63

Mínguez-Mosquera MI, Gandul-Rojas B, Garrido-Fernández J (1996) Preparation of Cu (II) complexes of oxidized chlorophylls and their determination by thin-layer and high-performance liquid chromatography. J Chromatogr 731:261–271

Mínguez-Mosquera MI, Gandul-Rojas B, Gallardo-Guerrero L, Roca M, Jarén-Galán M (2007) Chlorophylls. In: Hurst WJ (ed) Methods of analysis for functional foods and nutraceuticals, 2nd edn. CRC Press, Boca Raton, pp 337–400

Official Journal of the Commission of the European Communities (EC) (1994) Regulation No. 94/36/CE, L237, June 30

Pérez AG, Romero C, Yousfi K, García JM (2008) Modulation of olive oil quality using NaCl as extraction coadjuvant. J Am Oil Chem Soc 85:685–691

Psomiadou E, Tsimidou M (1998) Simultaneous HPLC determination of tocopherols, carotenoids, and chlorophylls for monitoring their effect on virgin olive oil oxidation. J Agric Food Chem 46:5132–5138

Psomiadou E, Tsimidou M (2001) Pigments in Greek virgin olive oils: occurrence a nd levels. J Sci Food Agric 81:640–647

Puspitasari-Nienaber NL, Ferruzi MG, Schwartz SJ (2002) Simultaneous detection of tocopherols, carotenoids, and chlorophylls in vegetable oils by direct injection C30 RP-HPLC with coulometric electrochemical array detection. J Am Oil Chem Soc 79:633–640

Rahmani M, Csallany AS (1985) Determination of chlorophyll pigments by high-performance liquid-chromatography (hplc), in vegetable-oils. Rev Fr Corps Gras 32:257–259

Rahmani M, Csallany AS (1991) Chlorophyll and ß-carotene pigments in maroccan virgin olive oils measured by high-performance liquid chromatography. J Am Oil Chem Soc 68:672–674

Ranalli A, Contento S, Schiavone C, Simone N (2001) Malaxing temperature affects volatile and phenol composition as well as other analytical features of virgin olive oil. Eur J Lipid Sci Technol 103:228–238

Ranalli A, Malfatti A, Lucera L, Contento S, Sotiriou E (2005) Effects of processing techniques on the natural colourings and the other functional constituents in virgin olive oil. Food Res Int 38:873–878

Roca M, Mínguez.-Mosquera MI (2003a) Involvement of chlorophyllase in chlorophyll metabolism in olive varieties with high and low chlorophyll content. Physiol Plant 117:459–466

Roca M, Mínguez.-Mosquera MI (2003b) Carotenoid levels during the period of growth and ripening in fruits of different olive varieties (Hojiblanca Picual and Arbequina. J Plant Physiol 160:451–459

Roca M, Mínguez-Mosquera MI (2001a) Change in the natural ratio between chlorophylls and carotenoids in olive fruit during processing for virgin olive oil. J Am Oil Chem Soc 78:133–138

Roca M, Mínguez-Mosquera MI (2001b) Changes in chloroplast pigments of olive varieties during fruit ripening. J Agric Food Chem 49:832–839

Roca M, Mínguez-Mosquera MI (2001c) Unusual carotenogenesis in fruits with pronounced anthocyanic ripening (*Olea europaea* var. Arbequina). J Agric Food Chem 49:4414–4419

Roca M, Gandul-Rojas B, Gallardo-Guerrero L, Mínguez-Mosquera MI (2003) Pigment parameters determining spanish virgin olive oil authenticity: stability during storage. J Am Oil Chem Soc 80:1237–1240

Roca M, Gandul-Rojas B, Minguez-Mosquera MI (2007) Varietal differences in catabolic intermediates of chlorophylls in Olea europaea (L.) fruits cvs. Arbequina and Blanqueta. Postharvest Biol Technol 44:150–156

Roca M, Gallardo-Guerrero L, Mínguez-Mosquera MI, Gandul-Rojas B (2010) Control of olive oil adulteration with copper-chlorophyll derivatives. J Agric Food Chem 58:51–56

Salisbury FB, Ross CW (1992) Photosynthesis: cloroplasts and light. In: Salisbury FB, Ross CW (eds) Plant physiology, 4th edn. Wadsworth, Belmont

Sánchez J, Harwood JL (2002) Biosynthesis of triacylglycerols and volatiles in olives. Eur J Lipid Sci Technol 104:564–573

Schick GA, Bocian DF (1987) Resonance Raman studies of hydroporphyrins and chlorophylls. Biochim Biophys Acta 895:127–154

Schwartz SJ, Woo SL, von Elbe JH (1981) High performance liquid chromatography of chlorophylls and their derivatives in fresh and processed spinach. J Agric Food Chem 29:533–535

Seppanen CM, Rahmani M, Csallany AS (2003) Simultaneous determination of chlorophylls, pheophytins, beta-carotene, tocopherols, and tocotienols in olive and soybean oils by high-performance liquid chromatography. J Food Sci 68:1644–1647

Serani A, Piacenti D (2001) Sistema analitico per l'identificazione di oli deodorati in oli vergini di oliva. Nota 1 – Analisi dei pigmento clorofilliani in oli vergini di oliva. Riv Ital Sostanze Grasse 78:459–463

Shen Y, Hu YM, Huang K, Yin SA, Chen B, Yao SZ (2009) Solid-phase extraction of carotenoids. J Chromatogr A 1216:5763–5768

Stancher B, Zonta F, Bogoni P (1987) Determination of olive oil carotenoids by HPLC. J Micronutr Anal 3:97–106

Stefanoudaki E, Koutsaftakis A, Harwood JL (2011) Influence of malaxation conditions on characteristic qualities of olive oil. Food Chem 127:1481–1486

Strasburger E et al (2004) Tratado de Botánica. 35ª Spanish edition: Sitte P. Barcelona (Spain): Ediciones Omega

United States Department of Agriculture (USDA) (2010) Standards for grades of olive oil and olive-pomace oil. Fed Regist 75(81):22363–22366

Van Breemen RB, Canjura FL, Schwartz SJ (1991) Identification of chlorophyll derivatives by mass spectrometry. J Agric Food Chem 39:1452–1456

Verzegnassi L, Riffe-Chalard C, Kloeti W, Gulacar FO (1999) Analysis of tetrapyrrolic pigments and derivatives in sediments by high performance liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. Fresen J Anal Chem 364:249–253

Ward K, Scarth R, Daun JK, Thorsteinson CT (1994) Effects of processing and storage on chlorophyll derivatives in commercially extracted canola oil. J Am Oil Chem Soc 71:811–815

Zissis KD, Dunkerley S, Brereton RG (1999) Chemometric techniques for exploring complex chromatograms: application of diode array detection high performance liquid chromatography electrospray ionization mass spectrometry to chlorophyll a allomers. Analyst 124:971–979

Zonneveld H, Klop W, Gorin N (1984) Analysis of unboiled and boiled beans for chlorophylls and pheophytins by thin-layer chromatography and fluorimetry comparison with spectrometry. Z Lebensm Unters For 178:20–23

Chapter 8
Chromatographic Methodologies: Compounds for Olive Oil Odor Issues

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Contents

8.1 Introduction

Among vegetable oils, virgin olive oil (VOO) has special characteristics because it is extracted from the fruit of the olive tree, *Olea europea* L, and consumed without a further refining process, thus retaining minor compounds that give rise to a fragrant and delicate flavor. Virgin oil can be considered the juice of the olive fruit.

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties*,
DOI 10.1007/978-1-4614-7777-8_8, © Springer Science+Business Media New York 2013

VOOs are graded, according to current European Union regulation (EC 2011), in function of sensory assessment and three chemical parameters (free acidity, peroxide value, and UV absorbance) (Tables 16.6 and 16.7).

Thus, odor plays an important role in VOO sensory quality (Chap. 14) and consumer acceptance (Chap. 15). But olfaction, which is often alluded to as multidimensional in the sense that there are approximately 1,000 different receptor types, each one tuned to particular odor aspects contributing to olfactory perception, is, however, unidimensional in humans because they can detect and discriminate many odorants but can identify few by name (Yeshurun and Sobel 2010). In fact, unfamiliar odors are harder to discriminate than familiar ones, and it seems that the ability to discriminate odorants in a mixture is limited to four (Livermore and Laing 1998). The main thing that humans can invariably say about an odor is whether it is pleasant or not (García-González et al. 2011), and this hedonicity depends on odor intensity and familiarity and varies across individuals and cultures and within individuals over time. In addition, it can also be significantly influenced by visual and verbal information (Yeshurun and Sobel 2010).

The characteristic flavors of food and beverages are generally the result of extremely complex multisubstance mixtures containing several hundred compounds with different chemical structures. In most cases, the flavor impression that is perceived as a single sensation is a complex sensory impression of many individual substances in a specific concentration ratio. These volatile components, which are present in only minor amounts (mg/Kg to ng/Kg range), decisively influence the enjoyment and the acceptance of foodstuffs. Only in rare cases are individual components (flavor impact compounds) responsible for odor and taste (Werkhoff et al. 2002)

Many researches have proven that volatile compounds are responsible for VOO aroma, and consequently the identification and quantification of the volatile compounds responsible for aroma has always been a milestone in quality control. VOO volatiles are mainly produced by the oxidation of polyunsaturated fatty acids (Chap. 4). It is widely agreed that endogenous plant enzymes, mainly through the lipoxygenase (LOX) cascade, are responsible for pleasant aromas (positive aroma perception) (Salas et al. 1999; Aparicio et al. 1996; Morales et al. 1999), whereas chemical oxidation (Morales et al. 1997) and exogenous enzymes, mostly due to microbial activity, are responsible for unpleasant aromas (sensory defects) (Morales et al. 2005).

Volatiles are low molecular weight compounds (< 300 Da) with high vapor pressure that vaporize at room temperature. Odorants are volatile chemical compounds that are carried by inhaled air to the olfactory epithelium, where they reach and bond with specific proteins of olfactory receptors to give an odor sensation. Volatiles share two common characteristics: they have enough hydrosolubility to diffuse into the mucus that covers the sensitive olfactory receptors and a good degree of liposolubility to dissolve in membrane lipids contiguous to protein receptors (Leffingwell 2001; Genovese et al. 2009). García-González et al. (2011) explain the link between the olfactory receptors and the neurons that results in the acceptability of VOOs.

The identification of the aroma characteristics of VOOs can be carried out by two procedures: sensory assessment (the so-called panel test) and analysis of volatile

compounds. The first one has the great disadvantage of being a lengthy and expensive methodology whose final result might be affected by many factors such as, for example, the panelists' training and an inherent subjectivity (García-González and Aparicio 2002a). The analysis of volatiles by headspace high-resolution gas chromatography (GC) is, however, an accurate technique, but it is laborious and cannot be applied online but at-line in the processes of storage and bottling of VOO (García-González and Aparicio 2002b). There is, however, another alternative, the use of chemosensors or an electronic nose, which has the advantages of low cost and easy sample handling but the disadvantages of poor selectivity, signal drift, and humidity dependence.

Searching for relationships between chemical compounds and VOO sensory descriptors is the main objective of the identification and quantification of volatiles. Chemical factors such as volatility, hydrophobicity, conformational structure, and position of functional groups, among others, seem to be more related to odor contribution than the concentration itself. The odor activity value (Grosch 1994) of volatiles and their synergism and antagonism are keys to knowing not only the contribution of the volatiles to aroma (Aparicio et al. 1996) but also the neuronal activity of the consumers when they taste VOOs (García-González et al. 2011).

The analytical procedures that have been developed in recent years to carry out the analysis of volatile compounds, and the approaches developed to establish relationships between chemical compounds and sensory analysis, are discussed in this chapter. The most recent results in the evaluation of sensory descriptors and research on brain activity induced by pleasant and unpleasant VOO aromas are analyzed as well.

8.2 Virgin Olive Oil Volatile Compounds

Almost 200 compounds belonging to several chemical classes have been identified and quantified in the different varieties and categories of VOOs. Table 8.1 shows a list of compounds that have been reported in several reviews (Morales and Tsimidou 2000; Angerosa 2002; Angerosa et al. 2004; Kalua et al. 2007), and Table 8.2 shows compounds identified by standards or GCMS. Although the volatiles detected in the aroma of VOOs partially depend on the methodology used for their determination (Sect. 8.3) and on the quality of the VOO, the aroma is, in general, attributed to aldehydes, alcohols, esters, ketones, furans, hydrocarbons, and other as yet unidentified compounds.

Olive oils obtained from sound olives, correctly harvested at their optimum ripeness (Chap. 2) and processed under adequate technological conditions (Chap. 3), show volatile profiles mainly produced by biogenic pathways. The main compounds are produced enzymatically from polyunsaturated fatty acids through the so-called LOX pathway (Chap. 4). The aroma of these oils contains abundant accumulation of C_6 aldehydes, C_6 alcohols, and their corresponding esters (Aparicio et al. 1997; Aparicio and Morales 1998; Kalua et al. 2007), together with smaller amounts of C_5 carbonyl compounds (Morales et al. 1999). Hexan-1-ol, hexanal, E-2-hexenal, and 3-methylbutan-1-ol, for example, have been found in the most common extra virgin olive oils (EVOOs) from the Mediterranean basin (Aparicio and Luna 2002),

Table 8.1 Volatile compounds identified in virgin olive oil aroma by MS

Volatile compound	OT[a]	SD (aroma)[b]	SPME R (%)[c]	Kovats I[d]	References[e]
Aldehydes					
Acetaldehyde	0.22	Pungent, sweet	12.6	714	1
Butanal	0.15	Green, pungent	150	832	2
3-Methylbutanal	5.4	Malty	100	910	1
2-Methylbutanal	5.2	Malty	100	912	1
Pentanal	240	Woody, bitter, oily	100	935	3
	300	Green, bitter almond	90	1,093	4
Hexanal	75	Green-sweet			4
	80	Green apple, grassy			3
	300	Green			1
E-2-Pentenal	300	Green, apple	20	1,117	3
Z-3-Hexenal	3	Green	75	1,151	4
	1.7	Leaflike			1
Heptanal	500	Oily, fatty, woody	25	1,174	3
E-2-Hexenal	424	Green, applelike	22	1,201	1
	420	Bitter almonds, green			3
	1,125	Green astringent			4
E-2-Heptenal	5	Oxidized, tallow, pungent	15	1,243	3
Octanal	320	Fatty, sharp, citruslike	7.6	1,280	3, 1
2,4-Hexadienal	0.27	Green, floral, citric	1.3	1,391	5, 6
Nonanal	150	Fatty, waxy, pungent	5.9	1,402	3
E-2-Octenal	4	Herbaceous, spicy	2.5	1,442	3
2,4-Heptadienal	3,620	Fatty, rancid	3.7	1,480	3
Decanal	650	Penetrating, sweet, waxy	7.6	1,484	3
Z-2-Nonenal	4.5	Green, fatty	–	1,510	1
E-2-Nonenal	900	Paperlike, fatty	1.5	1,527	1
E-2-Decenal	10	Painty, fishy, fatty	1.3	1,651	3
2,4-Decadienal	2,150	Strong, fatty	0.32	1,632	3
E,*E*-2,4-Nonadienal	2,500	Soapy, penetrating	–	1,705	3
	2,500	Deep fried			1
E,*Z*-2,4-Decadienal	10	Deep fried	0.2	1,758	1
E,*E*-2,4-Decadienal	180	Deep fried	0.1	1,832	1
E-4,5-Epoxy-E-2- decanal	1.3	Metallic	–	2,000	1
Alcohols					
Ethanol	30,000	Alcohol	40	936	3
Butan-2-ol	150	Winey	90	1,024	3
Propan-1-ol	30	Alcohol, fruity, pungent	128	1,036	7
2-Methyl-buten-3-ol	–	Oily, fruity, herbaceous	–	1,037	8
2-Methylpropan-1-ol	–	Wine, solvent	55	1,099	5
Pentanol	470	Fruity	100	1,107	4
	3,000	Strong, sticky, balsamic			3
Butan-1-ol	–	Sweet, fatty, medicine	120	1,145	6
1-Penten-3-ol	400	Pungent, butter	16	1,157	5, 6
3-Penten-2-ol	400	Perfumey, woody	–	1,180	3
2-Methyl butan-1-ol	480	Winey, spicy	80	1,205	3

(continued)

Table 8.1 (continued)

Volatile compound	OT[a]	SD (aroma)[b]	SPME R (%)[c]	Kovats I[d]	References[e]
3-Methyl butan-1-ol	100	Woody, whiskey, sweet	94	1,208	3
Heptan-2-ol	10	Earthy, mushroom	65	1,332	3
Hexanol	400	Fruit, banana, soft	75	1,360	10
	400	Undesirable			4
E-3-Hexen-1-ol	1,500	Green	20	1,379	10
Octan-1-ol	–	Toasted, herbal, fatty	20	1,388	5, 6
Octen-3-ol	1	Moldy, earthy	13	1,400	3
Z-3-Hexenol	6,000	Green	14	1,401	4
	1,100	Leaflike			1
E-2-Hexen-1-ol	5,000	Green grass, leaves	15	1,408	3
	8,000	Green, grassy, sweet			10
Octan-2-ol	100	Earthy, fatty	–	1,421	3
6-Methyl-5-hepten-2-ol	2,000	Perfumey, nutty	–	1,507	3
Nonan-1-ol	280	Fatty	8	1,619	2, 3, 5
Esters					
Methyl acetate	200	Solvent, fruit	45	864	9
Ethyl acetate	940	Sticky, sweet	100	898	3
Ethyl propanoate	100	Fruit, strong	–	951	3
Ethyl butanoate	30	Sweet, fruity	–	1,028	3
	28	Cheesy, fruity			1
Ethyl isobutyrate	1.2	Fruity	965	1,037	1
Ethyl 2-methylbutyrate	0.72	Fruity	–	1,056	1
Ethyl 3-methylbutyrate	0.62	Fruity	–	1,060	1
Butyl acetate	300	Green, fruity, pungent	92	1,075	3
Propyl butanoate	150	Pineapple, sharp	–	1,128	3
2-Methylpropyl butanoate	100	Unpleasant, winey, fusty	–	1,152	3
Hexyl acetate	1,040	Green, fruity, sweet	20	1,264	4
Z-3-Hexenyl acetate	750	Green	10	1,308	4
	200	Bananalike			1
Ethyl cyclohexyl carboxylate	0.16	Aromatic, fruity	–	–	1
Ketones					
Butan-2-one	40,000	Ethereal, fruity	85	881	3
l-Penten-3-one	50	Green	100	973	4
	0.73	Green, pungent			1
3-Pentanone	7,000	Sweet, fruity	100	983	2, 5
4-Methylpentan-2-one	300	Fruity, sweet	100	1,003	9
Heptan-2-one	300	Sweet, fruity	75	1,170	3
3-Octanone	–	Herb, butter	28	1,244	5, 10
Octan-2-one	510	Mold, green	35	1,285	3
Z-1,5-Octadien-3-one	0.45	Geraniumlike	–	1,312	1
l-Octen-3-one	10	Mushroomlike, mold, pungent	5	1,317	3
	10	Mushroomlike			1

(continued)

Table 8.1 (continued)

Volatile compound	OT[a]	SD (aroma)[b]	SPME R (%)[c]	Kovats I[d]	References[e]
6-Methyl-5-hepten-2-one	1,000	Pungent, green	–	1,336	3
2-Nonanone	–	Hot milk, soap, green	15	1,388	5, 6
E-β-Damascenone	11	Boiled applelike	–	1,832	1
Carboxylic acids					
Acetic acid	500	Sour, vinegary	40	1,452	3
	124	Vinegarlike			1
Propanoic acid	720	Pungent, sour	35	1,523	3
Butanoic acid	650	Rancid, cheese	25	1,619	3
3-Methylbutyric acid	22	Sweaty	–	1,686	1
Pentanoic acid	600	Unpleasant, pungent		1,721	3
Hexanoic acid	700	Pungent, rancid	12	1,829	3
Heptanoic acid	100	Rancid, fatty	3	1,930	3
Octanoic acid	3,000	Oily, fatty	1.3	2,083	3
Hydrocarbons					
Octane	940	Sweety, alcane	45	800	3
Ethylbenzene	–	Ethereal, floral, sweet	100	1,124	6, 8
Limonene	250	Citruslike, ethereal	43	1,234	6
Miscellany					
4-Methoxy-2-methyl-2-butanethiol	0.017	Black currantlike, catty	–	1,221	1
Guaiacol	16	Phenolic, burnt	–	1,859	1

[a]Odor threshold (μg/kg) in refined oil
[b]Aroma sensory descriptor
[c]Recovery (%) using SPME DVB/CAR/PDMS triple fiber
[d]Kovats Index from Flavornet & Pherobase databases
[e]Reference where the compound was cited
References: 1, Reiners and Grosch (1998); 2, Morales et al. (2005); 3, Aparicio and Luna (2002); 4, Morales and Aparicio (1999); 5, Flath et al. (1973); 6, Fedeli (1977); 7, Olías et al. (1980); 8, Morales et al. (1994); 9, Angerosa et al. (1998); 10, Aparicio et al. (2000b)

Table 8.2 Other volatile compounds also identified in virgin olive oil aroma by MS

Volatile compound	Reference[a]	Volatile compound	References[a]
Aldehydes		*Carboxylic acids*	
Propanal	1	2-Methyl propanoic acid	4
Z-2-Pentenal	1, 2	3-Methyl butanoic acid	4
Z-2-Hexenal	1, 2	Propanoic acid	4
Z-3-Nonenal	7	Butanoic acid	9
E-2-Undecenal	1, 2	Hexanoic acid	9
2-Methylbut-2-enal	2, 6	Heptanoic acid	9
Benzaldehyde	1, 2		
Phenylacetaldehyde	7	*Hydrocarbons*	
		n-Nonane	4
Alcohols		n-Decane	4
Methanol	8	n-Undecane	4
Heptan-1-ol	2, 3	n-Dodecane	4

(continued)

Table 8.2 (continued)

Volatile compound	Reference[a]	Volatile compound	References[a]
Decan-1-ol	3	Tridecane	4
Pentan-3-ol	2	Tetradecane	4
Octan-3-ol	4	Methyldecane	4
2-Penten-1-ol	6	Hexene	5, 6
4-Hexen-1-ol	3	Octene	3
3-Methyl-1-pentanol	3	Tridecene	3
2-Phenylethanol	2, 3	Limonene	3
		α-Copaene	3
Ketones		α-Murolene	3
Acetone	1	α-Farnesene	3
2-Hexanone	2	1,3-Hexadien-5-yne	6
3-Metylbutan-2-one	2	Ethylbenzene	3, 5
4-Methyl-3-penten-3-one	5	Diethylbenzene	3
2-Methyl-2-hepten-6-one	2	Trimethylbenzene	3
Acetophenone	2	Tetramethylbenzene	3
		Propylbenzene	3
Esters		Isopropylbenzene	3
Ethyl octanoate	2, 3	Xilene	3
Ethyl heptanoate	1, 3	Styrene	3
Ethyl decanoate	1, 3		
Methyl hexanoate	1, 2	*Miscellany*	
Methyl heptanoate	1, 2	2-Pentyl-3-methylfuran	1
Methyl octanoate	1, 2	Ethylfuran	6
Methyl nonanoate	6	3-(4-Methyl-3-pentenyl) furan	6
Methyl decanoate	6	Methoxybenzene	1, 2
Methyl oleate	1	1,2-Dimethoxybenzene	2
Methyl linoleate	1	Linalool	2
1-Octyl acetate	2	α-Terpineol	1, 2
Propyl propionate	2		
Ethyl 2-methylpropionate	2, 3		
Methyl 2-methylbutyrate	2		
Methyl 3-methylbutyrate	2		
3-Methyl-2-butenyl acetate	6		
2-Methyl-1-butyl acetate	2, 5		
3-Methyl-1-butyl acetate	2, 5		
2-Methylbutyl propanoate	6		
2-Methyl-1-propyl acetate	2, 6		
Methyl benzoate	3		

[a]Reference where the compound was cited

References: 1, Morales et al. (2005); 2, Flath et al. (1973); 3, Fedeli (1977); 4, Olías et al. (1980); 5, Morales et al. (1994); 6, Angerosa et al. (1998); 7, Montedoro et al. (1978); 8, Angerosa et al. (1999); 9, Guth and Grosch (1991)

although profiles vary when autochthonous Mediterranean cultivars are planted in other geographical regions (Tena et al. 2007). For example, diverse varietal olive trees (e.g., Picual, Frantoio, Koroneki) cultivated under the same environmental conditions produce VOOs with different profiles of volatiles (Aparicio and Luna 2002; Benincasa et al. 2003), which would also occur if the same cultivar (e.g., Arbequina) were cultivated under different environmental conditions (e.g., geographic areas) (García-González et al. 2010a, 2010b).

A VOO of poorer quality usually has a much more complex profile, with a higher number of volatiles. These VOOs present lower concentrations of C_6 and C_5 compounds than those detected in high-quality VOOs, and sometimes they are even completely absent. At the same time, some volatiles belonging to C_5 branched aldehydes and alcohols or to C_6-C_{10} dienals or to C_7-C_{11} monounsaturated aldehydes or to C_8 ketones may become important contributors to VOO organoleptic defects, such as fusty, muddy sediment, musty, rancid, and winey-vinegary (Morales et al. 2005). If the concentrations of the volatiles are below their olfactory threshold (Grosch 1994; Guth and Grosch 1991; Reiners and Grosch 1998), however, they do not contribute to VOO aroma, although they can be of interest as traceability markers of the olive oil from olive tree to consumer (Morales et al. 2012). In fact, cultivar, fruit ripeness, extraction method, and pedoclimatic conditions affect, in this order, the volatile composition (Chap. 12).

Olive fruit maturity is a decisive parameter in the formation of volatiles through the LOX pathway, whose activity decreases with ripeness (Salas et al. 1999). At early ripening stages, the amounts of C_6 aldehydes and C_6 alcohols, although low enough, are of the same order of magnitude, while almost all C_6 aldehydes reach their maximum concentrations when the olive skin turns from green to purple color (Aparicio and Morales 1998; Angerosa and Basti 2001). With increasing ripeness, the decrease is observed for most of the aldehydes produced through the LOX pathway – E-2-hexenal, one of the major volatile compounds, decreases in most of the cultivars – although some exceptions have been observed in preliminary studies about some cultivars planted in Australia (Kalua et al. 2004). A general exception is the increase in Z-3-hexenal with ripeness (Aparicio and Morales 1998).

Inadequate conditions in the processing of sound olives, harvested at their optimum ripeness level, affect both yield and VOO quality. Malaxation temperature and time are decisive parameters in the production of volatiles responsible for the aroma sensory attributes appreciated by consumers. Higher temperatures or longer malaxation times increase the concentration of volatiles responsible for sensory defects (e.g., 2- and 3-methyl butanol). Research has shown that the malaxation of olive paste should be carried between 25 °C and 35 °C in order to achieve an appreciated olive oil aroma (Morales and Aparicio 1999; Ranalli et al. 2001) without a substantial decreasing the oil yield (Aparicio et al. 1994c); the optimum is between 22 °C and 28 °C, according to a mathematical model based on a large database.

Few volatiles are present in the intact tissue of the fruit, and those are mainly hydrocarbons, which do not contribute to aroma, and secondary products not occurring (or occurring only in traces) within intact cells. These volatiles are formed very quickly during disruption of cell structure as the result of enzymatic reactions in the

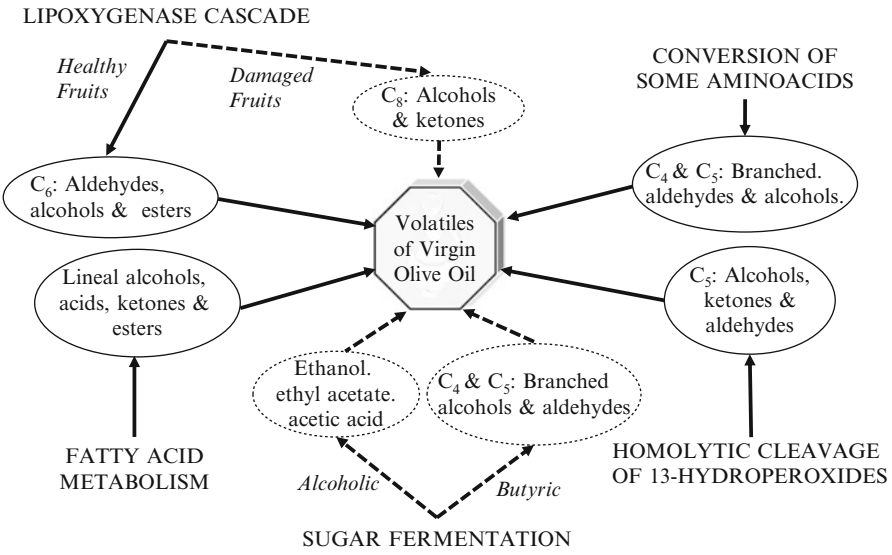

Fig. 8.1 Pathways involved in production of some virgin olive oil volatiles

presence of oxygen. Thus, the profile of volatiles is produced at the beginning of the malaxation process that influences much more in the concentration of some volatiles than the appearance of new ones (Morales and Aparicio 1999). In fact, the profile of volatiles depends on the enzymatic activity of the cultivars.

8.2.1 Biogenesis of Virgin Olive Oil Volatile Compounds

The volatiles responsible for VOO flavor, which come from the olive fruit, can be considered direct metabolites produced in plant organs by intracellular biochemical pathways. The growing unripe fruit synthesizes high molecular mass compounds such as lipids, proteins, and polysaccharides, of which the former is the main precursor of the volatiles. In the course of ripening, changes take place in the metabolism of the fruit (Prasanna et al. 2007). Thus, ethylene production, respiration rise, protein synthesis, increase in enzyme activities, and permeability of cell membranes influence the biogenesis of volatiles. However, the volatiles, which are produced in significant amounts when olives reach the climateric stage of ripeness, are mostly formed through the action of enzymes that are released when the fruit is crushed, and their concentrations increase substantially during the malaxation process (Chap. 3). Figure 8.1 shows the main pathways involved in the production of the volatile compounds responsible for VOO aroma.

The primary precursors of the formation of volatiles are fatty acids (particularly linoleic and α-linolenic) and amino acids (leucine, isoleucine, and valine). The main

biochemical pathways involved in VOO aroma biogenesis can be clustered as follows (Fig. 8.1):

- *Lipid metabolites*:
  - *Fatty acid metabolism*: Unripe fruits produce a variety of fatty acids and some minor volatile compounds (primary and secondary alcohols). During ripening, fruits develop the ability to convert some of the fatty acids into ketones, esters, and alcohols (Chap. 4). Aliphatic esters, alcohols, acids, and carbonyls derived from fatty acid metabolism can be found in VOO.
  - *Lipoxygenase pathway*: This is the most extensively studied biochemical pathway (Feussner and Wasternack 2002) directly involved in the formation of the major volatile compounds of VOOs (Kalua et al. 2007). It is responsible for the secondary volatile compounds that are usually found in all VOOs. The main precursors are linoleic and α-linolenic polyunsaturated fatty acids. It is well established that the formation of C_6 aliphatic volatile compounds from the 13-hydroperoxides of linoleic and linolenic acids are promoted in olives, catalyzed by the so-called hydroperoxide lyase (HPL), while C_9 compounds are practically absent. The presence of the LOX pathway in olives and a characterization of the different enzymes involved in the pathways are described in Chap. 4. E-2-hexenal, hexanal, and Z-3-hexenal are the major aldehydes found in VOOs. Z-3-hexen-1-ol, E-2-hexen-1-ol, and hexan-1-ol are usually found in high concentrations, with their balance being influenced by the stage of ripeness and variety of the olives (Aparicio and Morales 1998). Other C_6 volatile compounds such as Z-2-hexenal, E-3-hexenal, Z-2-hexen-1-ol, and E-3-hexen-1-ol are also produced through this pathway, but they are determined at lower concentrations.
  - *A cleavage reaction* of the 13-hydroperoxide of linolenic acid, mediated by LOX via an alkoxy radical (Salch et al. 1995), could give rise to the formation of stabilized 1,3-pentene radicals, which can later dimerize, leading to C10 hydrocarbons (pentene dimers), or couple with a hydroxy radical present in the medium producing C5 alcohols (Angerosa et al. 1998, 2004) such as 1-penten-3-ol and 2-penten-1-ol, which have been identified in VOOs. Further activation of alcohol dehydrogenase might be responsible for the formation of C_5 aldehydes.
  - *Amino acid metabolites*:
  - The structural skeleton of some volatiles is derived from the branched-chain amino acids (valine, leucine, and isoleucine) by a series of biochemical transformations (Wyllie et al. 1996; Schwab et al. 2008). Valine, leucine, and isoleucine are transformed into the branched aldehydes 2-methyl propanal, 3-methyl butanal, and 2-methyl butanal, respectively. Further activation of alcohol dehydrogenase converts the aldehydes so formed into the corresponding alcohols (Van der Hijden and Bom 1996). Later the action of alcohol acyl tranferase gives rise to ester formation (Wyllie et al. 1996; Dudareva et al. 2004).

Many of these pathways have not been well studied in olives until now, but their activity is confirmed by the presence of volatiles in VOO. Figure 8.2 shows the profile of volatiles in fresh high-quality VOOs from different single European varieties.

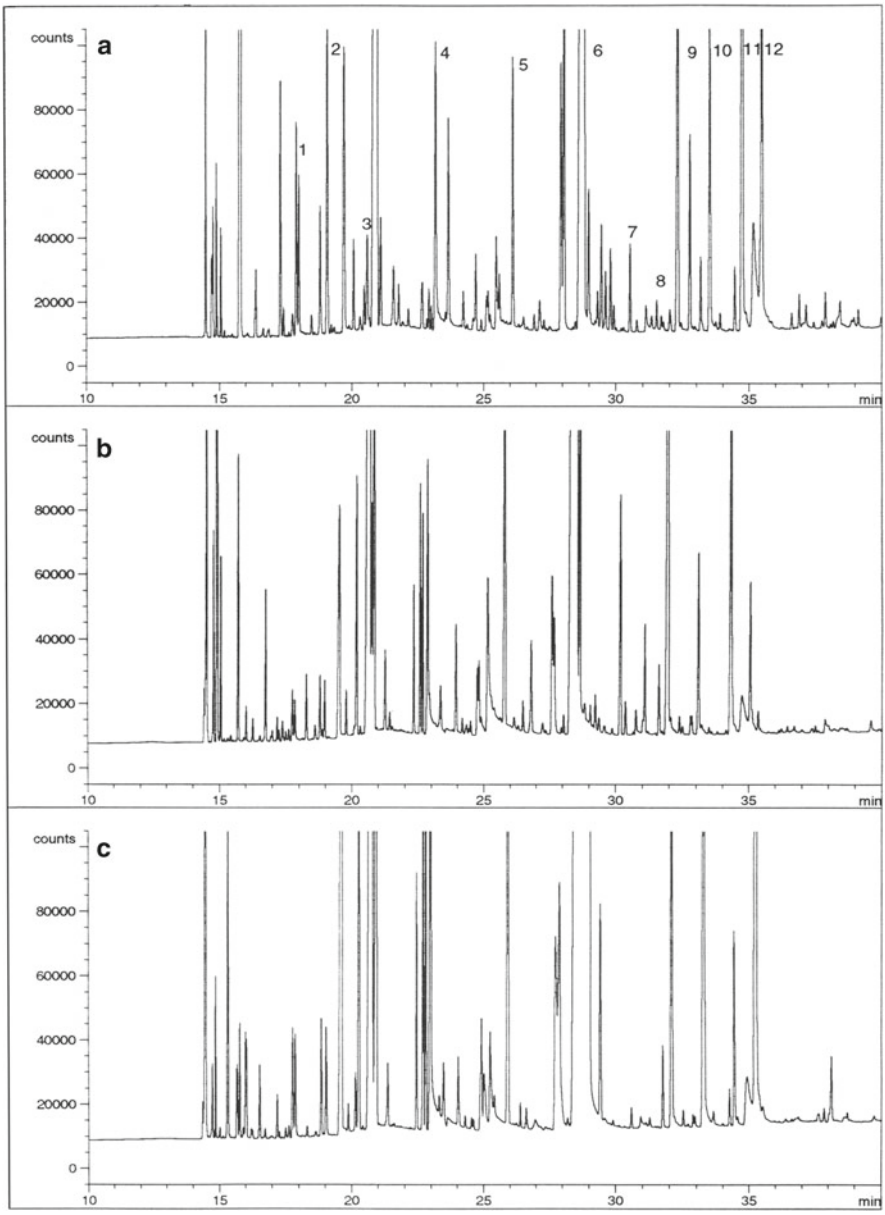

Fig. 8.2 Chromatograms of volatile compounds found in extra virgin olive oils from different European varieties. Codes: (**a**) Picual (Spain); (**b**) Koroneiki (Greece); (**c**) Coratina (Italy). (*1*) 3-methyl butanal, (*2*) ethyl propanoate, (*3*) 1-penten-3-one, (*4*) hexanal, (*5*) Z-3-hexenal, (*6*) = E-2-hexenal, (*7*) hexyl acetate, (*8*) Z-3-hexenyl acetate, (*9*) 2-penten-1-ol, (*10*) hexan-1-ol, (*11*) Z-3-hexen-1-ol, (*12*) = E-2-hexen-1-ol

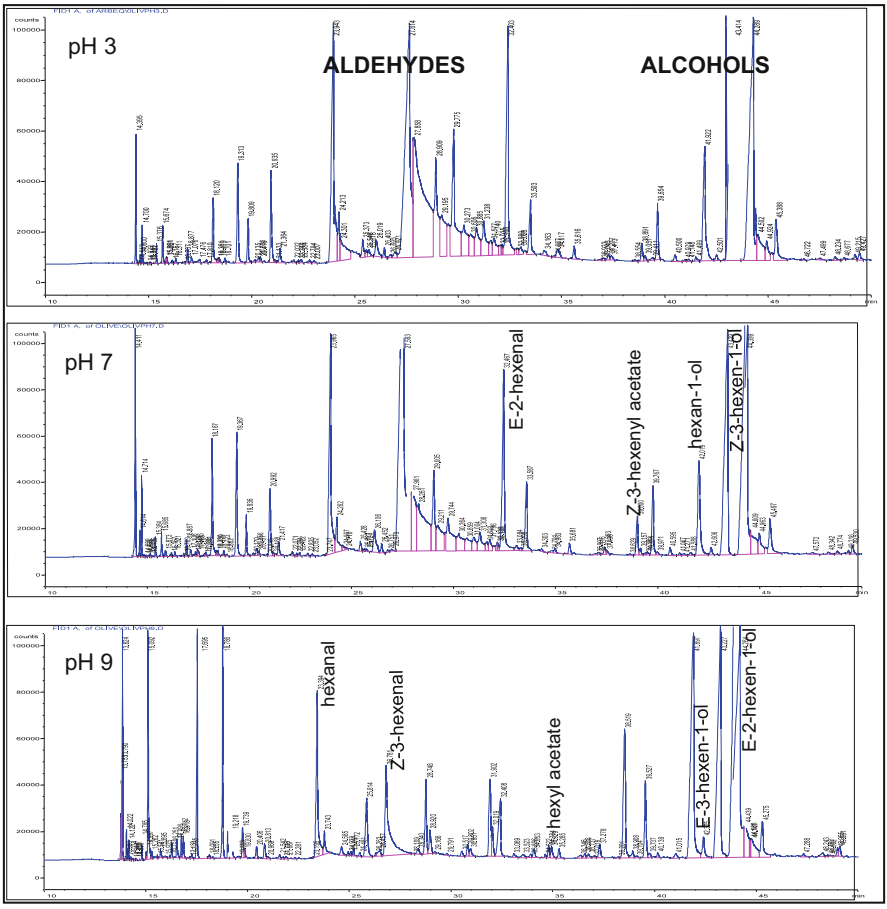

Fig. 8.3 Chromatograms of volatile compounds of olive oil *cv*. Arbequina. The milling and malaxation processes were carried out with different buffer solutions. pH values correspond to apparent pH of olive paste after buffer addition (Aparicio et al. (2000a), with permission of Kluwer Academic Publisher)

Although VOOs must be obtained by mechanical means exclusively, some experiments have been carried out to determine the influence of the pH of olive paste on the volatile profile in the course of the malaxation process (Aparicio et al. 2000a). Figure 8.3 shows how the effect of diverse pHs on the malaxation process, from acidic to basic, can alter the profiles of the volatile compounds of olive oil and, hence, the sensory descriptors qualifying the resulting VOOs. Thus, the concentration of aldehydes is promoted by low values of pH, while the production of alcohols is promoted by basic values of pH. These values agree with those found by biochemical studies (Salas 1999; Salas and Sánchez 1998; 1999) which show that the hydroperoxide lyase (HLP), the enzyme responsible for the formation of aldehydes, has its optimum pH at an acid value and alcohol dehydrogenase (ADH), the enzyme

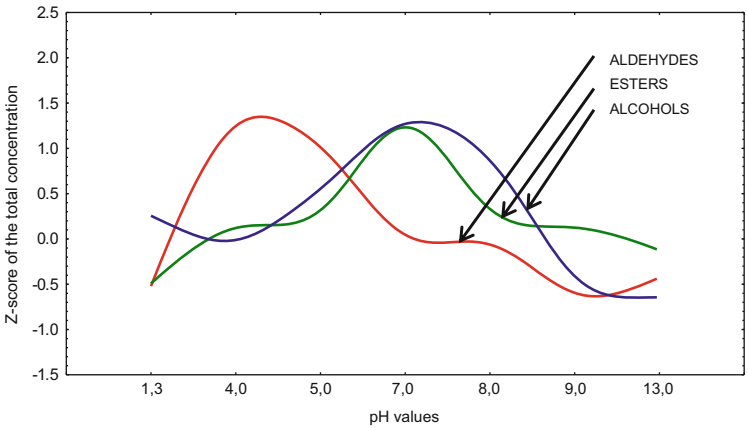

Fig. 8.4 Variation in content of total aldehydes, alcohols, and esters according to pH of olive paste during malaxation process

responsible for the formation of alcohols from aldehydes, has its optimum pH at a basic value. The study also pointed out that the formation of esters (e.g., hexyl acetate, Z-3-hexenyl acetate) seems to be favored by basic values while E-2-hexen-1-ol shows an unexpected shape in the pH range (Fig. 8.4).

Model systems, based on genetically modified potatoes (Salas et al. 2005) and *Arabidopsis thaliana* (Salas et al. 2007), have also been used to determine the importance of some enzymes of LOX cascade in the production of aroma and the alternatives that the plant looks for when LOX enzymes are knocked out. Thus, the high concentration of C_5 alcohols determined in model systems with a severe decrease in the HPL gene expression increased the sweet-fruity notes of the aroma, which contrasted with the predominant green notes found in the aroma of the wild plants. Therefore, these studies have pointed out the possibility of changing the flavor of edible oils by modifying the activity level of some of the enzymes of the LOX pathway.

8.3 Analysis of Volatile Compounds

In general, the analytical methods used for the evaluation of volatile compounds involve all the steps of the general analytical process such as sampling, sample preparation, separation, identification, quantification, and data analysis. Four points should be considered before dealing with the analysis of volatiles (Morales et al. 1992):

1. The concentration of volatiles in VOO is normally low, so that only a small amount will be obtained even from a large amount of sample.
2. The volatile fraction comprises many components of different molecular masses and chemical natures, and their concentrations may vary widely.

3. No direct relationship exists between the incidence of each compound in the food aroma and its concentration in the aromatic fraction because the detection thresholds vary greatly.
4. The formation of artifacts must be avoided during analysis because they may interfere and produce incorrect results.

Although every one of the preceding steps is important and should be carefully followed, it is not an exaggeration to say that the choice of an appropriate sample preparation procedure greatly influences the reliable and accurate analysis of VOO volatiles. Sample preparation is rather dependent on the instrumental technique subsequently used for separation, identification, and quantification of the volatile compounds. The separation and quantification of volatile compounds of VOO is usually carried out by HRGC. The other instrumental technique widely applied in recent years for the analysis of the aroma compounds of VOO are chemosensors that usually do not require a sample preparation step, although in certain cases some preconcentration procedure such as solid-phase microextraction (SPME) has been used.

8.3.1 Sample Preparation

Sample preparation prior to GC analysis of VOO volatile compounds usually involves two steps – isolation and preconcentration of the volatile fraction of the sample – that can be realized by different procedures clustered into two large groups (Morales et al. 1992):

1. Those not involving preconcentration

   Direct injection (DI)
   Static headspace (SHS)

2. Those involving preconcentration

   Distillation and simultaneous distillation-extraction (SDE)
   Dynamic headspace (DHS)
   Headspace with SPME (HS-SPME)
   Supercritical fluid extraction (SFE)
   Headspace sorptive extraction (HSSE)

8.3.1.1 Procedures Not Involving Preconcentration

As previously mentioned, the characteristic flavors of food and beverages are generally the result of complex multisubstance mixtures containing several hundred compounds with different chemical structures. Although most of the usual techniques for the quantification of volatile compounds involve an enrichment step, because of the very low concentration of most of them, the next two methods have been used, and are still used, in particular analyses of olive oil and other edible oils.

Direct Injection

This is the simplest technique for the analysis of volatile substances and the one requiring the least handling of the sample. It basically consists in placing a small amount of sample in a tube filled with glass wool fitted at the injector inlet (Dupuy et al. 1971). The sample is heated up to a predetermined temperature and purged with gas. The volatile compounds are then extracted and purged by the carrier gas into the column for their chromatography. Also called direct thermal desorption, it has been applied to olive oil volatile analysis by ramping the desorption temperature and holding the upper temperature in isothermal mode (Cavalli et al. 2003). Direct injection, with diverse modifications, has been used (Morales and Tsimidou 2000): (1) to predict the flavor stability of oils during storage; (2) to study the volatile compounds of oils oxidized under different conditions; (3) to study the effects of antioxidants, packing containers, and light on the quality of refined oils; and (4) to detect the presence of halogenated volatile compounds (e.g., trichloroethylene and tetrachloroethylene) in VOOs.

Several studies have reported the application of direct thermal desorption on olive oil samples (Hoffmann and Heiden 2000; Overton and Manura 1999; Manura and Overton 1999). Direct thermal extraction has been applied to several cooking olive oils to determine the volatile and semivolatile compounds contributing to flavor and off-flavor. The procedure can also be used for quality control and authenticity issues. Direct thermal desorption has been compared to other olive oil sample preparation procedures, proving useful for extracting volatile and especially semivolatile compounds, such as sesquiterpenes (Cavalli et al. 2003).

Direct injection is the least sensitive of the techniques due to the fact that it works with very low concentrations of volatiles, so that it often does not allow their detection, because it is volatile in concentrations below gas chromatographic detectability thresholds. The method also requires a high working temperature, and degradation products have been detected in analyses. An adequate cleaning process of the chromatographic column must be carried out after each analysis to avoid the memory effect (Morales and Tsimidou 2000).

Static Headspace

This simple way to analyze volatile fractions consists in analyzing an aliquot from the vapor phase, in equilibrium with the sample, in a sealed vial that is heated at a predetermined temperature over a certain time. Strict control of the temperature and sampling is necessary. The concentrations of the volatile compounds in both phases do not change with time once equilibrium is reached. They can be disturbed temporarily during sampling, so the methodology of obtaining the gaseous aliquot must be carefully considered.

The sample is usually injected by a syringe, although in certain cases multiple extractions have been used; in that case, several aliquots from the same vial are successively injected, allowing sufficient time for equilibrium to be reestablished

before each injection. The technique has as its main advantages that no foreign substance is introduced, changes due to possible chemical reactions are reduced to a minimum, and there are no losses of the more volatile substances. It does have, however, some disadvantages (Morales and Tsimidou 2000): (1) it is appropriate only for highly volatile compounds (i.e., those present in the headspace in analytically significant amounts); (2) the rubber of the stopper can absorb certain volatiles; and (3) leaks can occur during filling of the syringe.

SHS has been used (Morales et al. 1992; Escuderos 2011) to study the aroma of VOOs from different cultivars; to understand the sensory perception of fusty, winey, and rancid defects by consumers; to explain that the volatiles identified in refined oils came from autoxidation of the unsaturated fatty acids; to study the relationships between the contents of volatiles and fatty acids in thermoxidized vegetable oils; to study halogenated volatile compounds in VOOs; and to evaluate several sensory defects in VOO samples using direct coupling headspace-mass spectrometry and multivariate calibration techniques without any chromatographic separation (López-Feria et al. 2007).

8.3.1.2 Procedures Involving Preconcentration

The most commonly used procedures for the quantification of VOO volatile compounds involve an enrichment or preconcentration step because of the low concentration of most of the volatile compounds. The procedures require a careful analysis of the variables affecting the isolation and chromatographic determination of VOO volatiles in order to have comparable results among laboratories. The following variables and parameters are of great importance in a process of optimization:

- Temperature: The temperature of the enrichment step must be selected within a range allowing for most of the volatiles to be stripped in an effective way (>20 °C) and avoiding possible oxidative degradation of the oil sample (<45 °C).
- Adsorbent material: The number and amount of volatiles as well as the presence of artifacts depend on the sorbent material (the trap); there is no material that adsorbs volatiles in the entire range from low to high boiling points.
- Extraction parameters: The objective is to obtain the largest number of volatiles with concentrations higher than the GC detection threshold. Sample size, the geometry of the trap, and the flow rate of the carrier gas are decisive in an optimization process.
- Desorption step: Artifacts are related to the desorption process and should be avoided.

Distillation and Simultaneous Distillation-Extraction

Distillation is one of the most commonly used techniques for the isolation of volatile substances of foods. The methodologies most commonly used are vacuum and steam distillation. Reduced-pressure distillation is of great interest, as the working temperature is lower, thus minimizing possible changes in the sample. Generally,

the vapors from distillation are condensed on a refrigerant or trapped in different cryogenic traps or adsorbent materials, although the latter is less frequent. Once distillation is finished, the distillate can be injected directly into the chromatograph, but a concentration step is usually necessary because some compounds might be present at trace levels, making their detection impossible. This concentration is normally carried out by extracting the aromatic fraction of the distillate, drying the extract, and concentrating it.

A special distillation procedure is the so-called simultaneous distillation-extraction (SDE) that consists of separate distillations of a dilute aqueous solution of the sample and the solvent. They condense in the same area where extraction takes place. The two phases are then separated and recycled. Modifications of this original technique have been described; one obtains volatiles from fats and oils and gives water-free condensates, another is a micro version of the original apparatus that is used for analytical applications.

During the early 1970s, Flath and coworkers (Flath et al. 1973) carried out an exhaustive study of VOO volatiles. They used codistillation with water, followed by solvent extraction and dry-column chromatography, to obtain a polar concentrate, and they identified 77 volatiles by GCMS. The organoleptic assessment of some of these compounds indicated that several volatiles were significant contributors to aroma.

Another methodology of the vacuum distillation technique collects the volatiles in three cold traps (two cooled with liquid nitrogen and one with acetone/solid carbon dioxide), dissolves them in diethyl ether, and, finally, concentrates them by distillation and microdistillation (Ullrich and Grosch 1988). Another procedure consists of a vacuum codistillation of oil with diethyl ether that is collected in three traps cooled with liquid N_2 and then in a "cold finger" cooled with running water. The last fraction is extracted with ether and combined with the ether extracts collected in the cold traps. Finally, the extract is concentrated by distillation and microdistillation (Guth and Grosch 1989).

The technique has advantages, such as using small amounts of solvent, reducing the introduction of contaminants, obtaining a high concentration of volatiles in a short time, and minimizing thermal degradation by working at reduced pressure. It also has disadvantages, the main one being that it is not appropriate for thermolabile volatile compounds.

Dynamic Headspace

This technique was designed to determine the gases dissolved in aqueous samples. It is similar to SHS but there is a continuous flow of gas over the sample that carries away VOO volatiles. It consists of purging the volatiles of the sample, heated at a certain temperature, with an inert gas at a controlled flow, and passing them through a trap where they are retained. They are later desorbed and injected into the chromatograph for their separation. There are two kinds of techniques:

1. True DHS. This technique consists in sweeping the sample surface with the inert gas under stirring. It can use either adsorbent or cryogenic traps. The first one

consists of a glass tube filled with one or two adsorbent materials (e.g., Tenax) that can be directly desorbed in the injection port of the gas chromatograph. The cryogenic trap consists of a metallic tube refrigerated with liquid nitrogen. A procedure uses both kinds of traps using the cold trap to concentrate all the volatiles thermally desorbed from an adsorbent trap of Tenax TA prior to being injected into the gas chromatograph (Morales et al. 1994).

2. Purge-and-trap. This technique consists of bubbling the gas through the sample. This can be carried out in two different ways: open-circuit, in which the gas passes through the sample and trap and is then voided, or closed-circuit, in which the gas is recycled through the sample and trap.

Various factors affect the process, such as the diameter and length of the traps, the size and shape of the container used in the isolation, and the particle size of the adsorbent. The three fundamental controlling variables, however, are temperature, time, and purge flow (Morales and Aparicio 1993; Aparicio and Morales 1994). The latter two should be fixed beforehand because values that are too low can lead to a defective purge and values that are too high can result in a loss of volatiles. The temperature depends on the types of compound to be analyzed. If the working temperature is low (maximum 60 °C), the volatile compounds recovered are those actually present in the sample at the moment of analysis. The amounts are quite small, however, and the analysis is difficult. If higher temperatures are used (up to 160 °C), the compounds obtained are not only those actually present in the sample, but also volatiles formed by the thermal degradation of certain precursors. In this case, the amounts recovered are greater, and the analysis can be more easily carried out.

Generally, the gaseous samples obtained using this method consist basically of purge gas, water vapor, and a small amount of volatile substances. Thus, a concentration step using traps of adsorbent materials or cryogenic traps is necessary. These two types of traps can be described as follows:

1. *An adsorbent trap* consists of a glass or stainless steel tube filled with a certain amount of sorbent material (the trap). Volatiles are retained when the inert gas, charged with volatile compounds, passes through the trap. Various materials have been used.

   - *Activated carbon and graphitized sorbents*: the most commonly used materials in early research, these materials have a large specific surface and high adsorption capacities. They are highly appropriate for desorption with solvents but not for thermal desorption because the compounds are retained very strongly on their surface, so that a very high temperature is needed to obtain a quantitative desorption. Also, they have high water affinity and, in some cases, selective adsorption. The porous polymers, which retain little water and do not show irreversible adsorption, have the problem that they do not support as high temperatures as the other materials (Michel and Buszewski 2009).
   - *Tenax series*: despite having a small specific surface, these traps have a high adsorption capacity for many compounds and a high thermal stability, which allows desorption of substrates at high temperatures (up to 350 °C) without the formation of artifacts. They retain little water and are appropriate for

components of medium and high boiling points. Tenax TA is one of the most widely used polymers (Morales and Tsimidou 2000).
- *Other series*: other sorbent materials such as Porapak, Chromosorb, and Amberlite have been used for trapping volatile compounds from foodstuffs.
- All these traps require conditioning of the material to prevent interference in the analysis. The conditioning depends on the type of adsorbent; the procedure is described by the manufacturer.

2. *A cryogenic trap* is a length of column or capillary tube of a different type that is cooled to a very low temperature, normally with liquid nitrogen. Compounds passing through it condense inside. Desorption is carried out by passing a current of inert gas and raising the temperature so that the volatile compounds pass directly to the chromatographic column. This kind of trap has been used to analyze the volatile fraction of VOO samples in order to study the relationship between headspace composition and sensory analysis (Gasparoli and Fedeli 1987; Servili et al. 1995).

Both types of trap can be used at the same time. First, concentration is carried out with the use of a polymer, and second, the volatile compounds are passed through a cold trap where they condense. The desorption of the trapped volatiles is usually carried out by one of two procedures: with solvents or by means of thermal desorption.

Desorption with solvents is performed using a small volume of organic solvents so that the partition coefficient favors the eluent. The amount of solvent should be neither too small nor too great. An incomplete desorption can be produced in the former case; in the latter case, volatiles can be very diluted and require concentration, usually by evaporation. This procedure has disadvantages. For example, artifacts can be introduced into the sample, the solvent peak might mask part of the sample, and the method is not rapid and can cause losses on evaporation (Nuñez et al. 1984).

This technique has been widely applied to the analysis of VOO volatile compounds. Activated carbon and Tenax have been the most commonly used sorbent materials in VOO aroma analysis by DHS. Trapping on activated carbon with desorption using carbon disulfide (CS_2) as solvent was often used during the 1970s and 1980s to study the volatiles present in VOOs (Morales and Tsimidou 2000; Escuderos 2011). This research described various recovery devices, used large amounts of sample, and had long purge times. Some of the methods used three activated carbon traps in succession instead of a single trap. The first two traps were extracted jointly with CS_2 to give the main concentrate. The third trap, extracted separately, was used to check that the volatiles were present in the first two traps and to ensure that aromatic oils saturated them. Most of the research was aimed at identifying various volatiles present in VOOs. The technique of trapping on activated carbon and desorption with CS_2 has also been used to study thermoxidation of different types of oil.

Other solvents such as diethyl ether have been used to desorb activated carbon traps in experiments on the volatiles responsible for VOO rancidity, on volatiles related to cultivars and processing technologies (e.g., two- and three-phase centrifugation systems) (Morales and Tsimidou 2000), and on the changes in certain aroma components of VOOs related to degree of ripeness (Escuderos 2011).

Thermal desorption consists in heating the trap to a high temperature while passing a current of inert gas through it. Various devices have been used for the heating, some fitted to the injector and others using a modification of it. Among the disadvantages are thermal decomposition and possible impurities of the adsorbent, but the main disadvantage is that each sample can be injected only once. Thus, if it is necessary to repeat the injection, the whole process must be repeated.

The thermal desorption of a Tenax trap at 180 °C on a column previously cooled to −50 °C has been used to obtain fingerprint chromatograms of volatile compounds of eight types of oil at different oxidation levels (Snyder et al. 1985). A variant consists in collecting the volatile compounds on a Tenax trap, which is then desorbed thermally onto another smaller trap and reconcentrated on a third, from which the compounds are injected into the chromatograph. The use of this technique for the quality analysis of flavor in soybean and corn oils reduces analysis time and compares well with sensory evaluation (Raghavan et al. 1989). Other traps used are those containing various adsorbent materials: a trap of glass balls (to trap high-boiling-point compounds)/Tenax (medium boiling point)/Ambersorb (very volatile compounds) has been used in the analysis of stored soybean oil. In this case desorption was carried out thermally at 250 °C.

A DHS technique using Tenax TA traps as adsorbent material and further condensation on a cryogenic trap using thermal desorption has been widely applied to the analysis of VOOs. The technique has been widely used to study the volatile fraction of VOOs (Morales et al. 1994); to establish the relationship between volatile compounds and sensory attributes; to characterize different European varieties (Aparicio et al. 1997; Luna et al. 2006a), extraction systems (Aparicio et al. 1994b), and degrees of ripeness (Aparicio and Morales 1998); to study the influence of extraction conditions on VOO sensory quality; and to evaluate VOO sensory defects (Morales et al. 2005; Luna et al. 2006b).

Although this technique has disadvantages, it can be used with varying amounts of sample and over a wide range of temperatures. Because of these advantages, together with the concentration of volatiles in the appropriate traps, sensitivity is increased. Because the technique allows low working temperatures, it is also possible to study the volatiles actually present in the sample at the moment of analysis.

Microwave desorption has also been applied to olive oil volatile analysis. Either activated or graphitized carbon adsorption traps were used to concentrate the volatile compounds followed by microwave desorption and GC analysis. Hydrocarbons in olive oil (Almarcha and Rovira 1994) and aroma components of VOO (Bocci et al. 1992) have been analyzed by this method.

Headspace Solid-Phase Microextraction

An alternative to DHS analysis is HS-SPME, which integrates sample extraction and concentration in a single step that can be fully automated. This efficient, cheap, and simple technique consists of a fused-silica fiber coated with a liquid (polymer), a solid (sorbent), or a combination of both phases where the volatiles are retained, and

Table 8.3 Preconcentration and chromatographic conditions of a HS-SPME method

Preconcentration conditions	
Sample amount	2 g
Temperature	40 °C
Equilibrium time	10 min
Adsorption time	40 min
Stirring	Yes
Standard	4-methyl-2-pentanol
Analytical instrumentation	
Fiber	DVB/CAR/PDMS
Chromatograph	GC (GCMS)
Detector	FID
Column	DB-WAX (60 m × 0.25 mm × 0.25 μm)
Chromatographic conditions	
Injector/ detector temperatures	260 °C/280 °C
Desorption time	5 min
Carrier gas/flow rate	H_2/1 mL/min
Oven temperature program	40 °C (10 min) to 200 °C (3 °C/min)

the degree depends on the affinity that volatiles have to the absorbent. Nowadays, numerous fibers with diverse levels of polarity are available that allow analyzing volatiles ranging from polar to nonpolar. Fibers of 10 and 20 mm in length coated with a film thickness of 50/30 μm of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) or with 75 μm of polydimethylsiloxane/carboxen (PDMS/CAR) have been reported to have the best efficiency in sampling the volatiles present in VOOs (García-González et al. 2006).

The process consists in heating and stirring the oil sample contained in a thermostated vial sealed with a septum, for a preestablished period of time, till reaching equilibrium between vapor and liquid phases. Then the fiber is exposed to vapor phase to absorb volatiles. The analysis of the absorbed compounds is carried out by inserting the fiber directly into the GC injector held at a suitable temperature.

The main advantage of HS-SPME is its simplicity, reduced risk of cross contamination, good repeatability, acceptable linearity (although depending on the fiber), and percentages of recovery that vary too much between compounds, depending on the carbon atom number and the position of the functional groups (Angerosa et al. 1997). Table 8.3 shows the main characteristics of the sample pretreatment and chromatographic conditions of the experiments carried out with HS-SPME by the authors.

SPME has been profusely applied to the analysis of VOO volatile compounds. A comparative study on volatile analysis of EVOOs by DHS and SPME showed that the application of a DHS-TD Tenax TA procedure separated a higher number of compounds compared to the SPME-PDMS-DVB, which required shorter total times for the analysis (Kanavouras et al. 2005).

Supercritical Fluid Extraction

Supercritical fluids for analytical extractions provide a powerful alternative to traditional extraction techniques. They have been applied to the extraction of seed oils but have scarcely been used to study the volatile fraction of vegetable oils. One of the main disadvantages of analyzing volatiles is the problem of leaks that could cause the removal of part of the chemical compounds responsible for the aroma of the foodstuff. EVOO and olive fruits have been analyzed using supercritical CO_2 to isolate the volatile compounds (Morales et al. 1998). In order to collect the volatile components of these samples, the built-in extract trap of the SFE system was bypassed. Instead, the SFE extract, together with the total volume of venting carbon dioxide, was purged through a removable Tenax TA trap. The trap was then analyzed by thermal desorption–high-resolution gas chromatography–mass spectrometry (HRGC-MS). Applying different extraction conditions (temperature and pressure) different profiles can be obtained. Softer conditions (40 °C and 80 bars) allow one to obtain volatile compounds responsible for VOO flavor, while harder conditions (80 °C and 260 bars) permit one to obtain VOO off-flavors (Morales et al. 1998).

Headspace Sorptive Extraction

Headspace sorptive extraction (HSSE) is a solventless enrichment procedure developed to overcome some of the limits of the techniques described earlier, for example, the recovery of medium- to high-volatility analytes (David and Sandra 2007). The method was developed to extract volatile compounds from gaseous (headspace) or aqueous samples (stir bar sorptive extraction or SBSE) (Tienpont et al. 2000; David and Sandra 2007). HSSE is based on the sorption of analytes onto a thick film of polydimethylsiloxane coated on a stir bar (twister) although today there are new developments based on dual-phase twisters, which combine the preconcentration capabilities of two or more sampling materials, with which the precision and selectivity of the procedure have been enhanced (Bicchi et al. 2005). Extraction is controlled by the partitioning coefficient of the solutes between the polymer coating and the sample matrix and by the phase ratio between the polymer coating and the sample volume. After sampling analytes are recovered from the sorbent by thermal desorption. The procedure, however, has been scarcely applied to VOOs despite the fact that it yields results similar to those obtained by SPME (Cavalli et al. 2003).

Table 8.4 summarizes the advantages and disadvantages of the described methods according to the authors' experience.

Table 8.4 Advantages and disadvantages of most common techniques for quantification of VOO volatiles

Technique	Description	Advantages	Disadvantages
Without preconcentration			
Direct injection	Sample is placed in tube fitted to GC injector. Volatiles are purged by carrier gas into column	Rapid Simple	Very low sensitivity. Degradation products
Static headspace	Sample is deposited in sealed vial After equilibrium an aliquot of vapor phase is injected into gas chromatograph	Fine with multiple extractions Appropriate for volatiles with high Vapor pressure	Poor sensitivity and reproducibility Artifacts. Inappropriate for trace analysis Leaks during syringe filling
With preconcentration			
Distillation-extraction	Vapor from distillation is condensed on a refrigerant or trapped in different cryogenic traps or adsorbent materials and later injected into gas chromatograph	Small amount of solvents Rapid concentration process Low thermal degradation of volatiles	Not appropriate for thermolabile volatiles Leaks of analyte by co-evaporation
Dynamic headspace	An inert gas (e.g., N_2) sweeps headspace of sample, which is stirred or bubbled. Volatiles are trapped in Tenax	High adsorption capacity. Useful for almost all kind of volatiles	Less sensitive to some acids
Tenax traps			Temperature & flow-rate must be controlled
Thermal desorption and cold trap injection	Trap is thermally desorbed in gas chromatograph	Good recovery factors. Good repeatability. No artifacts	An analysis per sample
Headspace with SPME	A SPME fiber is exposed to sample vapor phase. Volatiles adsorbed on fiber are desorbed in gas chromatograph injection port	Rapid. Cheap. Easy to use All the steps in a single process when using automatic injector. Various kinds of fibers. Good repeatability	Differences in quantification of low molecular weight molecules. Less number of volatiles at low concentrations. Some of the disadvantages of the static headspace
Supercritical fluid extraction	Sample is placed in extraction cell A supercritical fluid passes through cell and extracts volatile compounds	Easy to apply Detection of volatiles in oil and olives Adequate for off-flavors Selection of the target analytes	Leaks Selective with some volatiles (oxygenate compounds & by molecular weight and polarity) Need of pre-concentration (e.g. Tenax)
Headspace sorptive extraction	Sorption of volatiles onto a thick film of polymer coated on a stir bar (twister) and thermal desorption	Increase the concentration capability of SPME Expected better results by using dual-phase twisters	Competence phenomena on the polymer surface Sensitivity of one phase PDMS twisters

Source: Morales and Tsimidou (2000)

8.3.2 Gas Chromatography

The separation and quantification of volatile compounds of VOO is usually carried out by HRGC using different chromatographic conditions (Escuderos 2011). GC is a powerful separative technique with a high capacity to separate complex mixtures of very similar components. It is relatively fast and has high resolution and very high precision, mostly when autosamplers are used. It requires only small amounts of sample, having excellent sensitivity to detect volatile organic mixtures at low concentrations. It is the most suitable analytical procedure for the analysis of volatile analytes, although HPLC has been used sometimes to analyze certain volatile compounds. The instrument is not very complex and has the advantage that it can be coupled to other techniques (e.g., MS, IR).

Detection is often carried out using flame ionization detectors (FID). Electron capture detectors can be used to indicate the presence of halogen atoms. HRGC-MS with different modalities has been widely applied to the identification of VOO volatile compounds.

GC-olfactometry is the most frequently used technique to assign the aroma impact zones of the chromatogram and to relate chemical compounds to sensory descriptors. The aroma of a food consists of many volatile compounds, only a few of which have sensory significance. An essential step in aroma analysis is the distinction of the more potent odorants from volatiles having low or no aroma activity. Different approaches have been developed, from calculation of the ratio of the concentration of an odorant to its odor threshold and denoted it as aroma value, which was the first attempt to estimate the sensory contribution of single odorants to the overall aroma of a food (Rothe and Thomas 1963), or the use of similar methods like calculation based on nasal odor thresholds (odor unit), using retronasal odor thresholds (flavor unit), and odor activity value (OAV), which is synonymous with odor unit and odor value (Grosch 2001). These concepts are appropriate for assigning sensory significance when a screening step for the most important aroma compounds is used, but they are time-consuming since they require identification and quantification of a large number of volatile compounds and determination of their threshold values (Blank 2002).

GC in combination with olfactometric techniques (GC-O) is a valuable method for the selection of aroma-active components from a complex mixture. In GC-O human subjects sniffing GC effluents assign the sensory properties of the different peaks/zones of the chromatogram. Many key aroma compounds occur at very low concentrations because their sensory relevance is due to low odor thresholds. The peak profile obtained by GC does not necessarily reflect the aroma profile of the food. Usually, it is difficult to assign a sensory relevance of volatiles from a single GC-O run (Blank 2002).

Several procedures have been developed to objectify GC-O data and to estimate the sensory contribution of single aroma components. Dilution techniques and time-intensity measurements are the two main GC-O methods, although several interesting approaches based on the application of multivariate statistical procedures to GC

and sensory data have been developed and applied to VOO and other fat-containing foods (Morales et al. 1995; Aparicio et al. 1996; García-González et al. 2008).

Time-intensity measurements. The OSME method analyzes the non-diluted aroma extract by GC-O (Miranda-Lopez et al. 1992), and the odor intensities perceived in replicate by several assessors are averaged, yielding a consensus aromagram. Olfactometry global analysis (OGA) is similar to Osme, but each peak in the aromagram is related not to the odor intensity of the volatile, but to its detection frequency.

Dilution techniques. Two methods based on dilution have been developed: CharmAnalysis (Acree et al. 1984) and aroma extract dilution analysis (AEDA) (Grosch 1993). Both evaluate the odor activity of individual compounds by sniffing the GC effluent of a series of dilutions of the original aroma extract, usually as a series of 1:1 or 1:2 dilutions, and each dilution is analyzed by gas chromatography–olfactometry (GC-O). In the case of AEDA, the result is expressed as a flavor dilution (FD) factor (Grosch 1993), which is the ratio of the concentration of the odorant in the initial extract to its concentration in the most dilute extract in which the odor is still detectable by GC-O. Consequently, the FD factor is a relative measure and is proportional to the OAV of the compound in air. Charm analysis constructs chromatographic peaks whose areas are proportional to the amount of the chemical in the extract (Acree 1993). In CharmAnalysis, the dilutions are presented in randomized order to avoid bias introduced by knowledge of the samples. The assessor detects the beginning and the end of each aroma perception and notes the sensory attributes. The primary difference between the two methods is that CharmAnalysis measures the dilution value over the entire time the compounds elute, whereas AEDA simply determines the maximum dilution value detected (Grosch 2001).

8.3.3 Chemosensors

The chromatographic techniques can assess sensory quality in routine tests but cannot be used in online processes because of the need for sample pretreatment or a volatile concentration step. An alternative is the use of sensors that have already been used in many fields of analytical chemistry.

Conceptually speaking, a sensor is a device that is able to give a signal proportional to the physical or chemical property to which the device responds. The electronic integration of various sensors inside one set constitutes an array of sensors, or the so-called electronic nose, which is a kind of low-cost structure commonly used to analyze the aroma of foodstuffs.

Nowadays, several commercial sensors are available on the market in addition to the large number of prototypes that are designed by research groups each year. All these types of sensors exhibit physical and chemical interactions with the chemical compounds when they flow over, or are in contact with, the sensors. The choice of sensor is hence quite important and not an easy matter, so that they have been classified into broad classes following an arborescent structure. Odor sensors, tasting

sensors, and biosensors constitute the first class. The last two groups are the result of very recent research. The sensors of the former group can be clustered into two large groups according to their working temperature: hot sensors and cold sensors. The group of so-called hot sensors mainly includes different kinds of metal oxide gas sensors (based on doped or undoped semiconductors), while cold sensors include piezoelectric crystal sensors (bulk acoustic wave sensors, surface acoustic wave sensors) and conducting organic polymer sensors. Recent research seems to be focused on designing instruments that combine different classes (e.g., conducting polymer sensors plus piezoelectric crystal sensors) to reduce drawbacks, and even going beyond by integrating electronic nose and electronic tongue.

8.3.3.1 Metal Oxide Semiconductor (MOS) Sensors

These sensors, which are the most common of the commercially available sensing systems, consist of a ceramic former heated by wire and coated with a metal oxide semiconducting film. The oxide coating may be either n-type or p-type semiconductors. The n-type semiconductors (e.g., SnO_2, ZnO, In_2O_3, WO_3, Fe_2O_3, Ga_2O_3, TiO_2) respond to oxidizing compounds and their thermal or photolytic excitation results in an excess of electrons, which increases the reactivity with oxidizing molecules. The p-type semiconductors (oxides of nickel or cobalt) respond to reducing compounds and their excitation promotes reactions with reducing compounds due to an electron deficiency in their valence band. These metal oxides (mostly SnO_2, Ga_2O_3, and TiO_2) can also be doped with metals (e.g., Cu, Pt, Sb, Bi, Fe, Au, Cd) or metal oxides (e.g., Co, Cr, La, Ce, Mg, Al, Ru, Rh, Ir) to improve the sensitivity and the selectivity as well as to diminish humidity and temperature dependence.

The mechanism of sensing of these sensors is related to the semiconductor behavior when exchanging oxygen between the volatiles and the metal film. Under nonoperating conditions, when only the carrier gas (e.g., synthetic air) flows in the sensor chamber, the oxygen is adsorbed on the surface and inside the metal coating, filling the sensor lattice vacancies, and later oxygen attracts free electrons from the semiconductor material, which results in increases of the sensor conductance. In operation, when adsorbed volatile molecules interact with the metal film take place, the conductance decreases due to the reaction with the adsorbed oxygen.

These small sensors are robust and have a quite good sensitivity, their signal processing is simple, and they have a very low cost. Their sensitivity is, however, affected by humidity. Although the sensors are made of materials relatively resistant to humidity and aging, humidity is still the main problem as the sensors sensitivity decreases when humidity increases. Another drawback is a temporary blinding effect, any other analytes present in the sample produced by the presence of ethanol. Other undesirable characteristics of these sensors are: their slow baseline recovery when compounds with high molecular mass are analyzed, their poor specificity and selectivity, the sensor drift mainly caused by sensitivity loss, and the nonlinear response to some chemical compounds.

The array of MOS sensors, the so-called electronic nose, has been applied with success to detect the presence of some of the most common defects in VOOs (e.g., vinegary, rancid, fusty) even before the official sensory assessment or panel test (García-González and Aparicio 2002b). Thus, the hypothesis that the sensor response depends on the amount and composition of volatile compounds had been demonstrated by the authors when analyzing sensory defects by canonical correlation (García-González and Aparicio 2002c). Based on these results, four steps were suggested for analyzing the data sets of responses of the sensors to VOO volatiles: (1) detection of multivariate outliers by principal component analysis (PCA), (2) training process of supervised procedure SLDA, (3) implementation of canonical equation in a discriminating model, and (4) validation of results with samples of an external test set (García-González and Aparicio 2004). The result has allowed, for instance, for the selection of commercial MOS sensors suitable to distinguish high-quality olive oils with irreproachable sensory descriptors from lampante olive oils, which are not suitable for human consumption, by means of a neural network with only 4.5 % error in the data set and with no error in an external validation set (García-González and Aparicio 2003).

The simultaneous analyses of volatiles by GC and an array of MOS sensors, and even a coupling of GC with a sensor array, are described in works in the bibliography (García González and Aparicio 2002a, 2010). The objectives were to correlate the intensity of sensor responses with the structure of volatile compounds, to analyze simple mixtures of volatiles (or to remove a masking component), and to check the pros and cons of a micromachined gas chromatographic column in tandem with an array of sensors (Zampolli et al. 2005). Nevertheless, the coupling of gas chromatography-sensor array (GC-EN) has been used only very recently to analyze the aroma of foods made by a complex mixture of volatile compounds such as in the case of VOOs.

The individual contribution of volatile compounds from VOO to the overall MOS sensor responses when they interact all together with the sensitive material of the sensors is unknown, and a classification of VOOs into different quality categories performed by merely processing the sensor response data requires elucidation on whether the classification is actually based on the volatiles that are responsible for the VOO sensory attributes. On the other hand, the possibilities of an array of sensors as an alternative to destructive FID chromatographic detectors need to be explored as well.

Because GC allows for both qualitative and quantitative determinations of the volatiles present in VOO headspace, the use of a sensor array, or electronic nose (EN), as a nondestructive detector in parallel with the FID makes it possible to know the volatiles that contribute to the sensor response as well as the sensor sensitivity to each volatile at its concentration in the sample headspace. Thus, when coupling GC-EN, the sensors respond to the volatiles that, eluted from the chromatographic column, are transferred separately to the sensor chambers by the chromatograph carrier gas.

Figure 8.5a shows the signal derived from a GC-EN system. The first changes in the intensity of the sensor responses are observed at 20 min (retention time),

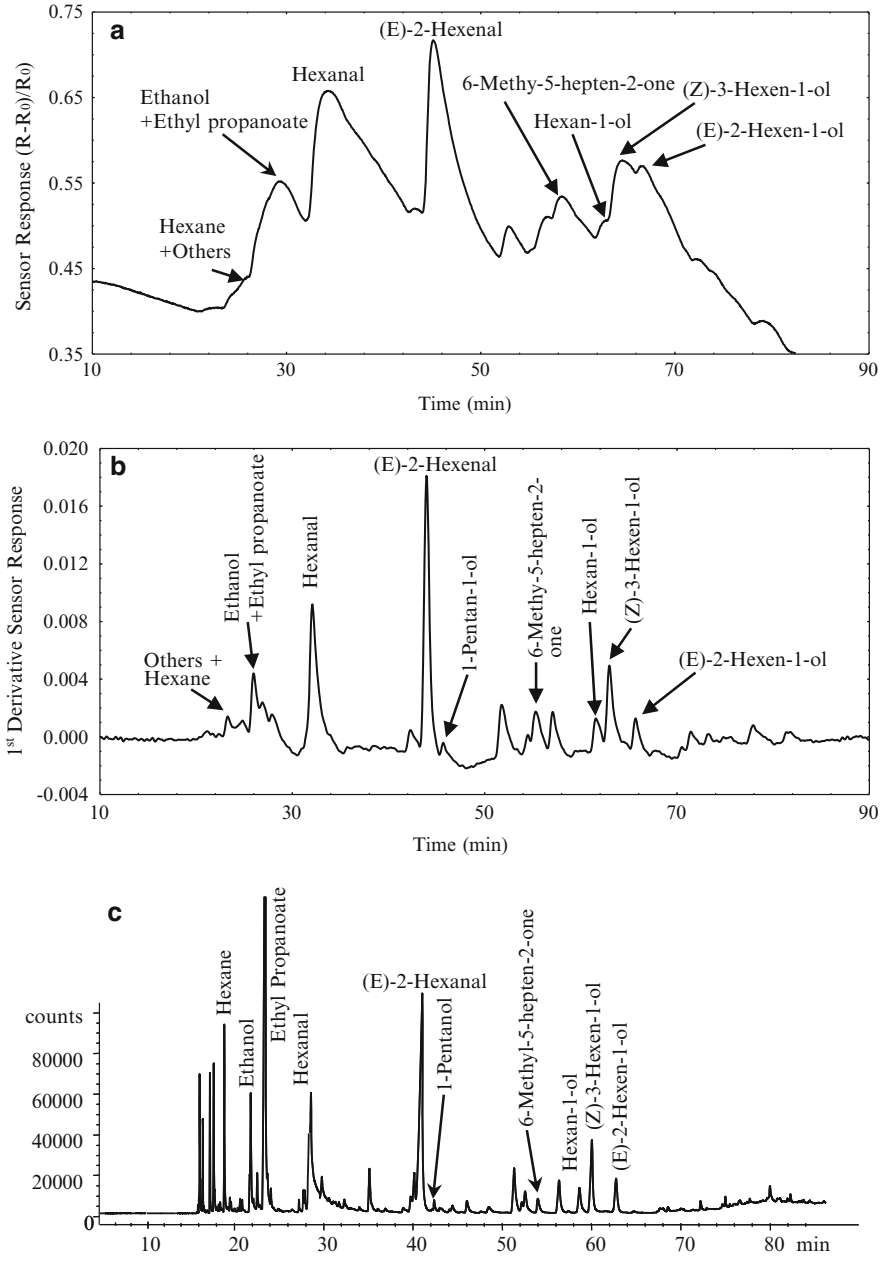

Fig. 8.5 Sensorgrams of raw response of Cr3O2 sensor (**a**), its first derivative (**b**), and SPME-GC chromatogram of volatiles (**c**) of a virgin olive oil (García-González and Aparicio (2010), with permission of Elsevier)

corresponding to hexane, octane, and methyl acetate. These compounds scarcely contribute to VOO aroma, and their low response in the sensorgram also shows that they hardly contribute to sensor response as well. The second broad peak, more intense than the previous one, corresponds to a group of compounds including ethanol, ethyl propanoate, and 3-pentanone, from all of them sensors are more sensitive to ethanol. Hexanal produces a very intense peak too. This volatile contributes to the perception of a sweet-green sensory note in EVOO when its concentration is higher than its odor threshold, but it contributes to the rancid perception when it is present at high concentrations (Morales et al. 1997). The following compounds observed in a sensorgram are E-2-hexenal and, only when present at high concentrations, pentan-1-ol. The first is characterized by a bitter-almond sensory note, while the second is characterized at high concentrations by an intense and unpleasant gluelike aroma (Morales et al. 2005) and by a pungent, strong, and balsamic aroma at medium or low concentrations (Luna et al. 2006b). The next peaks have been assigned to a series of compounds that are commonly responsible for pleasant fruity-green attributes (Aparicio et al. 1996), and they have close retention times in the chromatogram. These compounds are hexyl acetate, 6-methyl-5-hepten-2-one, and Z-3-hexenyl acetate, and the alcohols hexan-1-ol, Z-3-hexen-1-ol, and E-2-hexen-1-ol. Depending on their relative concentrations, some of these compounds may mask others, and hence the derivative of the raw sensor response is necessary to elucidate the contribution of each of them. The resulting sensorgram (Fig. 8.5b) resembles a GC chromatogram obtained using the SPME-GC methodology (Fig. 8.5c).

The main application of MOS sensors is, however, the detection of VOO samples with defective sensory attributes (García-González and Aparicio 2002b), which must be refined prior to being consumed. Previous studies showed that the area covered by the response curve was, in general, higher in defective VOOs than EVOOs, but it was not possible to know if the volatiles to which the sensor was sensitive contributed to VOO off-flavor. Coupling a GC column to an array of MOS sensors made it possible to determine whether the sensors were sensitive to compounds present at high concentrations in defective VOOs, and in addition, they were compounds that contribute to the perception of undesirable sensory attributes. Figure 8.6 shows the profiles of the sensorgrams of the most common defective VOOs: rancid, fusty, winey-vinegary, and musty-humidity. The highest response of the sensor in terms of total area under the curve was observed in rancid VOO followed by fusty and the other two defective oils (winey-vinegary and musty). This order fully agrees with the chromatograms of defective oils (Morales et al. 2005); in addition, the profiles of the sensorgrams are very similar to their respective chromatograms. Thus, a tentative assignation of peaks of rancid sensorgram made it possible to identify the main aldehydes (pentanal, hexanal, octanal, nonanal) and a zone where acids (butanoic, pentanoic, hexanoic, heptanoic acids) and 2,4-dienals (octadienal, nonadienal, decadienal) are located. The main contributors to a fusty defective sensory attribute are esters and acids (Morales et al. 2005), and butyl acetate and ethyl propanoate seem to be responsible for sensorgram peaks, together with hexanal, 6-methyl-5-hepten-2-one, and the acids (acetic, propanoic, butanoic, and pentanoic acids). The peaks of the musty-humidity sensorgram are much

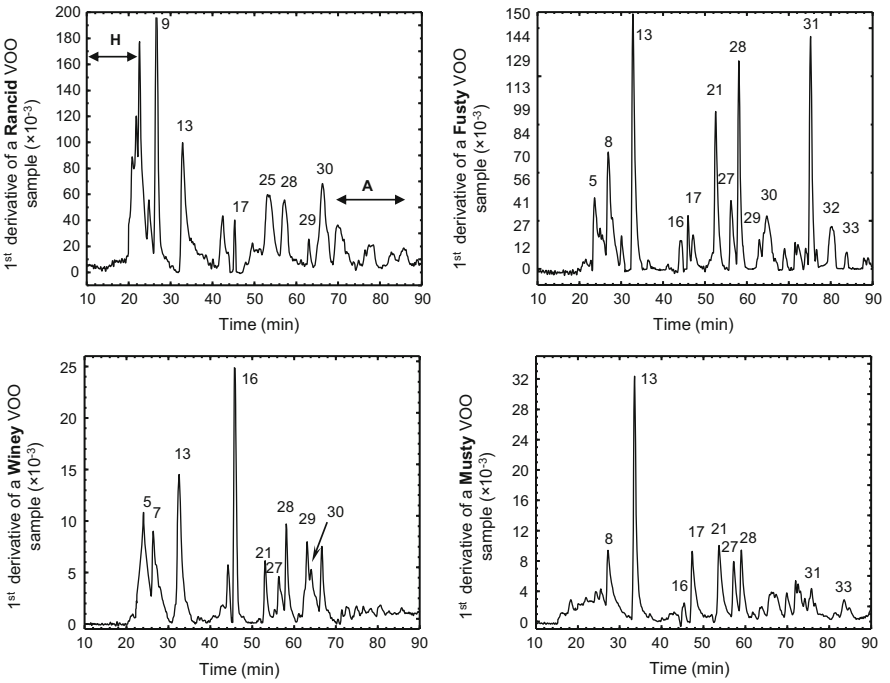

Fig. 8.6 Sensorgrams of Cr2O3 + Ti sensor of standards of virgin olive oil samples characterized by defects rancid, fusty, winey–vinegary, and musty–humid at intensity levels 8 out of 10. Note: (*5*) Ethyl acetate, (*7*) Ethanol, (*8*) Ethyl propanoate, (*9*) Pentanal, (*13*) Hexanal, (*16*) E-2-Hexenal, (*17*) Pentan-1-ol; (*21*) 6-Methyl-5-hepten-2-one, (*25*) Nonanal, (*27*) E-2-octenal, (28) Acetic acid, (*29*) Propanoic acid, (*30*) E-2-Decenal, (*31*) Butanoic acid, (*32*) Pentanoic acid, (*33*) Hexanoic acid, *A* Acids and 2,4-dienals, *H* Hydrocarbons (i.e., heptane, octane) (García-González and Aparicio (2010), with permission of Elsevier)

smaller and reflect the low concentration of E-2-hexenal in comparison with hexanal, the latter being the most significant compound in this kind of defective VOO. Other peaks might be due to the presence of pentanol, 6-methyl-5-hepten-2-one, and acetic, butanoic, and hexanoic acids. Despite the high concentration of ethyl acetate in VOOs qualified with a winey-vinegary attribute (Morales et al. 2005), this compound produces a relatively low response in Cr_3O_2 sensors, although the response is much more intense in SnO_2 sensors.

This new approach requires further research to improve the resolution of the peaks as well as to reduce the sensor desorption time. Solutions can come from new designs for the sensor chamber, the substitution of current capillary columns by micromachined gas chromatographic columns, and MOS sensors by surface acoustic wave (SAW) sensors that show a faster desorption time, although the robustness and repeatability of the latter sensors still need to be improved.

8.3.3.2 Conducting Polymer Sensors

Conducting polymer sensors(CPSs) are based on a measurable change in electrical conductivity when CPSs are exposed to volatile compounds. A fixed voltage among the electrodes produces a constant current that passes through the conducting polymer. When gas flows on the sensor, the volatile compounds adhere to its surface, and an adsorption-desorption process occurs on the polymer layer that alters the electron flow in the system and, hence, the sensor conductivity.

These sensors are made of a broad range of monomers that are polymerized by chemical or electrochemical methods. A thin film of polymer is deposited onto a sensing substrate formed by two parallel platinum- or gold-plated electrodes with insulating base such as oxidized silicon. The most common polymers are polypyrroles and polyanilines, although new polymers are being synthesized to extend these sensors to new applications. The nature of the dopants also modifies the initial properties of polymers, and this means the possibility of designing sensors tailored to specific series of volatiles (e.g., ketones, aldehydes, alcohols) or to a particular application (e.g., characterization or authentication). They are easily fabricated, their power consumption is low, and they are robust and can work at room temperature.

The random nature of the deposition process is, however, a problem for the production of reproducible sensors. The sensors also have other drawbacks such as their too long response time (20–40 s), in comparison with MOS, and their inherent drift over time or with changes in temperature. The main problem is, however, that they are extremely sensitive to moisture.

The great interest in the application of PCS to the evaluation of VOO aroma at the end of the millennium (Aparicio et al. 2000b; Stella et al. 2000; Escuderos et al. 2007) seems to have slammed on the brakes in the last decade, with MOS and SAW sensors having taken their place.

8.3.3.3 Acoustic Sensors

These sensors are based on the propagation of acoustic waves produced by piezoelectric materials (e.g., quartz, LiNbO3, Si-SiO2-ZnO) in a multilayer structure. Surface acoustic wave (SAW) and bulk acoustic wave (BAW) sensors are the most common acoustic sensors. The operation mode is based on the physical changes produced on their membranes made of chemically interactive materials (CIMs). The physical changes that can be detected are related to the piezoelectric materials used for the acoustic transduction or the mass density and (visco-)elastic properties of CIMs. From the sensing point of view, the presence of volatile compounds produces an adsorption of molecules on the sensor and, hence, changes on the properties of the CIM that affect both the phase velocity and the propagation loss of the acoustic wave. The result is a frequency shift whose magnitude is related to the amount of material adsorbed onto the layer sensor.

Acoustic sensors have various advantages over other sensors – such as, for instance, high sensitivity and short response time, low power consumption and size,

and robustness – but have common disadvantages such as temperature and humidity dependence and also particular drawbacks like poor reproducibility in the deposition of the coating material and a certain level of noise because of the oscillator high frequency.

Regarding polymer sensors, SAW sensors have not found enough support in the food industry to develop new instrumentation beyond the in-house designs of some research groups (García-González et al. 2004, 2006).

8.4 Contribution of Chemical Compounds to Virgin Olive Oil Sensory Quality

The establishment of the relationship between VOO chemical compounds and sensory attributes is the most complex aspect of the global study of flavor. Great advances in instrumentation have allowed the identification of a large number of chemical compounds of VOOs. The relationships between these compounds and the sensory attributes have been studied for years, and several methods have been applied to establish these relationships in VOOs.

The first attempts were focused on finding correlations between single compounds and sensory attributes, but this is not the right way to describe the aroma for several reasons. First of all, it is not always true that volatile compounds at higher concentrations are the main contributors to aroma. Their contributions depend on the calculation of the ratio of their concentrations to their flavor thresholds nasally and retronasally evaluated, which is known as the OAV. The determination, although laborious enough, is a powerful tool in determining which compounds do not contribute to VOO flavor due to factors like volatility, hydrophobicity, and molecular characteristics (e.g., shape, size, type, and position of functional groups), together with other factors like synergism and antagonism between compounds, due to the simultaneous stimulation of olfactory and gustative receptors of the assessors. The application of multivariate statistical procedures to data sets of volatile compounds and of the intensities of sensory attributes evaluated by means of the official sensory assessment (Chap. 14), combined with OAVs of selected volatile compounds, have produced the best results.

8.4.1 Relationships Between Volatile Compounds and Basic Sensory Perceptions

Not all the compounds of a volatile fraction have the same influence on the sensory quality. Their influence will depend not only on their concentration but also on their odor threshold. A GC sniffing technique has been widely applied to study the independent sensory impact of the different volatile compounds on sensory quality. Calculation of OAV has also been used to determine the volatiles that make the

largest contributions to VOO aroma (Guth and Grosh 1993; Morales et al. 1996; Aparicio and Morales 1998). This procedure is effective for the determination of the most important contributors to aroma, but the presence of other volatile compounds in the matrix of the oil should not be forgotten as they can contribute to the global flavor by synergism phenomena. Multivariate statistical procedures have proven to be powerful tools for pointing out these relationships because they work with sets of chemical compounds and VOO sensory attributes.

Thus, the artificial neural network (ANN) has been used to predict sensory assessments with a fine degree of accuracy (Angerosa et al. 1996a). Partial least squares (PLS) and PCA have widely been applied to achieve the relationship between the chemical composition and free-choice profiling Quantitative Descriptive Analysis (QDA) data (Servili et al. 1995). Multidimensional scaling (MDS) also has been applied to bring out inter–intra dissimilarities from data sets of volatile compounds and sensory attributes of VOOs (Morales et al. 1995).

An interesting approach is the statistical sensory wheel (SSW), which represents the global VOO flavor matrix, and is the most complete explanation of sensory descriptors by volatiles to date. The idea of the sensory wheel is not new (Noble et al. 1987) but is more than a subjective procedure in which each flavorist places the sensory descriptors at his or her convenience. SSW consists of the information about sensory attributes evaluated by six different European panels, for a total of 103 sensory descriptors (Table 8.5). All the panels used QDA (Chap. 14) in the evaluation of the oils. Three of the panels followed the EC regulations that were in force at that time (EC 1991) for VOO sensory evaluation. The other three panels used a free-choice profile that allowed qualifying VOO samples with enough sensory information (Aparicio et al. 1994a). The values of the sensory attributes after means calculation, selection of the main sensory attributes (based on their repeatability and embodying all of the terms expressed by the panels), outlier detection, and cross validation were analyzed by PCA. A circle of radius 1.0 was drawn at coordinates (0, 0) on a plot of the selected attributes by the first two components of PCA. The limits of each sensory wheel sector were calculated by circular standard deviation (Aparicio and Morales 1995). The correlations of each of the remaining sensory attributes with the first two factors of PCA were calculated, and these values were taken as its coordinates (x, y).

Applying this methodology, researchers constructed a wheel. A global sensory matrix of the VOO is represented inside the circle (Fig. 8.7). The global matrix was divided into sectors representing the main perceptions produced by VOO (Aparicio and Morales 1995). Seven main sectors were found: green, bitter-pungent, undesirable, ripe olives, ripe fruit, fruity, and sweet. Some miscellanies appeared between sectors because changes in perception are not sudden but progressive, and some zones could be the result of this progressive change.

The quantitative values of the volatile compounds (Table 8.6) analyzed by DHS-GC were projected onto the wheel. The correlations of each volatile compound with the first two components of PCA were taken as its coordinates (x, y) in the wheel. Thus, all the volatiles were standardized by the set of selected attributes representing different kinds of consumers.

Table 8.5 Sensory descriptors evaluated by panels with different methods of perception. The descriptors with "*" were selected for building the core of the statistical sensory wheel

Attribute	Perception	Code	Attribute	Perception	Code
Olive fruity (green)*	Flavor	1	Twig	Odor	53
Apple*	Flavor	2	Pine/Harshy	Odor	54
Other ripe fruits*	Flavor	3	Lemon	Odor	55
Green*	Flavor	4	Orange	Odor	56
Bitter*	Flavor	5	Soft fruits	Odor	57
Pungent*	Flavor	6	Candies (fruit)	Odor	58
Sweet	Flavor	7	Wild flowers in springtime	Odor	59
Undesirable*	Flavor	8	Fermenting fruit	Odor	60
Olive fruity (ripe)*	Flavor	9	Farm	Odor	61
Olive fruity (ripe and green)*	Flavor	10	Oil for salads (soybean oil)	Odor	62
Other ripe fruits	Flavor	11	Tallow	Odor	63
Green*	Flavor	12	Cod liver oil	Odor	64
Bitter*	Flavor	13	Nuts	Odor	65
Pungent*	Flavor	14	Medicine	Odor	66
Sweet*	Flavor	15	Earthy	Odor	67
Allowable	Flavor	16	Taste intensity	Taste	68
Undesirable*	Flavor	17	Sweet	Taste	69
Olive fruity (ripe and green)*	Flavor	18	Salty	Taste	70
Apple*	Flavor	19	Olives	Taste	71
Other ripe fruits	Flavor	20	Green leaf	Taste	72
Green*	Flavor	21	Grass	Taste	73
Bitter*	Flavor	22	Green banana (not ripe)	Taste	74
Pungent*	Flavor	23	Dried green herbs	Taste	75
Sweet*	Flavor	24	Minced pepper	Taste	76
Undesirable*	Flavor	25	Red chili pepper	Taste	77
Tomato*	Aroma	26	Cream/butter	Taste	78
Ripe black olives*	Aroma	27	Coconut	Taste	79
Green olives*	Aroma	28	Caramel	Taste	80
Cut green grassy*	Aroma	29	Grotty	Taste	81
Artichoke*	Aroma	30	Velvetlike	Mouthfeel	82
Apple*	Aroma	31	Sticky	Mouthfeel	83
Yeast*	Aroma	32	Slightly burned/toasted	Taste	84
Bitter*	Taste	33	Ashtray	Taste	85
Pungent*	Mouthfeel	34	Glue with ethylacetate	Taste	86
Astringent*	Mouthfeel	35	Refinery	Taste	87
Strength of olive	Odor	36	Bitter	Taste	88
Strength of olive	Flavor	37	Astringent	Mouthfeel	89
Banana skins*	Flavor	38	Green	Aftertaste	90
Tomato*	Flavor	39	Fruity	Aftertaste	91
Sweet*	Odor	40	Cooling/evaporating	After mouthfeel	92
Hay/composty*	Flavor	41	Glue with ethylacetate	Aftertaste	93
Perfumey	Odor	42	Cocoa butter/white choc	Aftertaste	94
Perfumey	Flavor	43	Putty/lineseed oil	Aftertaste	95

(continued)

Table 8.5 (continued)

Attribute	Perception	Code	Attribute	Perception	Code
Grassy*	Flavor	44	Used frying oil	Aftertaste	96
Almond*	Flavor	45	Trany	Aftertaste	97
Throatcatching*	Mouth feel	46	Dry wood	Aftertaste	98
Thickness	Mouth feel	47	Dusty	Aftertaste	99
Pungent	Flavor	48	Dry	After mouthfeel	100
Odor intensity	Odor	49	Sharp/etching	After mouthfeel	101
Sea breeze on the beach	Odor	50	Pungent/sharp throat	After mouthfeel	102
Prickling	Odor	51	Undesirable	Flavor	103
Apple	Odor	52			

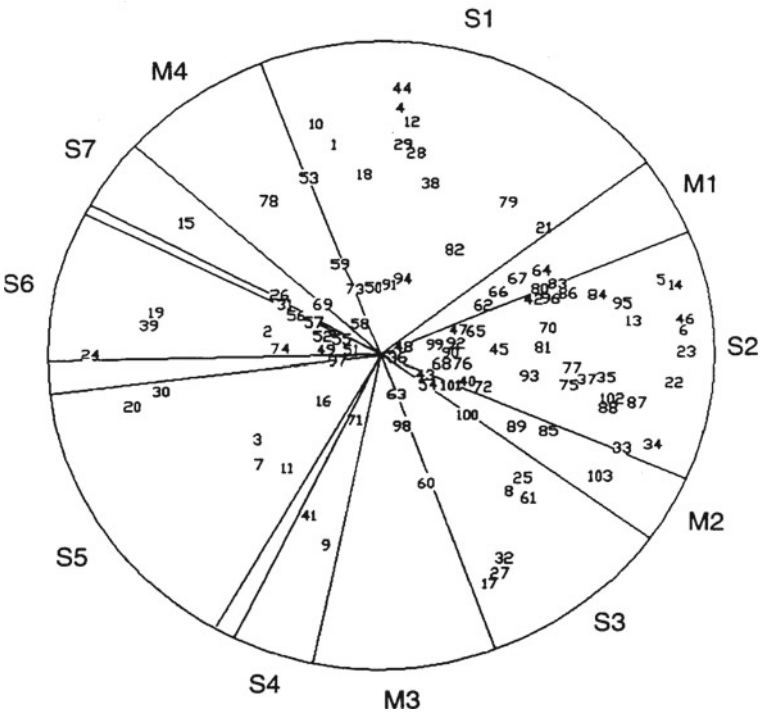

Fig. 8.7 Statistical sensory wheel showing relationship between different sensory attributes (Table 8.5) evaluated by different sensory panels. Sectors correspond to main sensory perceptions of virgin olive oil matrix: (S1) Green sector; (S2) Bitter-pungent sector; (S3) Undesirable sector; (S4) Ripe-olive sector; (S5) Ripe-fruit sector; (S6) Fruity sector; (S7) Sweet sector, (M1) Miscellany 1 (green-bitter), (M2) Miscellany 2 (bitter), (M3) Miscellany 3 (ripe-undesirable), (M4) (sweet-green) (Aparicio et al. (1996), with permission of Springer)

Table 8.6 Volatile compounds determined in virgin olive oil samples. Sensory characterization of volatiles by statistical sensory wheel

Code	Chemical compound	Sensory wheel	Code	Chemical compound	Sensory wheel
1	Methyl acetate	Green	33	Z-2-Hexenal	Green
2	Octene	Green	34	2-Methylbutan-1-ol	Miscellany 3
3	Ethyl acetate	Undesirable	35	3-Methyl butanol	Undesirable
4	Butan-2-one	Fruity	36	3-Methyl-2-butenyl acetate	Undesirable
5	3-Methylbutanal	Ripe fruit	37	Dodecene	Undesirable
6	1,3-Hexadien-5-yne	Green	38	Pentan-1-ol	Fruity
7	An alcohol	Fruity	39	Ethenylbenzene	Fruity
8	Ethylfuran	Miscellany 4	40	Hexyl acetate	Green
9	Ethyl propanoate	Miscellany 4	41	A C_8 ketone	Green
10	An alcohol + hydrocarbon	Miscellany 3	42	Octan-2-one	Ripe olives
11	3-Pentanone	Green	43	3-4-Methyl-3-pentenylfuran	Ripe olives
12	4-Methylpentan-2-one	Green	44	3-Hexenyl acetate	Green
13	Pent-1-en-3-one	Sweet	45	Z-2-Penten-1-ol	Green
14	2-Methylbut-2-enal	Undesirable	46	6-Methyl-5-hepten-2-one	Bitter
15	A hydrocarbon	Miscellany 4	47	Nonan-2-one	Miscellany 4
16	Methylbenzene	Ripe fruit	48	Hexan-1-ol	Undesirable
17	2-Methylbut-3-enol	Undesirable	49	E-3-Hexen-1-ol	Miscellany 2
18	Butyl acetate	Miscellany 4	50	Tridecene	Bitter
19	Hexanal	Sweet	51	Z-3-Hexen-1-ol	Green
20	A hydrocarbon	Miscellany 4	52	2-4-Hexadienal	Ripe fruit
21	2-Methylbutyl propanoate	Miscellany 2	53	E-2-Hexen-1-ol	Undesirable
22	2-Methyl-1-propanol	Green	54	Acetic acid	Undesirable
23	E-2-Pentenal	Green	55	Methyl decanoate	Miscellany 1
24	An alcohol	Undesirable	56	Hydrocarbon C_{11}	Bitter
25	Z-2-Pentenal	Miscellany 3	57	Hydrocarbon	Bitter-pungent
26	Ethylbenzene	Bitter	58	2-Methyl-4-pentenal	Bitter-pungent
27	E-3-Hexenal	Ripe fruit	59	1,2,4-Trimethylbenzene	Undesirable
28	Z-3-Hexenal	Green	60	4-Methyl-1-penten-3-ol	Ripe fruit
29	1-Penten-3-ol	Undesirable	61	Alcohol C_6 branched	Ripe fruit
30	3-Methylbutyl acetate	Bitter	62	Z-2-hexen-1-ol	Green
31	Heptan-2-one	Ripe fruit	63	2-Octenal	Green
32	E-2-Hexenal	Bitter	64	Propanoic acid	Miscellany 1
			65	Hydrocarbon	Green

The resulting plot is shown in Fig. 8.8. Volatile compounds (circled numbers) were placed inside different sectors according to the sensory perception they elicited. In most cases, the place in the SSW was in agreement with the sensory perception produced by the pure compound, which was determined by GC sniffing as well. When a disagreement between GC sniffing and SSW was detected, the pure compound was tasted at the same concentration as found in the VOO samples.

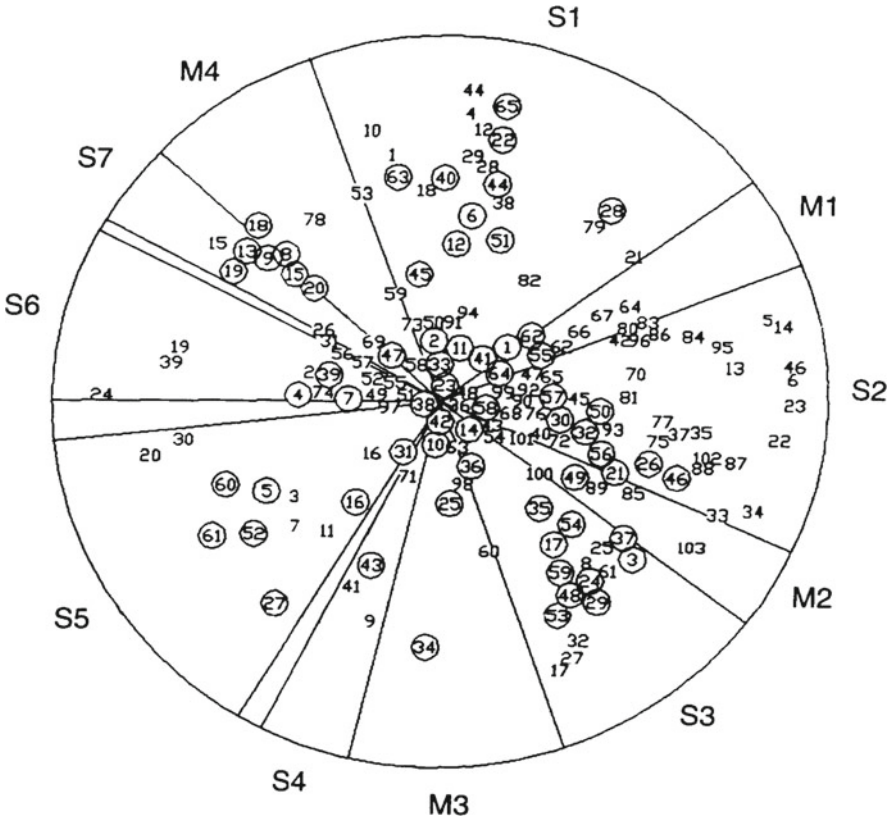

Fig. 8.8 Projection of volatile compounds on statistical sensory wheel. *Circled* numbers correspond to volatile compounds described in Table 8.6 (Aparicio et al. (1996), with permission of Springer)

Z-3-hexen-1-ol, Z-3-hexenal, hexyl acetate, and 3-hexenyl acetate were placed in the green sector close to green sensory attributes, thereby emphasizing their contribution to the green perception. Hexanal and pent-1-en-3-one were placed in the sweet sector. Various volatiles were placed in the undesirable sector, but most of these volatiles are usually present at low concentrations in VOOs.

The bitter-pungent sector can be considered the taste perception sector of VOOs. Few volatiles were located in this sector, and their presence was justified because they also contribute to taste as a result of their astringent, rough, and sharp sensory qualifications. Phenolic compounds, which are elicited from oleuropein glucoside and its aglycons, are also related to bitter and astringent sensory perceptions (Chap. 9); the taste of some aglycons, for example, has been described as having a stinking, biting, or sharp sensation that is associated with bitterness. Figure 8.9 shows the resulting plot of projecting phenolic compounds on the SSW (Aparicio et al. 1996). Aglycons were found to be more closely responsible for bitter and pungent sensory perceptions

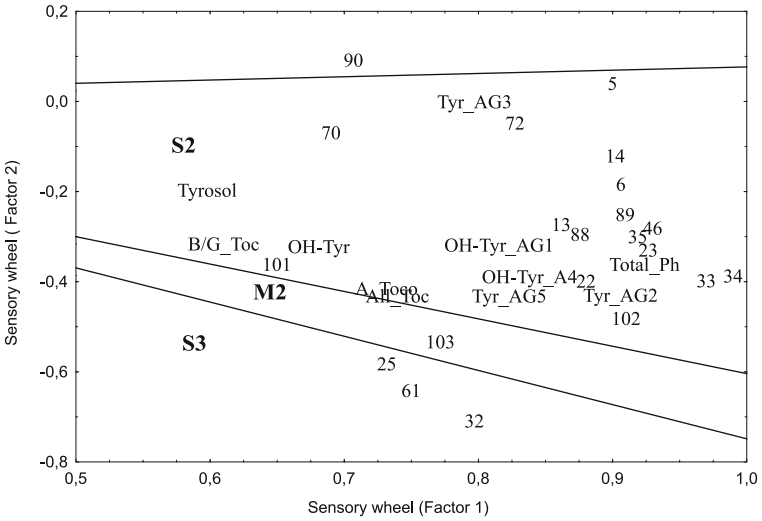

Fig. 8.9 Plot showing relationship between phenolic compounds and sensory attributes (codes according to Table 8.5). Zoom of statistical sensory wheel S2 sector (bitter-pungent)

than tyrosol and OH-tyrosol; in general, aglycons were located very close to most of the bitter and pungent sensory attributes. Total phenols (Total_Ph) are related to bitter-astringent sensory descriptors. A tyrosol aglycon (Tyr_AG3) seems to be characterized by a green-bitter descriptor because it was located in the vicinity of the green sector; α-tocopherol (A_Toc) and total tocopherols (All_Toc) were placed in the boundary with miscellany M2 far from bitter-pungent descriptors.

8.4.2 Sensory Defects of Virgin Olive Oil

VOOs obtained from unsound olives are characterized by undesirable sensory attributes (Chap. 2), the most common being fusty, musty-humidity, muddy sediment, winey-vinegary, rancid, and metallic (IOC 2011; EC 2008). The main reason for the detection of sensory defects is the presence of high concentrations of certain volatile compounds produced by overripening of the fruit, oxidation of the unsaturated fatty acids, or attack by molds and bacteria. This last named reason is usually the case when fruits have been stored for a long period of time prior to oil extraction. Thus, the profile of volatiles in a VOO with sensory defects (Table 8.7) is very different from that of an EVOO. Figure 8.10 shows the profiles of volatiles of an EVOO and four standard oils used in the training process for assessors to detect sensory defects. Each one of the standard oils is characterized by one of the following attributes: rancid, winey-vinegary, musty-humidity, and fusty.

Rancid off-flavor is undoubtedly the most studied sensory defect, and the number and concentration of volatiles depend on the kind and intensity of the alteration

Table 8.7 Main sensory defects produced in each of the processes of olive oil extraction; the defect causes and possible volatile compounds responsible for them

Process	Defect	Cause	Volatiles
Harvesting	Grubby	Infestation by Bactrocera oleae or prays	Ethanol and acids
	Hay-wood	Dried-out olives	
Washing	Muddy earth	Uncleaned ground picked olives	Heptan-2-ol
Olive storing	Fusty winey mustiness	Olives in advanced stage of anaerobic fermentation	Pentanoic, Butanoic and Acetic acids, Butan-2-ol,
		Olives stored in piles for a long time	2-Methyl-butan-1-ol
		Olives with fungi and yeasts	1-Octen-3-ol, 1-Octen-3-one
Crushing	Metallic	Uncleaned crusher in first days crop	Unknown
Mixing	Heated	Excessive temperature ($T \geq 35$ °C) or prolonged heating time ($t > 60$ min)	Aldehydes (Pentanal, Hexanal, Nonanal)
Pressure	Mats ("esparto")	Uncleaned mats or diaphragms	Not described
Decantation	Muddy sediment Vegetable water	Olive oil in contact with wastewater for a long time	Butyrates and 2-ethyl butyrates
Centrifugation	Greasy	Uncleaned horizontal decanters	Not described
Olive oil storage in deposits	Rancid	Inadequate storing conditions	Hexanal, Nonanal & long chain aldehydes and acids
Olive oil storage in bottles	Cucumber	Olive oil hermetically stored in plastic bottles for a long time	2,6-Nonadienal

Source: Angerosa (2002) and Morales et al. (2005)

(Morales et al. 1997). Thus, high concentrations of aldehydes are mostly produced by oxidation of the unsaturated fatty acids, while the presence of acids is due to the oxidation of the aldehydes previously formed; a high concentration of acids indicates a high level of alteration of the oil sample, as these compounds appear at the end of the oxidative process (Morales et al. 2005).

Table 8.8 shows the volatiles that have been selected as markers of different sensory defects together with their odor threshold and the sensory notes qualifying them, with which they contribute to a rancid perception.

The standard olive oil characterized as fusty is the next profile shown in Fig. 8.10. The activity of microorganisms (e.g., the Enterobacteriaceae *Aerobacter* and *Escherichia*, and genera *Pseudomonas*, *Clostridium*, and *Serratia*) found in olives after a long time of storage results in the presence of volatile compounds other than the usual ones, which are responsible for this defect. Table 8.8 also shows the volatile markers for this defect. The concentrations of octane (2.05 mg/kg), ethyl butanoate (3.70 mg/kg), butanoic (11.5 mg/kg) and propanoic (16.69 mg/kg), and 3-methyl-1-butanol (0.48 mg/kg) in the standard of fusty VOOs are much higher than their odor thresholds. The high concentrations of butanoic and propanoic acids in this defective

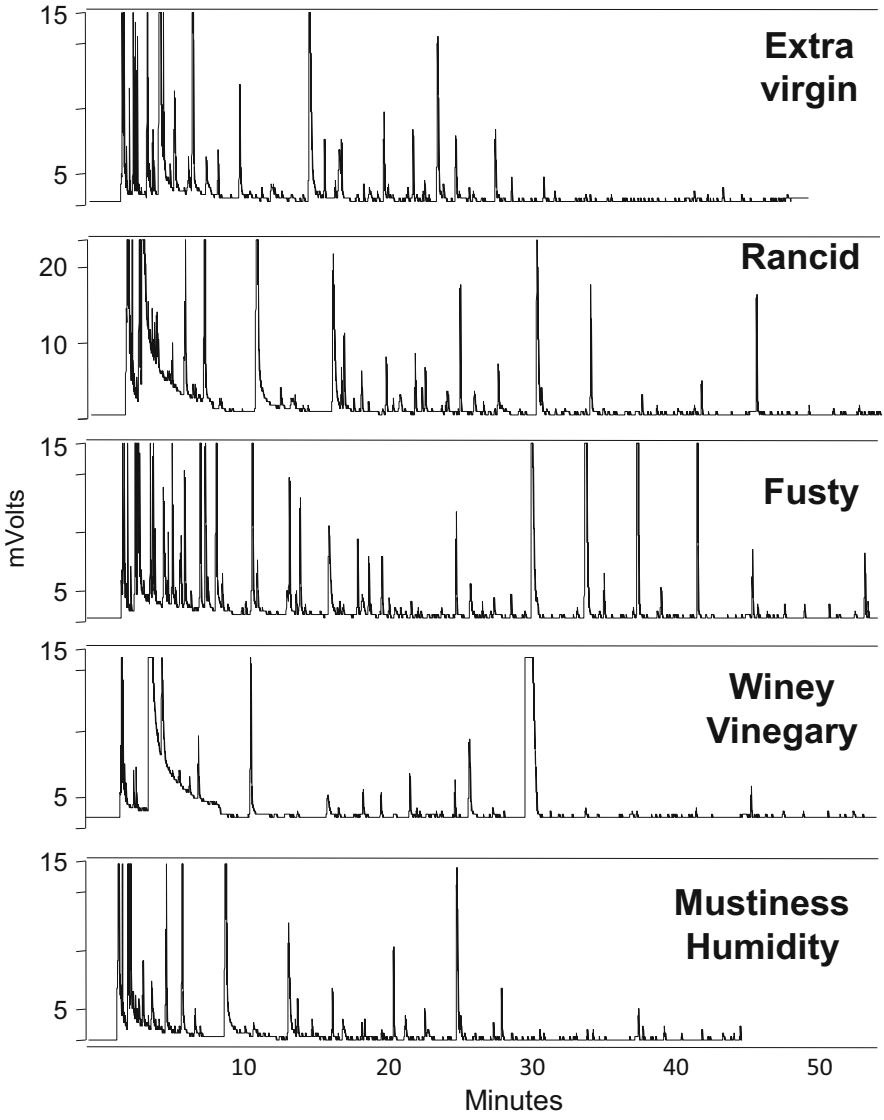

Fig. 8.10 Chromatograms of standard lampante virgin olive oils characterized by different sensory defects

standard oil, in comparison with their trace levels in EVOOs, can also be explained by processes induced by some species of *Clostridium* (Angerosa et al. 1996b).

The main reason a VOO acquires a winey-vinegary attribute is the production of off-flavors associated with a fermentative process due to microbial contamination of the olives. Lactic acid (*Lactobacillus*) and acetic acid bacteria have been detected on olives later used to obtain olive oils (Morales et al. 2005). This kind of microorganism induces a fermentative process in the olives, giving rise to the production of

Table 8.8 Chemical compounds responsible for the main sensory defects detected in virgin olive oils together with their odor thresholds to contribute to sensory defects with their particular sensory notes

Sensory defect	Chemical compound	Odor threshold (mg/kg)	Sensory note
Rancid	Pentanal	0.24	Oily
	Hexanal	0.08	Oily, fatty
	Heptanal	0.50	Oily, fatty
	E-2-Heptenal	0.04	Tallowy, oxidized
	Octanal	0.32	Fatty
	Nonanal	0.15	Waxy, fatty
	E-2-Decenal	0.01	Fishy, fatty
	Hexanoic acid	0.70	Rancid
Fusty	Octane	0.94	Alkane
	Ethyl butanoate	0.03	Fruity
	Butanoic acid	0.65	Fusty
	Propanoic acid	0.72	Sour, moldy
	3-Methyl-1-butanol	0.10	Winey
Winey, vinegary	Acetic acid	0.50	Vinegary, sour
	Ethyl acetate	0.94	Sticky
	3-Methyl-1-butanol	0.10	Whiskey
Musty, humid	1-Octen-3-ol	0.05	Mould, earthy
	1-Octen-3-one	0.01	Mushroom, moldy
	Ethyl acetate	0.94	Sticky
	Heptan-2-ol	0.01	Earthy
	Acetic acid	0.50	Vinegary, sour
	E-2-Heptenal	0.04	Tallowy, oxidized
	Propanoic acid	0.72	Sour, moldy
Muddy sediment	Heptan-2-ol	0.01	Earthy
	6-Methyl-5-hepten-2-one	1.00	Oily
	1-Pentene-3-one	0.04	Mustard
	Butan-2-ol	0.15	Winey
Metallic	1,5-Octadien-3-one	0.0005	Metallic, geranium
Burned, heated	Pentanal	0.24	Oily
	Hexanal	0.08	Oily, fatty
	Nonanal	0.15	Waxy, fatty
	E-2-Hexen-1-ol	5.00	Lawn
	Hexanol	0.40	Astringent
Bottled	2,6-Nonadienal	0.09	Cucumber

ethanol, ethyl acetate, and acetic acid, which are the principal volatiles responsible for the described sensory note (Fig. 8.10). Table 8.8 shows the thresholds of these volatile compounds, whose concentrations in the standard of this defect are notably higher: 12.4 mg/kg for acetic acid, 3.76 mg/kg for ethyl acetate, and 7.10 mg/kg for 3-methyl butan-1-ol.

The presence of several species of genus *Aspergillus*, together with ascomycetes, *Penicillium notatum*, and other fungi (*Alternaria, Fusarium, Rhizopus*), has been reported in olive fruits stored in piles under high-humidity conditions (Morales et al. 2005). These microorganisms have the ability to oxidize free fatty acids, producing

volatile compounds such as methyl ketones (2-heptanone, 2-nonanone) and other compounds responsible for the sensory note mustiness-humidity (Table 8.8). Other sensory defects that are usually detected in lampante VOOs (muddy sediment, burned/heated, metallic, and bottled) are also explained by volatile markers in Table 8.8.

Generally, EVOOs and VOOs have a low content of total volatile compounds, as do VOOs characterized by the mustiness-humidity sensory note, although their profiles are different enough. Winey-vinegary and fusty defect oils have higher contents of total volatile compounds, these being approximately twofold in the case of winey-vinegary defect and threefold in the case of the fusty defect with respect to EVOOs. Rancidity is the sensory defect that corresponds to the highest content of volatile compounds depending on the degree of alteration. A total concentration of approximately eightfold higher than EVOOs has been described (Morales et al. 2005).

8.5 Interface Between Volatiles and Sensory Descriptors

Flavor scientists have used increasingly sophisticated instrumental techniques to identify the volatile compounds responsible for VOO aroma, while sensory scientists have assessed the same VOO samples by means of panels of trained assessors. Today, however, there is still a frustrating black box at the interface between results of these two sciences: aroma input (volatiles) and output (descriptors reported by assessors). Several reasons explain the gap between instrumental analysis and sensory assessment.

The first reason is the high complexity of VOO volatiles, which constitutes a resolution challenge for the currently applied chromatographic techniques. The second reason is the kinetic component of the flavor release occurring during eating and swallowing and influenced by many factors such as saliva composition; odors are perceived through the nose (orthonasally) but also through the mouth (retronasally). Finally, little is known about the physiological mechanisms by which aroma compounds result in neural activation, and they ultimately cause a sensory perception. As soon as a subject has smelled the oil, the signals from olfactory and somatosensory receptors travel, via nerve afferents, to primary, secondary, and higher-order brain areas responsible for emotional responses to aroma experienced; electrical signals generated in the olfactory epithelium are transmitted by the cranial nerve I (olfactory nerve) to the olfactory bulb, which projects directly to the primary olfactory cortex without passing through the thalamus.

The application of medical techniques such as positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) to the field of food science has opened a new research field in flavor chemistry that complements chemical/sensory studies on food quality. Thus, fMRI, using the blood-oxygenation-level-dependent (BOLD) contrast, is noninvasive technique widely used for mapping brain activity. In fact, fMRI measures brain activity indirectly by detecting changes in cerebral hemodynamics that are associated with neural activation.

Data from fMRI experiments have been analyzed assuming the hypothesis of greater activity during a cognitive process compared to the rest state (García-González et al. 2011). Thus, the stimulus is sequentially presented to a subject

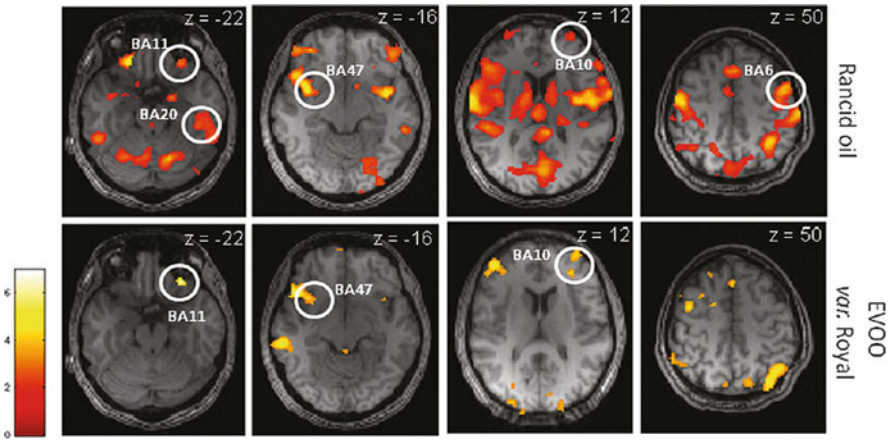

Fig. 8.11 Axial activations ($p<0.05$) in response to aromas of an extra virgin olive oil *var.* Royal (EVOO) and a rancid virgin olive oil. Note: *BA*, brodmann area (numbers 6, 10, 11, 20, and 47 are marked with circles). The third Talairach coordinate (z) is shown on each image (García-González et al. (2011), with permission of American Chemical Society)

alternating with rest periods in a block design with an ON/OFF frame. In addition to assuming a lower brain activity in rest periods, another important assumption is that the timing at which the neural responses are registered matches the time specified in the paradigm designed to present the samples to subjects.

García-González et al. (2011) assumed that it was possible to find differences in the brain activity during the smelling of pleasant versus unpleasant VOOs. Figure 8.11 shows the activity of some neuronal areas (Broadman areas) when subjects smelled standard lampante VOOs characterized by a rancid perception (Median=2,9, according to IOC) in comparison with the activity of those neuronal areas when the same subjects smelled an EVOO (var. Royal) (García-González et al. 2011).

The information resulting from this research is going to help in verifying the volatile compounds responsible for sensory defects according to physiological evidence in addition to sensory fundamentals. Furthermore, the procedure allows for an understanding of consumer preferences for certain varieties of VOOs from either aroma or taste perceptions.

8.6 Future Trends

The cherished VOO aroma is due to the presence of numerous volatile compounds, whereas the concentration of phenols is responsible for attributes related to taste (e.g., bitterness and pungency). Therefore, an objective measurement of VOO sensory quality should be based on the quantification of these compounds. The official method, however, is based on sensory assessment by trained assessors, the so-called panel test.

Any proposal to replace panel tests with an analytical procedure presently has two challenges. The first is the determination of the chemical compounds (volatiles and phenols) responsible for VOO flavor from the large set of currently identified compounds. The second challenge is to explain those important VOO sensory attributes that are described with considerable vagueness as fruity and green. These challenges must be addressed with a combined action of new analytical techniques and statistical procedures. However, some VOO sensory descriptors have not yet been satisfactorily explained due to the difficulty in interpreting the impact of volatile compounds on VOO aroma. The analytical techniques, for their part, are still needed for perfecting chromatographic resolution, computing the recovery percentage of compounds, and determining the odor thresholds. More powerful separation procedures, such as two-dimensional GC (GC×GC), will make it possible to achieve better separation of volatile compounds that cannot be well resolved nowadays.

The physiological processes implied in the olfaction and taste perceptions, as a result of chemical stimuli, have not yet been explored and are part of the black box associated with sensory assessment and consumers' decisions. New approaches are being developed to measure the hemodynamic response of the brain during smelling and tasting by fMRI. The application of this technique could open a new area of research that might lead to a full understanding of sensory perceptions and consumers' decisions.

In learning about sensory processes, another challenge is the elucidation of how the flavor compounds of VOO are distributed in the mucosa/saliva and in the air phase during consumption. The impact of the protein composition of saliva on flavor release is also poorly understood, and it may have a significant effect on the kind and intensity of perception. Research in this regard would allow us to understand what fraction of volatile compounds is available in the air phase and can reach olfactory receptors by means of retronasal delivery.

New researches based on chemical, sensory, and physiological approaches would make sensory assessment dispensable to some extent, and it would give coherence and unity to the sensory information, thereby avoiding the current confusion and fuzziness that affect the sensory assessment of VOOs.

References

Acree TE, Barnard J, Cunningham DF (1984) A procedure for the sensory analysis of gas chromatographic effluents. Food Chem 14:273–286

Acree TE (1993) Gas chromatography-olfactometry. In: Ho CT, Manley CH (eds) Flavor Measurement. New York: Marcel Dekker, p 77

Almarcha M, Rovira J (1994) Microwave-guide thermal desorption in food analysis. Tec Lab 16:699–703

Angerosa F (2002) Influence of volatile compounds on virgin olive oil quality evaluated by analytical approaches and sensor panels. Eur J Lipid Sci Technol 104:639–660

Angerosa F, Basti C (2001) Olive oil volatile compounds from the lipoxygenase pathway in relation to fruit ripeness. Ital J Food Sci 13:421–428

Angerosa F, Di Giacinto L, Vito R, Cumitini S (1996a) Sensory evaluation of virgin olive oils by artificial neural network processing of dynamic head-space gas chromatographic data. J Sci Food Agric 72:323–328

Angerosa F, Lanza B, Marsilio V (1996b) Biogenesis of "fusty"defect in virgin olive oils. Grasas Aceites 47:142–150

Angerosa F, Di Giacinto L, d'Alessandro N (1997) Quantitation of some flavor components responsible for the "green" attributes in virgin olive oils. J High Resol Chromatogr 20:507–511

Angerosa F, Camera L, d'Alessandro N, Mellerio G (1998) Characterization of seven new hydrocarbon compounds present in the aroma of virgin olive oils. J Agric Food Chem 46:648–653

Angerosa F, Lanza B, Marsilio V, Cumitini S (1999) Olive oil off odour compounds produced by *Aspergillus* and *Penicillium*. Acta Hortic 474:695–699

Angerosa F, Servili M, Selvaggini R, Taticchi A, Esposto S, Montedoro GF (2004) Volatile compounds in virgin olive oil: occurrence and their relationship with the quality. J Chromatogr A 1054:17–31

Aparicio R, Luna G (2002) Characterisation of monovarietal virgin olive oils. Eur Lipid Sci Technol 10:614–627

Aparicio R, Morales MT (1994) Optimization of a dynamic headspace technique for quantifying virgin olive oil volatiles. Relationships between sensory attributes and volatile peaks. Food Qual Pref 5:109–114

Aparicio R, Morales MT (1995) Sensory wheels: a statistical technique for comparing QDA panels. Application to virgin olive oil. J Sci Food Agric 67:247–257

Aparicio R, Morales MT (1998) Characterization of olive ripeness by green aroma compounds of virgin olive oil. J Agric Food Chem 46:1116–1122

Aparicio R, Alonso MV, Morales MT, Calvente JJ (1994a) Relationship between the COI test and other sensory profiles by statistical procedures. Grasas Aceites 45:26–41

Aparicio R, Alonso MV, Morales MT (1994b) Detailed and exhaustive study of the authentication of European virgin olive oils by SEXIA expert system. Grasas Aceites 45:241–252

Aparicio R, Calvente JJ, Alonso MV, Morales MT (1994c) Good control practices underlined by an on-line fuzzy control database. Grasas Aceites 45:75–81

Aparicio R, Morales MT, Alonso MV (1996) Relationship between volatile compounds and sensory attributes of olive oils by sensory wheel. J Am Oil Chem Soc 73:1253–1264

Aparicio R, Morales MT, Alonso V (1997) Authentication of European virgin olive oils by their chemical compounds, sensory attributes, and consumers' attitudes. J Agric Food Chem 45:1076–1083

Aparicio R, Morales MT, Luna G, Aparicio-Ruiz R (2000a) Biochemistry and chemistry of volatile compounds affecting to consumers' attitudes of virgin olive oil. In: Lanzotti V, Taglialatela-Scafati O (eds) Flavour and fragrance chemistry. Kluwer Academic, Dordrecht, pp 3–14

Aparicio R, Rocha SM, Delgadillo I, Morales MT (2000b) Detection of rancid defect in virgin olive oil by the electronic nose. J Agric Food Chem 48:853–860

Benincasa C, De Nino A, Lombardo N, Perri E, Sindona G, Tagarelli A (2003) Assay of aroma active components of virgin olive oils from southern Italian regions by SPME-GC/ion trap mass spectrometry. J Agric Food Chem 51:733–741

Bicchi C, Cordero C, Liberto E, Rubiolo P, Sgorbini B, David F, Sandra P (2005) Dual-phase twisters: a new approach to headspace sorptive extraction and stir bar sorptive extraction. J Chromatogr A 1094:9–16

Blank I (2002) Gas chromatography – olfactometry in food aroma analysis. In: Marsili R (ed) Flavor, fragrance, and odor analysis. Marcel Dekker, New York, pp 297–332

Bocci F, Frega N, Lercker G (1992) Preliminary research on the volatile component of extra virgin olive oil. Riv Ital Sostanze Grasse 69:611–613

Cavalli JF, Fernandez X, Lizzani-Cuvelier L, Loiseau AM (2003) Comparison of static headspace, headspace solid phase microextraction, headspace sorptive extraction, and direct thermal desorption techniques on chemical composition of French olive oils. J Agric Food Chem 51:7709–7716

David F, Sandra P (2007) Stir bar sorptive extraction for trace analysis. J Chromatogr A 1152:54–69
Dudareva N, Pichersky E, Jonathan GJ (2004) Biochemistry of plant volatiles. Plant Physiol 135:1893–1902
Dupuy HP, Fore SP, Goldblatt LA (1971) Elution and analysis of volatiles in vegetable oils by gas chromatography. J Am Oil Chem Soc 48:876
EC (1991) Official Journal of the Commission of the European Communities, regulation no. 2568/91, L248, Sept 5th
EC (2008) Official Journal of the Commission of the European Communities, regulation no. 640/2008, L178/11, July 4th
EC (2011) Official Journal of the Commission of the European Communities. Regulation no 61/2011, L 23/1, Jan 24th
Escuderos ME (2011) Olive oil aroma evaluation by gas chromatographic method: a critical review. Crit Rev Anal Chem 41:70–80
Escuderos ME, Uceda M, Sánchez S, Jiménez A (2007) Instrumental technique evolution for olive oil sensory analysis. Eur J Lipid Sci Technol 109:536–546
Fedeli E (1977) Caratteristiche organolettiche dell'olio di oliva. Riv Ital Sost Grasse 54:202–205
Feussner I, Wasternack C (2002) The lipoxygenase pathway. Annu Rev Plant Biol 53:275–297
Flath RA, Forrey RR, Guadagni DG (1973) Aroma components of olive oil. J Agric Food Chem 21:948–952
García González DL, Tena N, Aparicio-Ruiz R, Morales MT (2008) Relationship between sensory attributes and volatile compounds qualifying dry-cured hams. Meat Sci 80:315–325
García-González DL, Aparicio R (2002a) Sensors: from biosensors to the electronic nose. Grasas Aceites 53:96–114
García-González DL, Aparicio R (2002b) Detection of defective virgin olive oils by metal-oxide sensors. Eur Food Res Technol 215:118–123
García-González DL, Aparicio R (2002c) Detection of vinegary defect in virgin olive oils by metal oxide sensors. J Agric Food Chem 50:1809–1814
García-González DL, Aparicio R (2003) Virgin olive oil quality classification combining neural network and MOS sensors. J Agric Food Chem 51:3515–3519
García-González DL, Aparicio R (2004) Classification of different quality virgin olive oils by metal-oxide sensors. Eur Food Res Technol 218:484–487
García-González DL, Aparicio R (2010) Coupling MOS sensors and gas chromatography to interpret the sensor responses to complex food aroma: application to virgin olive oil. Food Chem 120:572–579
García-González DL, Barie N, Rapp M, Aparicio R (2004) Analysis of virgin olive oil volatiles by a novel electronic nose based on a miniaturized SAW sensor array coupled with SPME enhanced headspace enrichment. J Agric Food Chem 52:7475–7479
García-González DL, Barie N, Rapp M, Aparicio R (2006) A fuzzy filter to study the selectivity and sensitivity of a SPME enhanced SAW sensor system characterizing virgin olive oil aroma. Sens Act B 116:49–54
García-González DL, Romero N, Aparicio R (2010a) Comparative study of olive oil quality from single varieties cultivated in Chile and Spain. J Agric Food Chem 58:12899–12905
García-González DL, Tena N, Aparicio R (2010b) Quality characterization of the new virgin olive oil var. Sikitita by phenols and volatile compounds. J Agric Food Chem 58:8357–8364
García-González DL, Vivancos J, Aparicio R (2011) Mapping brain activity induced by olfaction of virgin olive oil aroma. J Agric Food Chem 59:10200–10210
Gasparoli A, Fedeli E (1987) Valutazione dei componenti volatili negli oli alimentari: un approccio alla tecnica "Purgue and Trap". Riv Ital Sost Grasse 64:453–460
Genovese A, Piombino P, Gambuti A, Moio L (2009) Simulation of retronasal aroma of white and red wine in a model mouth system. Investigating the influence of saliva on volatile compound concentrations. Food Chem 114:100–107
Grosch W (1993) Detection of potent odorants in foods by aroma extract dilution analysis. Trends Food Sci Technol 4:68–73

Grosch W (1994) Review: determination of potent odorants in foods by aroma extract dilution analysis (AEDA) and calculation of odour activity values (OAVs). Flavour Fragr J 9:147–158
Grosch W (2001) Evaluation of the key odorants of foods by dilution experiments, aroma models and omission. Chem Senses 26:533–545
Guth H, Grosch W (1989) 3-Methylnonane-2,4-dione – an intense odour compound formed during flavour reversion of soya-bean oil. Fat Sci Technol 91:225–230
Guth H, Grosch W (1991) A comparative study of the potent odorants of different virgin olive oils. Fat Sci Technol 93:335–339
Guth H, Grosch W (1993) Identification of potent odorants in static headspace samples of green and black tea powders on the basis of aroma extract dilution analysis (AEDA). Flavour Frag J 8:173–178
Hoffmann A, Heiden A (2000) Flavor profiling of different olive oils with rancidity-monitoring by thermal extraction GC/MS. Gerstel Appl. Note 9, pp 1–10
IOC (2011) Sensory analysis of olive oil. Method for the organoleptic assessment of virgin olive oil. COI/T.20/Doc. nº 15/Rev.4, International Olive Oil Council, Madrid, Nov 2011
Kalua CM, Bedgood DR, Bishop AG, Prenzler PD (2004) Monitoring changes in sensory quality of olive oil using SPME-GC. The 12th Annual RACI Analytical and Environmental Division Research and Development Topics, Melbourne
Kalua CM, Allen MS, Bedgood DR Jr, Bishop AG, Prenzler PD, Robards K (2007) Olive oil volatile compounds, flavour development and quality: a critical review. Food Chem 100:273–286
Kanavouras A, Kiritsakis A, Hernandez RJ (2005) Comparative study on volatile analysis of extra virgin olive oil by dynamic headspace and solid phase micro-extraction. Food Chem 90:69–79
Leffingwell JC (2001) Olfaction II. In: Leffingwell reports, vol 1 (no. 4), Sept 2001 pp 1–26
Livermore A, Laing DG (1998) The influence of odor type on the discrimination and identification of odorants in multicomponent odor mixtures. Physiol Behav 65:311–320
López-Feria S, Cárdenas S, García-Mesa JA, Fernández-Hernández A, Valcárcel M (2007) Quantification of the intensity of virgin olive oil sensory attributes by direct coupling headspace-mass spectrometry and multivariate calibration techniques. J Chromatogr A 1147:144–152
Luna G, Morales MT, Aparicio R (2006a) Characterisation of 39 varietal virgin olive oils by their volatile compositions. Food Chem 98:243–252
Luna G, Morales MT, Aparicio R (2006b) Changes induced by UV radiation during virgin olive oil storage. J Agric Food Chem 54:4790–4794
Manura JJ, Overton SV (1999) Comparison of sensitivity of headspace GC, purge and trap thermal desorption and direct thermal extraction techniques for volatile organics. SIS Appl. Note 39
Michel M, Buszewski B (2009) Porous graphitic carbon sorbents in biomedical and environmental applications. Adsorption 15:193–202
Miranda-Lopez R, Libbey LM, Watson BT, McDaniel MR (1992) Odor analysis of Pinot noir wines from grapes of different maturities by a gas chromatography-olfactometry technique (Osme). J Food Sci 57:985–993
Montedoro G, Bertuccioli M, Anichini F (1978) Aroma analysis of virgin olive oil by head space (volatiles) and extraction (polyphenols) techniques. In: Charalambous G, Inglett GE (eds) Flavor of foods and beverages. Academic Press, New York, pp 247–281
Morales MT, Aparicio R (1993) Optimization by mathematical procedures of two dynamic headspace techniques for quantifying virgin olive oil volatiles. Anal Chim Acta 282:423–431
Morales MT, Aparicio R (1999) Effect of extraction conditions on sensory quality of virgin olive oil. J Am Oil Chem Soc 76:295–300
Morales MT, Tsimidou M (2000) The role of volatile compounds and polyphenols in olive oil sensory quality. In: Harwood JL, Aparicio R (eds) Handbook of olive oil: analysis and properties. Aspen Publishers, Gaithersburg, pp 393–458
Morales MT, Aparicio R, Gutiérrez F (1992) Técnicas de aislamiento y concentración de volátiles de aceites vegetales. Grasas Aceites 43:164–173
Morales MT, Aparicio R, Rios JJ (1994) Dynamic headspace gas chromatographic method for determining volátiles in virgin olive oil. J Chromatogr A 668:455–462

Morales MT, Alonso MV, Rios JJ, Aparicio R (1995) Virgin olive oil aroma: relationship between volatile compounds and sensory attributes by chemometrics. J Agric Food Chem 43:2925–2931
Morales MT, Calvente JJ, Aparicio R (1996) Influence of olive ripeness on the concentration of green aroma compounds in virgin olive oil. Flavour Fragr J 11:171–178
Morales MT, Rios JJ, Aparicio R (1997) Changes in the volatile composition of virgin olive oil during oxidation: flavours and off-flavors. J Agric Food Chem 45:2666–2673
Morales MT, Berry AJ, McIntyre PS, Aparicio R (1998) Tentative analysis of virgin olive oil aroma by SFE-HRGC-MS. J Chromatogr A 819:267–275
Morales MT, Angerosa F, Aparicio R (1999) Effect of the extraction conditions of virgin olive oil on the lipoxygenase cascade: chemical and sensory implications. Grasas Aceites 50:114–121
Morales MT, Luna G, Aparicio R (2005) Comparative study of virgin olive oil sensory defects. Food Chem 91:293–301
Morales MT, García González DL, Aparicio R (2012) The role of the volatile compounds as traceability markers of virgin olive oil. Grasas Aceites 63:1–20
Noble AC, Arnold RA, Buechsenstein J, Leach EJ, Schmidt JO, Stern PM (1987) Modification of a standardized system of wine aroma terminology. Am J Enol Vitic 38:143–146
Nuñez AJ, Gonzalez LF, Janak J (1984) Pre-concentration of headspace volatiles for trace organic analysis by gas chromatography. J Chromatogr 300:127–162
Olías JM, Gutiérrez F, Dobarganes MC, Gutiérrez RR (1980) Componentes volátiles en el aroma del aceite de oliva. IV. Su evolución e influencia en el aroma durante el proceso de maduración de los frutos en las variedades picual y hojiblanca. Grasas Aceites 31:391–402
Overton SV, Manura JJ (1999) Comparison of cooking oils by direct thermal extraction and purge and trap GC/MS. SIS Appl. Note 30a
Prasanna V, Prabha TN, Tharanathan RN (2007) Fruit ripening phenomena–an overview. Crit Rev Food Sci Nutr 47:1–19
Raghavan SK, Reeder SK, Khayat A (1989) Rapid analysis of vegetable oil flavor quality by dynamic headspace capillary gas chromatography. J Am Oil Chem Soc 66:942–947
Ranalli A, Contento S, Schiavone C, Simone N (2001) Malaxing temperature affects volatile and phenol composition as well as other analytical features of virgin olive oil. Eur J Lipid Sci Technol 103:228–238
Reiners J, Grosch W (1998) Odorants of virgin olive oils with different flavor profiles. J Agric Food Chem 46:2754–2763
Rothe M, Thomas B (1963) Aroma of bread. Evaluation of chemical taste analyses with the aid of threshold value. Z Lebensm Unters Forsch 119:302–310
Salas JJ (1999) Ruta de la lipoxigenasa en aceituna: contribución a la biogénesis del aroma del aceite de oliva. Ph.D. thesis. University of Seville, Seville
Salas JJ, Sánchez J (1998) Alcohol dehydrogenases from olive (*Olea europaea*) fruit. Phytochem 48:35–40
Salas JJ, Sánchez J (1999) Hydroperoxide lyase from olive (*Olea europaea*) fruit. Plant Sci 143:19–26
Salas JJ, Williams M, Harwood JL, Sanchez J (1999) Lipoxygenase activity in olive (*Olea europaea*) fruit. J Am Oil Chem Soc 76:1163–1168
Salas JJ, Sánchez-Peña C, Aparicio R (2005) Impact of the suppression of lipoxygenase and hydroperoxyde lyase on the quality of the green odor in green leaves. J Agric Food Chem 53:1648–1655
Salas JJ, García-González DL, Aparicio R (2007) Volatile compound biosynthesis by green leaves from an *Arabidopsis thaliana* hydroperoxide lyase knockout mutant. J Agric Food Chem 55:8199–8205
Salch YP, Grove MJ, Takamura H, Gardner HW (1995) Characterization of a C-5,13-cleaving enzyme of 13(S)-hydroperoxide of linolenic acid soybean seed. Plant Physiol 108:1211–1218
Schwab W, Davidovich-Rikanati R, Lewinsohn E (2008) Biosynthesis of plant-derived flavor compounds. Plant J 54:712–732
Servili M, Conner JM, Piggott JR, Withers SJ, Paterson A (1995) Sensory characterisation of virgin olive oil and relationship with headspace composition. J Sci Food Agric 67:61–70

Snyder JM, Frankel EN, Selke E (1985) Capillary gas chromatographic analyses of headspace volatiles from vegetable oils. J Am Oil Chem Soc 62:1675–1679

Stella R, Barisci JN, Serra G, Wallace GG, De Rossi D (2000) Characterisation of olive oil by an electronic nose based on conducting polymer sensors. Sens Act B 63:1–9

Tena N, Lazzez A, Aparicio-Ruiz R, García-González DL (2007) Volatile compounds characterizing Tunisian Chemlali and Chétoui virgin olive oils. J Agric Food Chem 55:7852–7858

Tienpont B, David F, Bicchi C, Sandra P (2000) High capacity headspace sorptive extraction. J Microcol Sep 12:577–584

Ullrich F, Grosch W (1988) Flavour deterioration of soya-bean oil: identification of intense odour compounds formed during flavour reversion. Fat Sci Technol 90:332–336

Van der Hijden HTWM, Bom IJ (1996) Enzymes involved in the methabolic pathway leading to 3-methylbutanal in tomato fruit. In: Mottram DS, Taylor AJ (eds) Flavor science. Recent developments. The Royal Society of Chemistry, Cambridge, pp 130–133

Werkhoff P, Brennecke S, Bretschneider W, Bertram HJ (2002) Modern methods for isolating and quantifying volatile flavor and fragrance compounds. In: Marsili R (ed) Flavor, fragrance, and odor analysis, 2nd edn. Marcel Dekker, New York, pp 139–204

Wyllie SG, Leach DN, Nonhebel HN, Lusunzi I (1996) Biochemical pathways for the formation of esters in ripening fruit. In: Mottram DS, Taylor AJ (eds) Flavor science. Recent developments. The Royal Society of Chemistry, Cambridge, pp 130–133

Yeshurun Y, Sobel N (2010) An odor is notworth a thousand words: from multidimensional odors to unidimensional odor objects. Annu Rev Psychol 61:219–241

Zampolli S, Elmi I, Stürmann J, Nicoletti S, Dori L, Cardinali GC (2005) Selectivity enhancement of metal oxide gas sensor using a micromachined gas chromatographic column. Sens Act B 105:400–406

Chapter 9
Analytical Methodologies: Phenolic Compounds Related to Olive Oil Taste Issues

Maria Z. Tsimidou

Contents

9.1 Introduction

The International Olive Council took the initiative in the 1970s of including sensory assessment to assist standardization of the market for virgin olive oil (VOO). In fact, the overall acceptance of VOO has always been related to flavor in all consumer studies, although for some authors (Monteleone et al. 1997) taste outweighs odor. Bitterness, pungency, and astringency are the main VOO taste attributes, and they are related to the presence of secondary metabolites, which contain at least one phenolic moiety, conjugated or not. These phenolic compounds present in the oil originate from those encountered in the fruit, although their chromatographic profiles show differences not only quantitative but qualitative, too, as discussed in the next two sections.

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties*,
DOI 10.1007/978-1-4614-7777-8_9, © Springer Science+Business Media New York 2013

The phenolic composition is not exclusively circumscribed to explain the taste sensory descriptors since certain volatile phenolic compounds (quaiacol, phenol, *o*-cresol, *p*-cresol, *m*-cresol, 4-ethylguaiacol, 4-ethylphenol, 4-vinylguaiacol, 4-vinyl phenol) have tentatively been associated with fusty and musty undesirable odors (Vichi et al. 2008), which result from unsuitable practices during olive oil production (See Chaps. 3, 8 and 14).

The systematic training of panelists for VOO sensory assessment is not always rigorous enough so that its scores often become an issue for disagreements between interested parties. The casual link between the concentration of phenols and the detection of sensory descriptors, however, has not produced yet an objective procedure, based on analytical techniques, for VOO sensory evaluation despite numerous analytical procedures developed recently.

Furthermore, study of the contribution of the phenolic profile to the aesthetics and quality of olive products and the use of olive leaves in phytomedicines are still goals in the area of olive and olive oil research.

9.2 Phenolic Compounds of Olive Fruit

Generally speaking, phenol is a term that defines substances that possess a benzene ring bearing one or more hydroxy groups, including functional derivatives, and can be divided into nine groups (Harborne 1989): (1) phenols, phenolic acids, phenylacetic acids; (2) lignans; (3) lignins; (4) flavonoids; (5) cinnamic acids, coumarins, isocoumarins, and chromones; (6) tannins; (7) benzophenones, xanthones, and stilbenes; (8) quinones; and (9) betacyanins.

Olea europaea L., however, contains a number of unusual phenolics including various oleosides that derive from the shikimate pathway and phenylpropanoid metabolism. The amounts and types of phenolics vary markedly between olive leaf and fruit, although the latter accumulates mainly glucosidic forms of flavonoids and secoiridoids that are found in all of its parts (epicarp, mesocarp, and seed) (Damtoft et al. 1993) and simple phenols (though they are encountered to a lesser extent).

Within *Olea europea* fruits, the main phenolic components are (1) oleuropein, an heterosidic ester of elenolic acid with 3,4-dihydroxyphenethyl alcohol (hydroxytyrosol, HYTY); (2) demethyl oleuropein, the acid derivative of oleuropein; (3) ligstroside, a heterosidic ester of elenolic acid with 4-hydroxyphenethyl alcohol (tyrosol, TY); and (4) verbascoside, an heterosidic ester of caffeic acid with hydroxytyrosol.

The oleuropein compound has attracted the interest of researchers for many decades since its presence and content are clearly related to the ripening stage of the drupe. Furthermore, oleuropein contributes to the bitter sensory perception and its concentration depends heavily on cultivar as well (Amiot et al. 1986, 1989). Recent analytical innovations, and the extended application of liquid chromatography-mass spectrometry (LC-MS) techniques, have shown the presence not only of oleuropein but also of compounds biosynthetically related to it such as the precursors oleoside-11 methyl ester, its glucoside, ligstroside, as well as a variety of catabolites formed

due to the enzymatic activity of esterase (oleoside-11 methyl ester) and β-glycosidase (elenolic acid and aldehydic forms) (Gutiérrez-Rosales et al. 2010). Ligstroside, for its part, is present in young green olive fruits, although its concentration decreases as the fruit develops (Bianchi 2003), and only traces are detected in black olive fruits (Romero et al. 2002a).

The presence of verbascoside in the olive tree leaves – it has been detected in olive oil at trace level – and of nützenide in the seed is currently acknowledged in a majority of publications. Less information is available, however, about other phenolic constituents, e.g., flavones (luteolin-7-glucoside, apigenin-7-glucoside, luteolin-5-glucoside), flavonols (e.g., quercetin-3-rutinoside), or other components such as anthocyanins in black olives.

Centered on the evolution of phenols with olive ripeness, and the interest in knowing the so-called technological optimum for phenol content for determining the olive harvest time, most of the published information compares the concentration of individual phenols in green versus black olives (Amiot et al. 1986; Bianchi 2003; Bianco et al. 2001; Cardoso et al. 2005; Esti et al. 1998; Gomez-Rico et al. 2008; Jemai et al. 2009; Morello et al. 2004; Romero et al. 2002a, b; Ryan et al. 1999a, 2003). Thus, authors have detected that oleuropein and its aglycone increase their concentrations from green to black olives by around 30 % and 40 %, the concentration of demethyloleuropein increases up to 75 %, free hydroxytyrosol – another major polyphenol in olive fruits – increases its concentration by 20 % during olive ripeness, while tyrosol doubles its concentration in black olives. The contents of hydroxytyrosol elenolate and 3,4-DHPEA-EA (a derivative of oleuropein aglycone) are, however, almost halved in black olives. Phenolic acids have also been quantified in green and black olives. Thus, the concentration of sinapic acid is 75 % lower in black olives, the contents of *m*-coumaric and syringic acids respectively have 2.0 and 4.5 times higher concentrations in black olives, while the content of *o*-coumaric acid is 20 times lower in black olives. Other phenolic acids quantified in olive fruits are ferulic, caffeic, protocatechuic, p-coumaric, 4-hydroxybenzoic, p-hydroxyphenyl propanoic, 4-hydroxyphenylacetic, and 3-methoxy-4-hydroxyphenylacetic acids. Concerning black olives, it is well known that they contain anthocyanins like cyanidins (there are seven times more cyanidin 3-O-rutinoside than cyanidin 3-O-glucoside), quercetins (the content of quercetin 3-O-rutinoside is ten times higher than that of quercetin 3-O-rhamnoside), and flavonones (luteolin 7-O-glucoside being the most important, although luteolin, luteolin 6-C-glucoside, apigenin 7-O-glucoside, and apigenin 7-O-rutinoside have also been detected).

The changes in concentrations of individual compounds not only are influenced by the olive ripeness, which lasts several months, but also depend on cultivar, water availability, temperature, and farming practices (Morello et al. 2004). Thus, the content of tyrosol increases in water-deficit-stressed olives while, in contrast, the concentrations of vanillic acid and vanillin increase in irrigated olive trees (Gomez-Rico et al. 2006; Marsilio et al. 2006; Romero et al. 2002b).

The determination of phenols in olive fruits is dependent critically on the extraction procedure. The effect of both acid and base treatment to liberate simple phenols from complex forms, for example, was investigated by Ryan et al. (2001), who pointed out

the importance of this step of analysis, which is rather overlooked, whereas the "emphasis is being placed upon the highly sophisticated instrumental end of the analysis". The four selected methods described below summarize many analytical procedures described in the literature in the past 25 years.

Amiot et al. (1986) used a homogenized lyophilized powder (1 g) in 80 % ethanol in the presence of metabisulphite (2 %). After agitation at 4 °C for 20 min, followed by filtering, the residue is treated in the same way. Aqueous-alcohol extracts are collected, and ethanol is evaporated under vacuum. Pigments and lipids are removed with four successive petroleum-ether extractions. The phenolic compounds are then extracted by ethyl acetate (×3) in the presence of ammonium sulfate (20 %), metaphosphoric acid (2 %), and methanol (20 %). The ethyl acetate is eliminated, and the dry residue is dissolved in appropriate solvent for further analysis.

Servili et al. (1999) suggest that olives be peeled and destoned. Then the peel, pulp, and stone are placed in liquid nitrogen and freeze-dried to inhibit enzymatic activities. After freeze-drying, the stones can be crushed to recover the seed and freeze-dried samples stored at −30 °C before analysis. Then, 50 mL of 80 % methanol and 20 mg g^{-1} sodium diethyldithiocarbamate at 25 °C are added to the peel (2 g), the pulp (5 g), and the seed (5 g) of olive fruit. The mixture is homogenized in a mixer for 30 s at 16,000 g and filtered through a filter paper. The extraction is repeated six times. The combined methanol extract is evaporated under vacuum under nitrogen at 35 °C. The aqueous extract obtained is then subjected to solid-phase extraction using a C_{18} cartridge to purify the phenol fraction that is eluted using methanol.

Approximately 1 g of ground olive flesh (from 25 olives) is mixed with 40 mL of hexane, in duplicate, and agitated for 4 min using the methodology proposed by Morello et al. (2004). The upper phase is recovered, and the residue is extracted twice more to remove pigments and most of the lipids. The phenolic compounds are then extracted with 80 mL of 80 % (v/v) methanol containing 400 ppm of sodium metabisulphite. The mixture is homogenized for 30 s using a suitable homogenizer to separate the aqueous methanol phase. This procedure is repeated twice. The aqueous methanol phases are combined and filtered (0.45 μm nylon syringe filter).

Ríos and Gutiérrez-Rosales (2010) describe a method whereby lyophilized pulp (0.8 g) or a fresh one (1.0 g) is mixed with 15 mL 80 % aqueous ethanol (v/v) and 1 mL of an internal standard solution *p*-hydroxyphenylacetic acid (IS) at a concentration of 150 mg/kg. This mixture is homogenized for 4 min at 9,500 rpm and at a low temperature (0–4 °C) using a suitable homogenizer. The upper phase is collected and the procedure is repeated twice more. The extracts obtained are then sonicated for 30 s, immediately centrifuged at 15,000 rpm and 0 °C for 15 min, and then vacuum filtered. The volume is reduced under vacuum at 35–40 °C. Traces of ethanol are removed using a nitrogen flow. The aqueous extract is washed twice with hexane to eliminate lipids and pigments. Residual hexane is then removed using nitrogen. The extract is then brought to a final volume of 25 mL by adding a water–methanol mixture (70:30 v/v) and transferred to screw-top scintillation vials for storage under nitrogen atmosphere.

9.3 Olive Oil Phenolic Compounds

Phenolic compounds are transferred to oil during processing. Thus, VOO contains five different classes of phenols clustered into phenolic acids, simple phenols, complex oleuropein derivatives, flavonoids, lignans, and hydroxy-isocromans (Table 9.1). Phenolic acids, which were the first series of phenols determined in VOO, have a basic structure of cinnamic or benzoic acids, breakdown products of oleuropein and ligstroside form the majority of the phenolic fraction, the most abundant secoiridoids are the dialdehydic form of elenolic acid linked to hydroxytyrosol and an isomer of the oleuropein aglycone, the main phenolic alcohols are hydroxytyrosol and tyrosol, and lignans (1-acetoxypinoresinol, pinoresinol, 1-hydroxypinoresinol) are prevalent phenolic compounds in VOO.

The quantitative composition of phenols in various VOOs is affected by agronomic, climate, and technological extraction conditions. Thus, studies on the effect of major parameters such as cultivar, geographical origin, irrigation practice, organic versus conventional agricultural practices, stage of ripeness upon harvesting, and extraction systems without and with sophisticated procedures (e.g., destoning, enzyme use, leaf addition, drupe heating, nitrogen flashing) published since 2000 are numerous. The same applies to those addressing changes in phenolic compounds during storage in the dark or under light exposure at ambient or elevated temperatures or after heat treatment of VOO. A great number of these studies, though, do not address the same aims and contain information about material obtained with defective experimental designs (e.g., few samples, one harvest period, no fully described sampling or conditions). Some useful review articles and book chapters that compile knowledge for all of the

Table 9.1 Phenolic and related compounds in virgin olive oil

Simple phenolic acids and related compounds
Syringic acid; vanillic acid; *p*-coumaric acid; *o*-coumaric acid; gallic acid; caffeic acid; protocatechuic acid; ferulic acid; *p*-hydroxybenzoic acid; cinnamic acid; benzoic acid
Simple phenols
(3,4-Dihydroxyphenyl) ethanol (3,4 DHPEA) hydroxytyrosol; hydroxytyrosol acetate; (*p*-hydroxyphenyl) ethanol (*p*-HPEA), tyrosol; tyrosol acetate; (3,4-dihydroxyphenyl) ethanol-glucoside; vanillin
Complex oleuropein derivatives
Dialdehydic form of decarboxymethyl elenolic acid linked to 3,4-DHPEA (3,4 DHPEA-EDA); dialdehydic form of decarboxymethyl elenolic acid linked to *p*-HPEA (*p*-HPEA-EDA); oleuropein aglycon (3,4 DHPEA-EA); ligstroside aglycon; p-HPEA-derivative; dialdehydic form of oleuropein aglycon; dialdehydic form of ligstroside aglycon; elenolic acid (free and glycoside)
Flavonoids
Apigenin; luteolin
Lignans
(+)-1-Acetoxypinoresinol; (+)-pinoresinol
Hydroxy-isocromans

above aspects about VOO phenols are those of Tsimidou (1998, 1999), Morales and Tsimidou (2000), Servili et al. (2004), Carrasco-Pancorbo et al. (2005a), Boskou et al. (2006), De Castro and Japón-Luján (2006), Bendini et al. (2007), and Segura-Carretero et al. (2010).

9.3.1 Analysis of Olive Oil Phenolic Compounds

For more than four decades (Montedoro and Cantarelli 1969) total phenol content estimation or characterization of individual phenolic compounds has been carried out by applying a variety of techniques to the so-called polar extract of VOO. This fraction, being devoid of fatty derivatives or other unsaponifiables, is obtained mainly by liquid-liquid (LLE) or solid-phase extraction (SPE) and then used for further analysis using different separation and spectrophotometric techniques. Direct phenol analysis is currently achievable using sophisticated nuclear magnetic resonance (NMR) and infrared (IR) spectroscopic techniques and capillary electrophoresis. The method established over the years for phenol fingerprinting that still deserves a proper validation procedure by international bodies is to use reversed-phase high-performance liquid chromatography (HPLC) coupled to diode array plus other detectors.

The analytical procedure for the determination of individual phenols present in VOOs involves three steps: sample preparation involving extraction of phenols from VOO samples, analytical separation by a great variety of techniques, and the method of quantification.

9.3.1.1 Sample Preparation

Morales and Tsimidou (2000 and references therein) reviewed in detail the characteristic steps and difficulties that researchers must be aware of when phenolic compounds are to be extracted from an oil matrix. No doubt, LLE (methanol: water, 80:20 or 60:40, v/v) remains the most popular in practice when around 5 g of sample can be devoted for this reason despite the fact that SPE is faster and of equivalent performance for total phenol, simple phenol, or complex phenol content recovery (Pirisi et al. 2000; Bendini et al. 2003; Hrncirik and Fritsche 2004; Servili et al. 2004). The latter authors pointed out that the recovery of "bitter" phenolic compounds increases as follows: SPE_{C18} (with methanol) < SPE_{diol} (with methanol) < LLE (60 % aqueous methanol). Nevertheless, LLE seems to be more quantitative for the extraction of oxidized phenolic compounds (Armaforte et al. 2007). It is noteworthy that the COI/T.20/Doc No. 29 (IOC 2009) method for determination of biophenols in olive oils by HPLC adopts a methanol: water system, 80:20, v/v for extraction suggested as the optimum by the pioneers Montedoro and Cantarelli since 1969.

The addition of an internal standard, a procedure commonly used in the past to check recovery, is not widely followed nowadays. The oil amount (100, 30, 10, 5, 2.5 g) used for the extraction is continually reduced. The addition of hexane or other organic

solvents to the oil before extraction did not yield significant differences in the recovery of phenols. Oil/extraction solvent volume is a critical factor. Extract cleanup procedures are always used to eliminate interfering components (pigments, lipids).

For beginners, the well-appreciated paper of Montedoro et al. (1992a) on the study of all the factors influencing phenol extraction is recommended. Moreover, as shown in one of the latest publications (Papoti and Tsimidou 2009), small amounts of VOO can also be used: VOO (2.500 ± 0.001 g) is dissolved in 5 mL of *n*-hexane and 5 mL of MeOH are added. The mixture is vortexed and then centrifuged at 3,500 rpm for 10 min. The polar extract brought to dryness at around 35 °C under vacuum is then redissolved in MeOH-H_2O (60:40, v/v), washed with hexane (3 × 1 mL) to completely remove lipids and pigments, and finally filtered through a 0.45 μm PTFE filter just before analysis.

The extraction procedure is critical for the methodology used subsequently for identification and quantitation. Thus, Brenes et al. (2000) showed, using N,N-dimethylformamide (DMF) as the extraction solvent, that this could extract the entire array of olive oil phenolics much more easily than previously reported procedures, though in much smaller amounts. This meant that coupling to an electrochemical detector became a prerequisite. Direct determination of phenolic compounds in oil without any sample pretreatment requires powerful analytical techniques for their determination (e.g., NMR).

9.3.1.2 Total Polar Phenol Content Estimation

The colorimetric method broadly applied for the determination of phenols in an aqueous methanol extract is based on the use of Folin-Ciocalteau reagent and gives a good indication of resistance of VOO to oxidation. Results are usually expressed as caffeic acid equivalents (milligrams of caffeic acid/kilograms of oil), though other phenols (tyrosol, gallic acid) have also been used as standards. Standard curves prepared using different phenolic compounds, bearing trihydroxy, catecholic, guaiacolic, or monohydroxy groups, differ in slope and intercept values (Blekas et al. 2002). Despite the years of application, no general agreement has been reached on the matter. The procedure also gives a measure of the redox potential of the extract tested and is included among methods for antioxidant activity assessment. Further discussion is presented below where objective criteria for bitterness evaluation are discussed.

Papoti and Tsimidou (2009) proposed a fluorimetric procedure as an alternative to the Folin-Ciocalteu (F-C) assay that can be applied effectively to the estimation of the total phenol content in VOO, olive fruit, and leaf polar extracts. Phenol content was determined at excitation/emission wavelengths set at 280/320 nm. The validated in-house method deserves some attention as it claims to be more sensitive than the F-C one (limit of detection [LOD] and limit of quantitation [LOQ] values tenfold lower), is three times faster, needs no reagents, and most importantly, is not destructive for the sample, which can then be further used in HPLC or other assays. Data for VOO extracts correlated well with the colorimetric ones ($r = 0.69$, $n = 65$).

9.4 Separation Techniques

Separation of olive oil phenolic compounds has been achieved over the years by application of all types of liquid chromatography, gas chromatography, and, more recently, by capillary electrophoresis. Undoubtedly, HPLC coupled to a diode array detection system has become part of the armor of a modern olive oil laboratory.

9.4.1 Liquid Chromatography

Thin-layer chromatography (TLC) and normal-phase high-performance chromatography of olive oil phenols can be considered obsolete procedures for the aim of the present book, so they are not presented. TLC is sometimes used complementarily in the identification of compounds fractionated using preparative HPLC or SPE. Reversed-phase HPLC was the most successful chromatographic technique for profiling olive oil phenols for more than 30 years and is, therefore, detailed.

9.4.1.1 Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC)

Separation of phenols in reversed-phase mode (C_{18}, 250×4 or 4.6 mm i.d.) is carried out with water–methanol or water–acetonitrile mixtures enriched with acetic, phosphoric, or sulfuric acid (up to 3 %, pH<2) to suppress phenol dissociation and improve peak asymmetry. Analysts should pay attention to the various pKa values of individual phenols. Gradient elution using simple isocratic procedures have also been developed. Elution time is generally long (more than 40 min in most applications). The starting composition of eluent uses a small amount of organic phase (approximately 5 %). By the end of the run this amount is usually rather high, though it should never reach 100 % for practical reasons. The conditioning times from run to run that are needed to ensure reproducible retention data are long. Careful search reveals that most investigators practiced similar elution protocols mainly consisting of acidified aqueous methanol gradients despite the higher backpressures expected at certain ratios with water. Degassing must be constant to avoid bubbles that disrupt the long runs. Coupling to MS demands some changes in the composition of the eluent (volatile acids such as TFA are preferable). An autosampler facility is advantageous when large numbers of samples are to be examined.

For a laboratory that plans to run phenol analysis regularly, some rules of thumb include careful selection of packing material and then purchase of a couple of spare columns of the same batch; careful monitoring of backpressure levels (usually high in the presence of water) and of the water quality used for the analysis is also essential. Coupling with a suitable detection system must combine the benefits of a diode array system with those of a fluorescence system. UV detection at 280 nm is the standard wavelength, though others (225, 240, 254 nm) are also useful for the constituents of olive oil polar fractions. Flavonoids are detected at higher wavelengths (340 nm) and elenolic acid (nonphenolic) at 240 nm.

Morales and Tsimidou (2000) presented in detail protocols published up until the first edition of this handbook. In the last decade there have been fewer publications that deal with analytical aspects of separation. The work of Pirisi et al. (2000) is recommended because of its detailed evaluation of certain aspects (elution on ODS-2 analytical columns, Spherisorb, 250×4.6 mm i.d., 3 μm, with a 1.0 cm guard cartridge C_{18}, 10 μm, was performed under conditions named methods 1a, 1b, and 2). The methods presented in that paper were as follows: *Method 1*: mixture of A (H_2SO_4 [10–3 M]) and B (CH_3CN) at a flow rate of 1.0 mL min^{-1}. The elution profile (%) was as follows: *t:* 0 min, (A) 85, and (B) 15; *t*: 35 min, (A) 34, and (B) 66; *t*: 35.1–40 min, (A) 85, and (B) 15. *Method 1a* was with the detector set at 225 nm and a loop of 20 μL, and *Method 1b* was with the detector set at 280 and a loop of 40 μL. *Method 2*: mixture of H_2O with 0.5 % H_3PO_4 (A) and 50:50 CH_3CN/CH_3OH (v/v, B) at 1.2 mL min^{-1} with the following elution profile (%): *t*: 0, (A) 96, and (B) 4; *t*: 1 min, (A) 96, and (B) 4; *t*: 26 min, (A) 70, and (B) 30; *t*: 36 min, (A) 40, and (B) 60; *t*: 66 min, (A) 2, and (B) 98; *t*: 70 min, (A) 96, and (B) 4; *t*: 80 min, (A) 96, and (B) 4. The detector was set at 280 nm. Injection volume was 10 μL (concentrated samples 20×) or 40 μL (samples concentrated 5×). These conditions can be considered a good start for any newcomer in the analysis of phenolics.

Figure 9.1 presents HPLC chromatograms obtained using the method by Montedoro et al. (1992a) for VOO samples containing various levels of total polar phenols determined colorimetrically. As recommended by Servili et al. (2004), "the chromatographic profiles of VOO phenolic compounds show strong differences that may be related to the agronomic and technological aspects of production." Nevertheless, until now it was difficult to conclude which of the two factors prevailed when the sample was unlabeled. Most authors in recent years have avoided technological effects by examining olive oils produced in the laboratory using an Abencor or similar type of oil mill so as to facilitate studies concerning genetic or other effects.

Application of ultra-performance liquid-chromatography (UPLC) to the determination of phenolic compounds in VOO on columns packed with 1.7 μm particles is promising for various reasons. Suárez et al. (2008) obtained results using UPLC-MS/MS (retention times, linearity, reproducibility, LOD, and LOQ) for the analysis of 14 phenolic compounds in standard solutions, which were then compared with those obtained by HPLC–fluorescence and UPLC–diode array detection (DAD). LODs and LOQs were lower in UPLC–MS/MS than the other two methodologies for all of the analytes, with the exception of vanillic acid and pinoresinol, where values of LODs and LOQs by HPLC–fluorescence were similar to the values obtained by UPLC–MS/MS. The LLE–UPLC–MS/MS was successfully applied to analyze phenolic compounds in a VOO sample within 18 min.

9.4.2 Gas Chromatography and Other Separation Techniques

The qualitative and quantitative determination of phenolic components can also be accomplished by gas chromatography (GC). The determination requires the preparation of volatile derivatives, mainly trimethylsilyl (TMS) ethers, and high oven temperatures

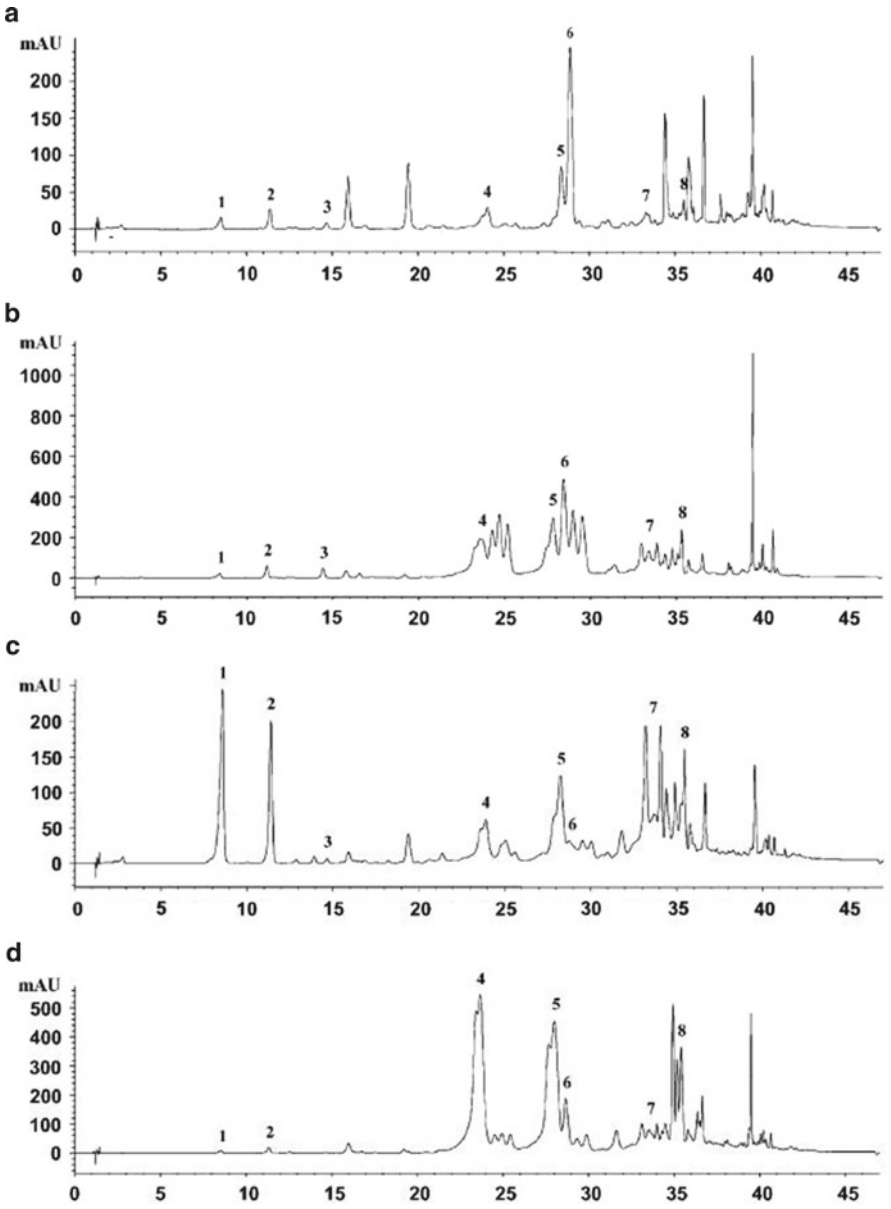

Fig. 9.1 Typical HPLC chromatograms of VOO characterized by different total phenol contents, evaluated by colorimetric method. HPLC and colorimetric evaluations of phenolic compounds were performed as reported previously by Montedoro et al. (1992a). Chromatograms: (**a**) 43 mg/kg; (**b**) 626 mg/kg; (**c**) 262 mg/kg; (**d**) 551 mg/kg. Peaks: (*1*) 3,4-DHPEA, (*2*) p-HPEA, (*3*) vanillic acid, (*4*) 3,4-DHPEA-EDA, (*5*) p-HPEA-EDA, (*6*) (+)-1-acetoxypinoresinol, (*7*) 3,4-DHPEA-EA, (*8*) ligstroside aglycon (*Source*: Servili et al. (2004), with permission of American Chemical Society)

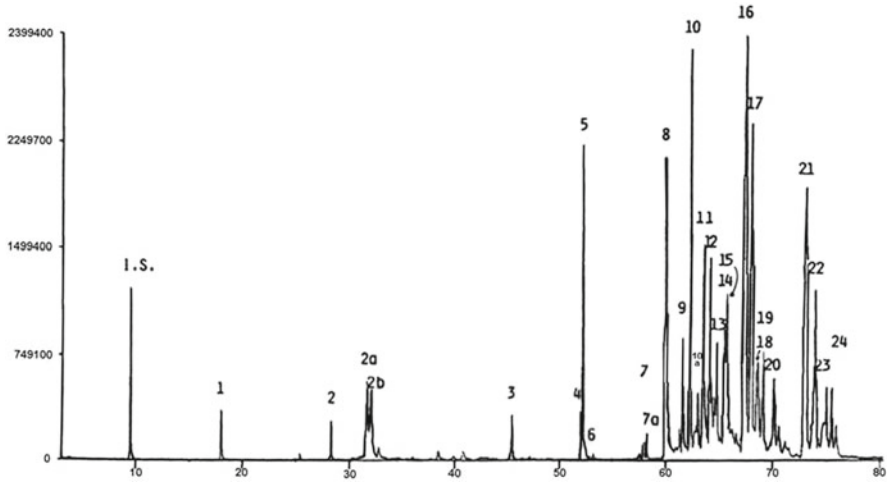

Fig. 9.2 Gas chromatographic profile of a derivatized olive oil methanolic extract. Peaks: (*1*) tyrosol (*TY*), (*2*) hydroxytyrosol (*HYTY*), (*2a*) and (*2b*) unknown, (*3*) palmitic acid, (*4*). linoleic acid, (*5*) oleic acid, (*6*) stearic acid, (*7*) and (*7a*) unknown, (*8*) dialdehydic form of ligstroside aglycon containing no carbomethoxy group, (*9*, *10*, *10a*, *14*, *16*, *17*, *18*, and19) linked phenols containing TY, (*11*, *13*, *15*, *20*, *21*, *22*, *23*, and *24*) linked phenols containing HYTY; 12, monoglyceride (*Source*: Angerosa et al. (1995), with permission of American Chemical Society)

on suitable columns (SB-5, ZB-5, and equivalents). Figure 9.2 shows 20 phenolic compounds separated by a GC on-column injection system and flame ionization detector (FID) at 315 °C within 80 min.

GC analysis of olive oil phenols was commonly used before LC-MS found extensive use (Angerosa et al. 1995; Ranalli and Serraiocco 1996). Currently the two techniques are complementarily performed to elucidate the identity of the most complex constituents (Del Carlo et al. 2004; Ríos et al. 2005) because the availability of standards is extremely limited and the GC mass spectra libraries are richer than the corresponding LC ones. Loss of information about compounds present in the polar fraction such as glucosides can be expected due to the derivatization process. High temperatures reduce the accuracy of quantitative results. Nevertheless, ongoing advancements in coupled techniques permit analysts to extract new information as recently reported for GC–atmospheric pressure chemical ionization–time of flight mass spectrometry (GC-APCI-TOF MS) for olive oil phenol profiling (García-Villalba et al. 2011).

Although the identification and quantification of phenolic compounds are successfully carried out by HPLC and by GC, these techniques are time consuming (more than 90 min) and need precise sample preparation. The need for faster analytical techniques with a good balance of simplicity and adequate analytical resolution has increased the interest in capillary electrophoresis (CE) in recent years, to the point where CE has become an alternative to HPLC for the analysis

of phenolic compounds in a large variety of foodstuffs (Bonoli et al. 2003; Carrasco-Pancorbo et al. 2004). Capillary electrophoresis (CE) is a high-resolution technique for plant metabolites requiring only small amounts of sample and buffer and short times of analysis and can be coupled to various types of detectors. Bendini et al. (2007) and Hurtado–Fernández et al. (2010), in two recent extensive reviews, provide abundant tabulated information on CE conditions such as phenol extraction system and amounts that are critical for method sensitivity, instrumental variables (detection means, voltage [kV], temperature, capillary dimensions [i.d. μm; l_{ef}, cm], and injection time [s]), and chemical variables (type of buffer, buffer concentration, pH). Electrophoretic conditions are in general optimized in terms of migration behavior, sensitivity, analysis time, and peak shape. For example, electrophoretic separation of compounds within all of the known classes of olive oil phenols was carried out using an aqueous CE buffer system consisting of 60 mM NH_4OAc at pH 9.5 with 5 % of 2-propanol, a sheath liquid containing 2-propanol/water 60:40 v/v and 0.1 % v/v triethylamine within 10 min, whereas the corresponding RP-HPLC runs demand at least 80 min. It remains to be seen whether or not the penetration of CE methods into the olive oil phenol analysis field in coming years will be significant.

9.4.3 Detection, Identification, and Quantification Practices After Separation of Phenolic Compounds

The detection, identification, and quantification techniques after separation of phenolic compounds depend on the aim of the study and available facilities. In the last decade LC-MS techniques have found extended application. No doubt this is the ultimate tool for every researcher. Still, routine analysis is less demanding regarding instrumentation. Thus, UV-visible spectrophotometry and spectrofluorimetry address a wide range of questions that are raised during the identification of olive oil phenolic compounds in combination with retention time data (preferable relative retention time data).

9.4.3.1 Color Tests

Among methods for detecting phenols on paper or TLC, the F-C reagent followed by ammonia vapors, spraying with p-toluenesulfonic acid plus vanillin, and heating at 105 °C for 10 min or leaving the layers in the air and evaluating the browning of the spots after 24 h of exposure at room temperature have been used (Montedoro et al. 1992a, b).

9.4.3.2 UV Spectrophotometry

UV spectrophotometry is extremely helpful in the identification of phenols and monitoring the purity of HPLC peaks. The spectrum region commonly scanned is between 200 and 290 nm, where simple phenols and phenolic acids exhibit one or

Table 9.2 Absorbance data for standard phenolic compounds in 3 % aqueous acetic acid-methanol, 94:6, v/v (pH = 2.64)

Phenol	λ_{max} (nm)	$\varepsilon\lambda_{max}$	ε_{280}
Gallic acid	270.6	9112.6	9112.6
Protocatechuic acid	259.0/293.0	10670.1/5412.4	4329.9
Tyrosol	275.2	1519.3	1519.3
p-hydroxyphenylacetic acid	274.4	1700.5	1700.5
Vanillic acid	260.2/291.4	16685.4/8595.5	7471.9
Homovanillic acid	278.4	2785.4	2785.4
Caffeic acid	319.4	28558.6	5225.2
Syringic acid	273.4	10646.8	5225.2
p-coumaric acid	308.6	16776.6	10989.0
Ferulic acid	321.4	20686.3	9803.9
o-coumaric acid	276.2/321.8	17352.9/8676.5	17352.9

Source: Tsimidou et al. (1996), with permission of Grasas y Aceites

two maxima (Montedoro et al. 1993). Hydroxycinnamic acids have a characteristic maximum at 310–332 nm. Elenolic acid, the acid originating from oleuropein and ligstroside, absorbs at 240 nm in acidified aqueous methanol solution (Montedoro et al. 1992a). Differences in maximum wavelength and molar absorption values among the various phenols (Table 9.2) influence quantification and should be considered when the "true" levels are investigated. For example, quantification of the same quantity of homovanillic acid at 280 and 325 nm gave values three times higher in the first case (Tsimidou et al. 1996).

The wide use of diode array systems coupled to HPLC offers a significant advance in phenol identification and, consequently, for the optimization of quantification due to the variability in λ max. Mateos et al. (2001) quantified hydroxytyrosol derivatives at 240 nm, other phenols, cinnamic acid, and lignans at 280 nm and flavones and ferulic acid at 335 nm. Two internal standards were used, *p-hydroxyphenylacetic* acid (280 nm) and *o*-coumaric acid (335 nm). Rotondi et al. (2004), using LLE-HPLC-UV/DAD, determined phenolic acids, phenols, and secoiridoids at 280 nm and flavonoids at 350 nm. Pirisi et al. (2000), working on three separation conditions and DAD, concluded that quantification of total and selected conjugated phenols (3,4-DHPEA-EA, 3,4-DHPEA-EDA, p-HPEA-EDA, demethyl oleuropein aglycon) is preferable at 225 nm rather than at 280 nm, though some of the conjugated forms also present a second maximum wavelength above that one (Table 9.3). The same authors proposed the expression "tyrosol equivalents" instead of using "gallic acid or caffeic acid equivalents" as the obtained values are higher in the former case. In general, caffeic acid, gallic acid, resorcinol, and syringic acid are used as internal standards for phenol quantification. Quantification using the external standard technique with one or more standard curves is preferred by many investigators. An interlaboratory study (17 laboratories, six VOO samples) conducted by IOC (2009) recommends detection at 280 nm and for quantification an internal standard approach using syringic acid and a response factor of syringic acid/tyrosol ratio (5.1 ± 0.4) that allows expression of results in tyrosol equivalents.

Table 9.3 Spectrometric characteristics of VOO phenolic and nonphenolic compounds that may be used in objective estimation of taste attributes

Compound	λ_{max}	($\lambda_{exc}/\lambda_{emiss.}$)
Hydroxybenzoic acids		
Vanillic acid	261; 293	308/360 or 264/354 or 280/353
Syringic acid	275	320/372 or 310/430 or 264/354
Gallic acid	270	412/496 (unstable) or 278/366
Protocatechuic acid	260; 295	304/365 (unstable) or 270/358
p-hydroxybenzoic acid	256	291, 340 or 264, 354
Hydroxycinnamic acids		
p-coumaric acid	306	310/430 or 280/453
o-coumaric acid	277; 325	–
caffeic acid	323; 300	262/426 or 310/430
ferulic acid	321	310/430 or 264/354
Nonphenolic compounds present in polar fraction		
Cinnamic acid	280	310/430
Elenolic acid	240	–
Simple phenols and derivatives		
Hydroxytyrosol	230 or 278 or 280 or 281	280/320 or 280/353
Tyrosol	220 or 273 or 276	280/320 or 280/353
Tyrosol acetate	230; 280	280/320
Hydroxytyrosol acetate	230 or 277.8 or 280	280/320
Secoiridoids		
3,4-DHPEA-EDA	223; 282; 330 or 235; 285	280/320 or 280/353
p-HPEA-EDA	225; 277; 330 or 235; 280	280/320 or 280/313
3,4-DHPEA-EA	229; 278 or 235; 285	280/320 or 280/353
p-HPEA-EA	235; 285	280/313
Lignans		
Pinoresinol	232; 280	280/320 or 280/339
1-Acetoxypinoresinol	240; 282	280/320 or 280/339
Hydroxychromans		
	No data for UV absorbance	No data for fluorescence
Flavones		
Apigenin	230; 270; 340	280/320 or 280/340
Luteolin	255; 350	280/320 or 280/340

9.4.3.3 Spectrofluorimetry

The introduction of fluorescence detection in line or not with DAD became a valuable tool in recent years as it is more specific and sensitive than spectrometric detection and of reasonable cost (Cartoni et al. 2000; Brenes et al. 2002; Selvaggini et al. 2006). According to (Ryan et al. 1999b), fluorescence at 320 nm (λ_{exc} = 280 nm) is "*the best compromise for general profiling*" for olive fruit phenolic compounds. Cartoni et al. (2000) detected vanillic *acid* (λ_{exc} = 264 nm, λ_{emiss} = 354 nm), p-coumaric, caffeic, and

ferulic acids (λ_{exc} = 310 nm, λ_{emiss} = 430 nm). Specificity is reported for tyrosol, lignans (pinoresinol in particular), and the "bitter" 3,4-DHPEA-EA, whereas the peak intensity for 3,4-DHPEA-EDA is less satisfactory due to a loss of planarity of the compound (Brenes et al. 2002; Selvaggini et al. 2006). Brenes et al. (2002) detected pinoresinol and 1-acetoxypinoresinol in VOOs from Picual and Arbequina at (λ_{exc} = 280 nm/ λ_{emiss} = 320 nm) as well as oleuropein and ligstroside aglycons. Table 9.3 gives some characteristic absorbance of the well-identified phenolic compounds that may be useful for the objective estimation of taste attributes.

9.4.3.4 Mass Spectroscopy

Mass spectroscopy (MS) in various modes is currently coupled to the aforementioned high-resolution separation techniques. Extensive use in routine analysis is not common. Research papers offer an extremely high volume of data on fragments derived from phenolic compound analysis under the various modes or within each mode of mass spectrometry. According to the literature, bitter attributes are related to the presence of the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA), the aldehydic form of oleuropein aglycon (AOA), and oleuropein aglycon (3,4-DHPEA-EA) as the main compounds, bitter and pungent notes (back of the tongue) to ligstroside derivatives such as (p-HPEA-EDA), and astringency to tyrosol. LC-MS, LC-MS/MS, GC-MS, and CE-MS give information on the m/z of these compounds as well as of $[M\text{-}H]^-$, $[M+H]^+$, and other fragments (Ryan et al. 2002; Gutiérrez-Rosales et al. 2003; Bendini et al. 2003; Andrewes et al. 2003; Rotondi et al. 2004; De la Torre-Carbot et al. 2005; Carrasco-Pancorbo et al. 2005b, 2007; Suárez et al. 2008; Jemai et al. 2009; Saitta et al. 2009; Ouni et al. 2011). Quantitative data are also produced on the basis of fragment abundance.

9.4.3.5 Nuclear Magnetic Resonance (NMR) Spectroscopy and NMR-MS Coupling

NMR is a powerful method for the structural elucidation of isolated phenolic compounds (Chap. 11). Nevertheless, it gives two more options: (a) in situ analysis of phenolic composition in the polar fraction and (b) coupled to LC-SPE in conjunction with other detection means (diode array, MS) or not. Addition of a postcolumn (SPE) system to replace loop collection improves the signal-to-noise ratio by up to a factor of 4 in natural phenolic extracts (Exarchou et al. 2003). Such sophisticated techniques have become important for the complete elucidation of isomers present in the polar fraction that are not detectable by other means, not even using MS or MS/MS techniques (Pérez-Trujillo et al. 2010). NMR cannot be considered in routine analysis but, nevertheless, may support functional properties of stereoisomers related to health or sensorial properties of particular compounds. The most representative publications over the last decade are those of the group of Dais

Table 9.4 Phenolic content (μmol/100 g) of virgin olive oil samples from Messinia determined by ^{1}H-NMR or ^{31}P-NMR

Phenolic compound	^{1}H-NMR	^{31}P-NMR
Total hydroxytyrosol	34.03	32.49
Total tyrosol	25.06	24.89
p-coumaric acid	0.71	0.78
Apigenin	0.31	0.31
Luteolin	0.80	0.90
(+) Pinoresinol	1.61	1.61
(+) 1-Acetoxypinoresinol	2.63	2.63
Syringaresinol	0.64	0.77
Vanillia	0.29	nd
Vanillic acid	0.00	nd
Homovanillil alcohol	0.00	nd
Aldehydic form of oleuropein	2.37	nd
Aldehydic form of oleuropein	1.17	nd
Aldehydic form of oleuropein and ligstroside	7.26	nd
Dialdehydic form of oleuropein and ligstroside lacking a carboxymethyl group	15.11	nd

Source: Abstracted from Christophoridou and Dais (2009), with permission of Elsevier
nd not detected

(Christophoridou et al. 2005; Christophoridou and Dais 2006; Dais et al. 2007; Christophoridou and Dais 2009). The authors exploited various NMR techniques to develop ^{1}H-NMR or ^{31}P-NMR procedures for the characterization of polar fractions (Table 9.4), but they have not yet correlated their findings (qualitative and quantitative) with taste aspects.

9.5 Objective Criteria for Evaluation of Taste Attributes

Bitterness is closely related to the presence of oleuropein and ligstroside derivatives (Andrewes et al. 2003; Cerretani et al. 2008; Gutiérrez-Rosales et al. 2003; Mateos et al. 2004; Siliani et al. 2006; Esti et al. 2009). The perception of bitterness in VOO seems to be influenced by the large quantity of monounsaturates (García-Mesa et al. 2008). Astringency and pungency are other taste attributes that are of interest in VOO organoleptic quality. The total polar phenol level and the presence of catecholic groups in a molecule (oleuropein derivatives) are the critical factors due to protein-phenol interactions in the saliva. Nevertheless there are reports (Favati et al. 1995) presenting data according to which differences in bitterness and astringency among oils from different cultivars (Coratina, Koroneiki, Picual, and Arbequina) did not always coincide with the total polar phenol content. On the basis of these results, for example, VOO from unripe Koroneiki olives was more bitter and astringent than a corresponding Coratina oil containing double the amount of total polar phenols. The publicity attached to oleocanthal, the dialdehydic form of

Table 9.5 Sensory qualities, taste threshold, and tentative identifications of phenolic components isolated from extra virgin olive oil

Phenol (tentative identification)	Sensory qualities	Threshold[a]
Hydroxytyrosol[b]	–	–
Tyrosol	Sticking astringency, not bitter	4.4–18
Deacetoxy-oleuropein aglycon (dialdehydic form)	Astringent, bitter, burning/stinging/numbing mostly on tongue	0.4–1.6
Derivative of oleuropein aglycon	Bitter, sour, astringent, sweet, cooling, peppery	0.2–0.8
Not identified	Bitter, astringent (dry teeth)	0.2–0.8
Derivative of oleuropein aglycon	Bitter, astringent, bit burning	0.1–0.4
Deacetoxy-ligstroside aglycon (dialdehydic form)	Strong burning mostly at back of throat, slightly bitter, astringent	0.4–1.6
Isomer of ligstroside aglycon	Astringent, bit burning, bitter	0.05–0.2
Isomer of ligstroside aglycon	Dry mouth, not bitter	0.4–1.6
Derivative of oleuropein aglycon	Bitter, astringent, salt	0.1–0.4
Isomer of oleuropein aglycon	Very bitter, very astringent	0.05–0.2
Hydrophobic polyphenols (fractions 70–90)	Strong bitter, astringent	–
Very hydrophobic polyphenols (fractions 90–120)	Astringent, bitter, bit sour, bit burning, salt	–

Source: Adapted from Andrewes et al. (2003), with permission of American Chemical Society
[a]Estimated taste threshold in mM
[b]Although synthetic hydroxytyrosol was available, it was not evaluated by the panel because the sample was not of sufficient purity to be safely evaluated

decarboxymethyl elenolic acid linked to tyrosol (Beauchamp et al. 2005), had as a result that consumers – familiar with its peppery, hot, and burning sensations at the back of the throat –also appreciated the importance of the anti-inflammatory properties it exerts. Nevertheless, it was Andrewes et al. (2003) who addressed the detailed taste attributes of VOO phenols using a taste dilution analysis (TDA) protocol. Their method used a tedious three-step sample preparation procedure (liquid-liquid extraction, reversed-phase HPLC phenol separation, fraction collection). The complexity of taste attributes for each of the isolated phenols and the threshold limits found in this work revealed why VOOs with similar levels of total phenols but different in individual phenol content are judged differently by panelists (Table 9.5).

On account of the limitations inherent in sensory analysis, in addition to difficulties related to the training and sustainability of panel test groups in objectivity (Angerosa et al. 2000), reproducible evaluation of certain taste attributes between among laboratories is difficult but highly desirable. The "bitter index," or K_{225} value, proposed by Gutiérrez et al. (1992) is estimated through measurement of absorbance at 225 nm of polar extracts obtained using a certain protocol. Thus, SPE (C_{18} cartridges) is used for the preparation of the polar extract, phenols are eluted with a methanol–water mixture (60:40, v/v), and the bitter index is calculated from the equation $K_{225} = A_{225}/C$, where C = g oil/100 mL, A = absorbance at 225 nm. The relevance of such measurements to bitter attributes has often been questioned because the particular wavelength is not selective for bitter compounds. Interference of nonbitter

molecules like elenolic acid, hydroxytyrosol acetate, hydroxytyrosol, tyrosol, and lignans is to be expected. Inarejos-García et al. (2009) proposed a more objective expression of K_{225} values in terms of oleuropein equivalents because (a) this glycoside is the only olive bitter compound commercially available and (b) bitterness of VOO is officially defined as the "characteristic taste of oil from green olives or olives turning colour" (EC 2002). The authors named that value the oleuropein score. Fluorescence is a more specific and sensitive characteristic of phenolic compounds that can prove useful in taste attribute estimation. Thus, in situ estimation of total fluorescence of the polar extract of VOO samples, expressed as oleuropein equivalents (Papoti and Tsimidou 2009), is well correlated with the oleuropein score ($R = 0.70$, $p < 0.05$, $n = 65$) and is free from interference due to the presence of elenolic acid (Inarejos-García et al. 2009). Moreover, RP-HPLC coupled to fluorimetry gives a phenol profile free of noise that is apparent at 225 nm and can thus be used as an extra tool in the quantification of taste-related molecules such as tyrosol, 3,4-DHPEA-EDA, oleocanthal, 3,4-DHPEA-EA, and p-HPEA-EA.

New sensor applications, in terms of electronic nose or other devices, are expected to add increased objective criteria for taste appreciation (Rodríguez-Méndez et al. 2010).

Another challenge is the elucidation of how the taste compounds of VOO are distributed in the mucosa/saliva. The impact of the protein composition of saliva on flavor release is barely known, and it may have a significant effect on the kind and intensity of perception. Research in this regard would allow us to understand which fraction of the phenols that remains in the proteic/aqueous phase of saliva could be responsible for taste and posttaste perceptions (e.g., bitterness, pungency, astringency). Research based on chemical, sensory, and physiological approaches would make sensory assessment dispensable to some extent, and it would give coherence and unity to sensory information, thus avoiding the current confusion and fuzziness that affect the sensory assessment of VOOs (García-González and Aparicio 2010).

9.6 Future Trends and Perspectives

Polyphenols are strongly related to the quality of VOOs. In addition, their contribution to VOO protection against oxidation is widely known. Thus, the analysis of VOO phenolic compounds is a multifaceted area of research depending on the aim of the study. Concerning information on taste issues, separation techniques are pivotal. RP-HPLC seems to be an unequivocal choice. The availability of different detectors may facilitate or burden the work of analysts. Nevertheless, since the taste and aroma aspects of VOO are vital for consumer choice and may add value to products bottled by different types of companies, there is a need for a correlation between sensory attributes and levels of selected phenolic compounds. The recently accepted health claims about phenolic compounds in VOO and various preparations originating from olive products and wastes are expected to accelerate the procedures for inter-laboratory studies on suitable methods for estimating phenols in oil and assist in the

standardization of formulations. Moreover, optional labeling permitted through EU Regulation 1019/2002 (EC 2002) such as "pungent," "fruity: ripe or green," "bitter," "intense," "medium," "light," "well balanced," and "mild oil" needs substantiation that is related, to a great extent, to the presence of specific phenolic compounds or to the total phenol level.

References

Amiot MJ, Fleuriet A, Macheix JJ (1986) Importance and evolution of phenolic compounds in olive during growth and maturation. J Agric Food Chem 34:823–826

Amiot MJ, Fleuriet A, Macheix JJ (1989) Accumulation of oleuropein derivatives during olive maturation. Phytochemistry 28:67–69

Andrewes P, Busch JL, De Joode T, Groenewegen A, Alexandre H (2003) Sensory properties of virgin olive oil polyphenols: Identification of deacetoxy-ligstroside aglycon as a key contributor to pungency. J Agric Food Chem 51:1415–1420

Angerosa F, D'Alessandro N, Konstantinou P, Di Giacinto L (1995) GC-MS evaluation of phenolic compounds in virgin olive oil. J Agric Food Chem 43:1802–1807

Angerosa F, Mostallino R, Basti C (2000) Virgin olive oil odor notes: their relationships with volatile compounds from the lipooxygenase pathway and secoiridoid compounds. Food Chem 68:283–287

Armaforte E, Mancebo-Campos V, Bendini A, Salvador MD, Fregapane G et al (2007) Retention effects of oxidized polyphenols during analytical extraction of phenolic compounds of virgin olive oil. J Sep Sci 30:2401–2406

Beauchamp GJ, Keast RSJ, Morel D, Lin J, Pika J et al (2005) Ibuprofen-like activity in extra-virgin olive oil. Nature 437:45–46

Bendini A, Bonoli M, Cerretani L, Biguzzi B, Gallina-Toschi T et al (2003) Liquid-liquid and solid-phase extraction from phenols from virgin olive oil and their separation by chromatographic and electrophoretic methods. J Chromatogr A 98:425–433

Bendini A, Cerretani L, Carrasco-Pancorbo A, Gómez-Caravaca AM, Segura-Carretero A, Fernández-Gutiérrez A, Lercker G (2007) Phenolic molecules in virgin olive oils: a survey of their sensory properties, health effects. Antioxidant activity and analytical methods. An overview of the last decade. Molecules 12:1679–1719

Bianchi G (2003) Lipids and phenols in table olives. Eur J Lipid Sci Technol 105:229–242

Bianco A, Coccioli F, Guiso M, Marra C (2001) The occurence in olive oil of a new class of a new class of phenolic compounds: hydroxy-isochromans. Food Chem 77:405–411

Blekas G, Psomiadou E, Tsimidou M, Boskou D (2002) On the importance of total polar phenols to monitor the stability of Greek virgin olive oil. Eur J Lipid Sci Technol 104:340–346

Bonoli M, Montanucci M, Gallina-Toschi T, Lercker G (2003) Fast separation and determination of tyrosol, hydroxytyrosol and other phenolic compounds in extra-virgin olive oil by capillary zone electrophoresis with ultraviolet-diode array detection. J Chromatogr A 1011:163–172

Boskou D, Tsimidou MZ, Blekas G (2006) Polar phenolic compounds. In: Boskou D (ed) Olive oil: chemistry and technology, 2nd edn. AOCS Press, Champaign, pp 73–92

Brenes M, García A, García P, Garrido A (2000) Rapid and complete extraction of phenols from olive oil and determination by means of a coulometric electrode array system. J Agric Food Chem 48:5178–5183

Brenes M, García A, Ríos JJ, García P, Garrido A (2002) Use of 1-acetoxypinoresinol to authenticate Picual olive oils. Int J Food Sci Technol 37:615–625

Cardoso SM, Guyot S, Marnet N, Lopes-da-Silva JA, Renard CM, Coimbra MA (2005) Characterisation of phenolic extracts from olive pulp and olive pomace by electrospray mass spectrometry. J Sci Food Agric 85:21–32

Carrasco-Pancorbo A, Cruces-Blanco C, Segura-Carretero A, Fernández-Gutiérrez A (2004) Sensitive determination of phenolic acids in extra-virgin olive oil by capillary zone electrophoresis. J Agric Food Chem 52:6687–6693

Carrasco-Pancorbo A, Cerratani L, Bendini A, Segura-Carretero A, Gallina-Toshi T, Fernández-Gutierréz A (2005a) Analytical determination of polyphenols in olive oils. J Sep Sci 28:837–858

Carrasco-Pancorbo A, Cerretani L, Bendini A, Segura-Carretero A, Del Carlo M et al (2005b) Evaluation of the antioxidant capacity of individual phenolic compounds in virgin olive oil. J Agric Food Chem 53:8918–8925

Carrasco-Pancorbo A, Neusüß C, Pelzing M, Segura-Carretero A, Fernández-Gutiérrez A (2007) CE- and HPLC-TOF-MS for the characterization of phenolic compounds in olive oil. Electrophoresis 28:806–821

Cartoni GP, Coccioli F, Jasionowska R, Ramires D (2000) HPLC analysis of the benzoic and cinnamic acids in edible vegetable oils. Ital J Food Sci 12:163–173

Cerretani L, Salvador MD, Bendini A, Fregapane G (2008) Relationship between sensory evaluation performed by Italian and Spanish official panels and volatile and phenolic profiles of virgin olive oils. Chemosens Percept 1:258–267

Christophoridou S, Dais P (2006) Novel approach to the detection and quantification of phenolic compounds in olive oil based on 31P nuclear magnetic resonance spectroscopy. J Agric Food Chem 54:656–664

Christophoridou S, Dais P (2009) Detection and quantification of phenolic compounds in olive oil by high resolution 1H nuclear magnetic resonance spectroscopy. Anal Chim Acta 633:283–292

Christophoridou S, Dais P, Li-Hong T, Spraul M (2005) Separation and identification of phenolic compounds in olive oil by coupling high-performance liquid chromatography with postcolumn solid-phase extraction to nuclear magnetic resonance spectroscopy (LC-SPE-NMR). J Agric Food Chem 53:4667–4679

Dais P, Spyros A, Christophoridou S, Hatzakis E, Fragaki G et al (2007) Comparison of analytical methodologies based on 1H and 31PNMR spectroscopy with conventional methods of analysis for the determination of some olive oil constituents. J Agric Food Chem 55:577–584

Damtoft S, Franzyk H, Jensen SR (1993) Biosynthesis of secoiridoids in *Oleaceae*. Phytochemistry 34:1291–1299

De Castro LMD, Japón-Luján R (2006) State-of-the-art and trends in the analysis of oleuropein and derivatives. Trends Anal Chem 25:501–510

De la Torre-Carbot K, Jauregui O, Gimeno E, Castellote AI, Lamuela-Raventós RM et al (2005) Characterization and quantification of phenolic compounds in olive oils by solid-phase extraction, HPLC-DAD and HPLC-MS/MS. J Agric Food Chem 53:4331–4340

Del Carlo M, Sacchetti G, Di Mattia C, Compagnone D, Mastrocola D et al (2004) Contribution of the phenolic fraction to the antioxidant activity and oxidative stability of olive oil. J Agric Food Chem 52:4072–4079

Esti M, Cinquanta L, La Notte E (1998) Phenolic compounds in different olive varieties. J Agric Food Chem 46:32–35

Esti M, Contini M, Moneta E, Sinesio F (2009) Phenolics compounds and temporal perception of bitterness and pungency in extra-virgin olive oils: changes occurring throughout storage. Food Chem 113:1095–1100

European Communities (EC) (2002) Official Journal of the Commission of the European Communities. Regulation no.796/2002, L.128, May 6th pp 8–28

Exarchou V, Godejohann M, van Beek TA, Gerothanassis IP, Vervoort J (2003) LC-UV-solid phase extraction-NMR-MS combined with a cryogenic flow probe and its application to the identification of compounds present in Greek oregano. Anal Chem 75:6288–6294

Favati F, Caporale G, Monteleone E, Bertuccioli M (1995) Rapid extraction and determination of phenols in extra virgin olive oil. In: Charalambous G (ed) Food flavors: generation, analysis and process influence. Elsevier Science BV, Amsterdam, pp 429–452

García-González DL, Aparicio R (2010) Research in olive oil: challenges for the near future. J Agric Food Chem 58:12569–12577

García-Mesa JA, Pereira-Caro G, Fernández-Hernández A, García-Ortíz A et al (2008) Influence of lipid matrix in the bitterness perception of virgin olive oil. Food Qual Prefer 19:421–430

García-Villalba R, Pacchiarotta T, Carrasco-Pancorbo A, Segura-Carretero A, Fernández-Gutiérrez A et al (2011) A gas chromatography-atmospheric pressure chemical ionization-time of flight mass spectrometry for profiling of phenolic compounds in extra virgin olive oil. J Chromatogr A 1218:959–971

Gomez-Rico A, Salvador MD, la Greca M, Fregapane G (2006) Phenolic and volatile compounds of extra virgin olive oil (Olea europaea L. cv. Cornicabra) with regard to fruit ripening and irrigation management. J Agric Food Chem 54:7130–7136

Gomez-Rico A, Fregapane G, Salvador MD (2008) Effect of cultivar and ripening on minor components in Spanish olive fruits and their corresponding virgin olive oils. Food Res Inter 41:433–440

Gutiérrez F, Perdiguero S, Gutiérrez R, Olias JM (1992) Evaluation of the bitter taste in virgin olive oil. J Am Oil Chem Soc 69:394–395

Gutiérrez-Rosales F, Ríos JJ, Gómez-Rey ML (2003) Main polyphenols in the bitter taste of virgin olive oil. Structural confirmation by on-line high-performance liquid chromatography electrospray ionization mass spectrometry. J Agric Food Chem 51:6021–6025

Gutiérrez-Rosales F, Romero MP, Casanovas M, Motilva MJ, Mínguez-Mosquera MI (2010) Metabolites involved in oleuropein accumulation and degradation in fruits of *Olea europaea L.* Hojiblanca and Arbequina varieties. J Agric Food Chem 58:12924–12933

Harborne JB (1989) Methods in plant biochemistry. In: Dey PM, Harborne JB (eds) Series of plant phenolics: volume 1. Academic Press, London, pp 1–28

Hrncirik K, Fritsche S (2004) Comparability and reliability of different techniques for the determination of phenolic compounds in virgin olive oil. Eur J Lipid Sci Technol 106:540–549

Hurtado-Fernández E, Gómez-Romero M, Carrasco-Pancorbo A, Fernández-Gutiérrez A (2010) Application and potential of capillary electroseparation methods to determine antioxidant phenolic compounds from plant food material. J Pharm Biomed Anal 53:1130–1160

Inarejos-García AM, Androulaki A, Salvador MD, Fregapane G, Tsimidou MZ (2009) Discussion on the objective evaluation of virgin olive oil bitterness. Food Res Int 42:279–284

International Olive Council (IOC) (2009) Method COI/T.20/Doc.No 29. Determination of biophenols in olive oils by HPLC, Madrid

Jemai H, Bouaziz M, Sayadi S (2009) Phenolic composition, sugar contents and antioxidant activity of Tuinisian sweet olive cultivar with regard to fruit ripening. J Agric Food Chem 57:2961–2968

Marsilio V, D'Andria R, Lanza B, Russi F, Iannucci E et al (2006) Effect of irrigation and lactic acid bacteria inoculants on the phenolic fraction, fermentation and sensory characteristics of olive (Olea europaea L. cv. Ascolana tenera) fruits. J Sci Food Agric 86:1005–1013

Mateos R, Espartero JL, Trujillo M, Ríos JJ (2001) Determination of phenols, flavones, and lignans in virgin olive oils by solid-phase extraction and high-performance liquid chromatography with diode array ultraviolet detection. J Agric Food Chem 49:2185–2192

Mateos R, Cert A, Pérez-Camino C, García JM (2004) Evaluation of virgin oil bitterness by quantification of secoiridoid derivatives. J Am Oil Chem Soc 81:71–76

Montedoro GF, Cantarelli C (1969) Indagini sulle sostanze fenoliche presenti negli oli d'oliva. Riv Ital Sost Grasse 46:3–12

Montedoro GF, Servili M, Baldioli M, Miniati E (1992a) Simple and hydrolyzable phenolic compounds in virgin olive oil. 1. Their extraction, separation, and quantitative and semiquantitative evaluation by HPLC. J Agric Food Chem 40:1571–1576

Montedoro GF, Servili M, Baldioli M, Miniati E (1992b) Simple and hydrolyzable phenolic compounds in olive oil: 2. Their extraction, separation, and quantitative and semi-quantitative separation and evaluation by HPLC. J Agric Food Chem 40:1577–1580

Montedoro GF, Servili M, Baldioli M, Selvaggini R, Miniati E et al (1993) Simple and hydrolyzable phenolic compounds in olive oil: 3. Spectroscopic characterization of the secoiridoid derivatives. J Agric Food Chem 41:2228–2234

Monteleone E, Carlucchi A, Caporale G (1997) Use of slope analysis to characterize consumer preference for virgin olive oil. Ital J Food Sci 9:133–140

Morales MT, Tsimidou M (2000) The role of volatile compounds and polyphenols in olive oil sensory quality. In: Harwood J, Aparicio R (eds) Handbook of olive oil: analysis and properties. Aspen Publishers, Gaitesburg, pp 393–438, and references therein

Morello J, Motilva M, Tovar M (2004) Changes in commercial virgin olive oil (cv. Arbequina) during storage, with special emphasis on the phenolic fraction. Food Chem 85:357–364

Ouni Y, Taamalli A, Gómez-Caravaca AM, Segura-Carretero A, Fernœndez-Gutiérrez A et al (2011) Characterisation and quantification of phenolic compounds of extra-virgin olive oils according to their geographical origin by a rapid and resolutive LC–ESI-TOFMS method. Food Chem 127:1263–1267

Papoti VT, Tsimidou MZ (2009) Looking through the qualities of a fluorimetric assay for the total phenol content estimation in virgin olive oil, olive fruit or leaf polar extract. Food Chem 112:246–252

Pérez-Trujillo M, Gómez-Caravaca AM, Segura-Carretero A, Fernández-Gutiérrez A, Parella AT (2010) Separation and identification of phenolic compounds of extra virgin olive oil from Olea europaea L by HPLC-DAD-SPE-NMR/MS. Identification of a new diastereoisomer of the adehydic form of oleuropein aglycone. J Agric Food Chem 58:9129–9136

Pirisi FM, Cabras P, Falqui Cao C, Migliorini M, Muggelli M (2000) Phenolic compounds in virgin olive oil. 2. Reappraisal of the extraction, HPLC separation, and quantification procedures. J Agric Food Chem 48:1191–1196

Ranalli A, Serraiocco A (1996) Evaluation of characteristics of olive oil produced by innovative or traditional processing technologies. Riv Ital Sost Grasse 73:303–314

Ríos JJ, Gutiérrez-Rosales F (2010) Comparison of methods extracting phenolic compounds from lyophilised and fresh olive pulp. LWT- Food Sci Technol 43:1285–1288

Ríos JJ, Gil MJ, Gutiérrez-Rosales F (2005) Solid-phase extraction gas chromatography-ion trap-mass spectrometry qualitative method for evaluation of phenolic compounds in virgin olive oil and structural confirmation of oleuropein and ligstroside aglycons and their oxidation products. J Chromatogr A 1093:167–176

Rodríguez-Méndez ML, Apetrei C, De Saja J (2010) Electronic tongues purposly designed for the organoleptic characterization of olive oils. In: Preedy VR, Watson RR (eds) Olives and olive oil in health and disease prevention. Elsevier Academic Press, Amsterdam, pp 525–532

Romero C, Garcia P, Brenes M, Garcia A, Garrido A (2002a) Phenolic compounds in natural black Spanish olive varieties. Eur Food Res Technol 215:489–496

Romero MP, Tovar MJ, Girona J, Motilva MJ (2002b) Changes in the HPLC phenolic profile of virgin olive oil from young trees (*Olea europaea* L. *cv*. Arbequina) grown under different deficit irrigation strategies. J Agric Food Chem 50:5349–5354

Rotondi A, Bendini A, Cerretani L, Mari M, Lercker G et al (2004) Effect of olive ripening degree on the oxidative stability and organoleptic properties of *cv.* Nostrana di Brisighella extra virgin olive oil. J Agric Food Chem 52:3649–3654

Ryan D, Robards K, Lavee S (1999a) Changes in phenolic content of olive during maturation. Int J Food Sci Technol 34:265–274

Ryan D, Robards K, Lavee S (1999b) Determination of phenolic compounds in olives by reversed-phase chromatography and mass spectrometry. J Chromatogr A 832:87–96

Ryan D, Lawrence H, Prenzler PD, Antolovich M, Robards K (2001) Recovery of phenolic compounds from *Olea* europaea. Anal Chim Acta 445:67–77

Ryan D, Antonovich M, Prenzel P, Robards K, Lavee S (2002) Biotransformations of phenolic compounds in *Olea europaea*. Sci Hortic 92:147–176

Ryan D, Prenzler PD, Lavee S, Antolovich M, Robards K (2003) Quantitative changes in phenolic content during physiological development of the olive (*Olea euro*paea) cultivar Hardy's Mammoth. J Agric Food Chem 51:2532–2538

Saitta M, Salvo F, Di Bella G, Dugo G, La Torre GL (2009) Minor compounds in the phenolic fraction of virgin olive oils. Food Chem 112:525–532

Segura-Carretero A, Carrasco-Pancorbo A, Bendini A, Cerretani L (2010) Analytical determination of polyphenols in olive oil. In: Preedy VR, Watson RR (eds) Olives and olive oil in health and disease prevention. Elsevier Academic Press, Amsterdam, pp 509–523

Selvaggini R, Servili M, Urbani S, Esposto S, Taticchi A et al (2006) Evaluation of phenolic compounds in virgin olive oil by direct injection in high-performance liquid chromatography with fluorometric detection. J Agric Food Chem 54:2832–2838

Servili M, Selvaggini R, Esposto S, Taticchi A, Montedoro G et al (2004) Health and sensory properties of virgin olive oil hydrophilic phenols: agronomic and technological aspects of production that affect their occurrence in the oil. J Chromatogr A 1054:113–127

Servilli M, Baldioli M, Salvaggini R, Macchioni A, Montedoro JF (1999) Phenolic compounds of olive fruit: one- and two-dimensional Nuclear Magnetic Resonance characterization of nűzhenide and its distribution in the constitutive parts of fruit. J Agric Food Chem 47:12–18

Siliani S, Mattei A, Innocenti LB, Zanoni B (2006) Bitter taste and phenolic compounds in extra virgin olive oil: an empirical relationship. J Food Qual 29:431–441

Suárez M, Macià A, Romero MP, Motilva MJ (2008) Improved liquid chromatography tandem mass spectrometry method for the determination of phenolic compounds in virgin olive oil. J Chromatogr A 1214:90–99

Tsimidou M (1998) Polyphenols and quality of virgin olive oil in retrospect. Ital J Food Sci 10:99–116

Tsimidou M (1999) Analysis of virgin olive oil polyphenols. Semin Food Anal High-Perform Liq Chromatogr 4:13–29

Tsimidou M, Litridou M, Boskou D, Pappa-Louisi A, Kotsifaki F et al (1996) On the determination of minor phenolic acids of virgin olive oil by RP-HPLC. Grasas Aceites 47:151–157

Vichi S, Romero A, Tous J, Tamames E, Buxaderas S (2008) Determination of volatile phenols in virgin olive oils and their sensory significance. J Chromatogr A 1211:1–7

Chapter 10
Infrared, Raman, and Fluorescence Spectroscopies: Methodologies and Applications

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Contents

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties*,
DOI 10.1007/978-1-4614-7777-8_10, © Springer Science+Business Media New York 2013

Abbreviations

ANN	Artificial neural network
ATR	Attenuated total reflectance
CCD	Coupled charge device
CV	Coefficient of variation
EEFS	Excitation-emission fluorescence spectroscopy
FF	Front face
FT	Fourier transform
IRE	Internal reflection element
MIR	Mid-infrared
MLR	Multiple linear regression
MSE	Multiple standard error
NIR	Near-infrared
NMR	Nuclear magnetic resonance
OPL	Optical path length
PCA	Principal component analysis
PCR	Principal component regression
PLSR	Partial least-squares regression
PLS	Partial least squares
PLSDA	Partial least-squares discriminant analysis
RA	Right angle
RHM	Resampling by half-means
SD	Standard deviation
SFS	Synchronous fluorescence spectroscopy
SHV	Smallest half-volume
SLDA	Stepwise linear discriminant analysis
SMD	Squared Mahalanobis distances
SMLR	Stepwise multiple linear regression
SVM	Support vector machines

10.1 Introduction

Spectroscopic techniques have emerged in food analysis as rapid and very useful tools for determining a great variety of chemical parameters. They provide elegant solutions to face analytical challenges. In spite of the intense research on

spectroscopic techniques during the twentieth century, the application of such techniques has been delayed due to the spread of chromatography, which allows an easy quantitative interpretation of results, and the lack of suitable sample presentation techniques, chemometric tools to calibrate and standardize instruments, intuitive chemometric tools, and standardized protocols for spectroscopy. However, the necessity of reducing the analytical time and cost, the high number of parameters and properties to be simultaneously controlled at the different steps of the food and feed chains, and the increasing demand for online techniques, as well as the relative limit of traditional techniques to solve some analytical questions faced by the control laboratories and industries, have rekindled interest in spectroscopy techniques. Furthermore, instrumental improvements such as the introduction of interferometry methodology and the diode array detector, the availability of new sample-handling accessories, the miniaturization of instruments, the computer facilities, and the existence of software specially designed to extract and to use the information contained in spectra have contributed to the development of near-infrared (NIR), mid-infrared (MIR), Raman, and fluorescence spectroscopies. These significant improvements have led to less complicated and expensive instruments that could be used on a regular basis at any laboratory without requiring any special skills or training.

In the field of fats and oils quantitative applications are relatively recent compared to qualitative methodologies. These quantitative procedures have benefited from new ways of calibration (e.g., signal-transduction calibration), adapted accessories for sample presentation (e.g., ATR, IR cards, and mesh cells), and adopted new procedures of spectra interpretation (e.g., 2D correlation spectroscopy). The great variety of optical materials and sampling approaches makes the spectroscopic techniques much more versatile than other methodologies, which explains the growing interest in developing quantitative applications. Although there are only few standard methodologies for olive oil analysis based on spectroscopy (e.g., determination of dienes and trienes by ultraviolet spectroscopy, COI/T.20/Doc. No 19/Rev. 2), a spectroscopic technique such as Fourier transform infrared spectroscopy (FT-IR) can be applied to determine the unsaturation degree, oxidation state, moisture content, *trans* double bonds, free fatty acids, and the presence of impurities or other edible oils, among many others. Such applications require more research to improve calibration performance without losing the advantageous feature of being rapid methods. Such research might deliver methodologies that could be eligible as standard methods in the future to alleviate complex olive oil analysis.

In this chapter, the second and third sections briefly present the theory and instrumentation currently used in IR, Raman, and fluorescence spectroscopies for the analysis of oils. The fourth section describes data acquisition, and the fifth section is dedicated to interpretation of oil spectra. The assignment of the most noteworthy bands and the correlation between absorption (or scattering) intensities and chemical indices are discussed. Part of this chapter (the sixth section) is devoted to the data treatment of IR and Raman spectra. In this section, a mathematical model construction in quantitative and qualitative analyses is presented. Finally, the results obtained in the determination of chemical values and indices are surveyed in the seventh section.

10.2 Theory

The importance of spectroscopy becomes apparent from a reading of the classic text published by Herzberg (1945). However, it was not until recently that dramatic progress was made with the advent of IR lasers (e.g., IR circular dichroism) and inteferometric methods, the introduction of high-power and pulsed lasers (e.g., hyper-Raman and coherent anti-Stokes-Raman scattering), or attenuated total reflection (ATR) spectroscopy, among others. Thus, spectroscopy is not a static field; it is a quite dynamic and innovative area. Regarding vibrational spectroscopy, the basic theory has been described in ten or so classic books on spectroscopy, some of which are compilations of data while others are comprehensive texts (Wilson et al. 1955; Williams and Norris 2001; Li-Chan et al. 2010a, b). Most practical books usually emphasize the correlation between molecular structural features and frequencies (Socrates 1994), while textbooks are devoted to explaining the theory of vibrational spectroscopy (Williams and Norris 2001; Diem 1993). Concerning fluorescence spectroscopy, modern manuals explaining the fundamentals and applications illustrate the increasing interest in this technique for developing applications beyond basic research (Valeur 2002).

The following section will briefly describe those theoretical aspects of IR, Raman, and fluorescence spectroscopies that are basic for understanding spectroscopic analyses.

10.2.1 Infrared Spectroscopy

Infrared spectroscopy is a technique in which the interaction of electromagnetic radiation with a sample is studied to obtain both qualitative and quantitative chemical information. The IR region lies between the red end of the visible spectrum and the microwave region. It comprises wavelengths (λ) between 800 and 2.5×10^5 nm. The IR region of the electromagnetic spectrum is subdivided into NIR (λ=0.8–2.5 μm), MIR (λ=2.5–25 μm), and far-IR (λ=50–1,000 μm). These distinctions are based on the nature of the absorptions giving rise to the corresponding spectra, as well as differences in instrumental design and experimental approach. All are parts of vibrational spectroscopy and arise from transitions between vibrational energy levels (Banwell 1994).

The simplest approach to explaining the phenomenon occurring in vibrational spectroscopy is to consider the bond between two atoms of masses m_1 and m_2 as behaving as a tiny spring of "strength," or force constant k ($N^* m^{-1}$). The system will vibrate at some natural resonance frequency ν (s^{-1}) given by Hooke's law:

$$\nu = \frac{1}{2\pi}\sqrt{\frac{\kappa}{\mu}},$$

where μ is the "reduced mass" $[m_1m_2/(m_1+m_2)]$. This approach is used to explain the observed difference in absorption frequencies between different functional groups on the basis of different force constants or reduced masses.

However, the quantum theory needs to be considered here. The energy E (J) of a photon of wavelength λ (m) is

$$E = h\nu = h\frac{c}{\lambda}.$$

In this equation h (Js) is Planck's constant and c (ms^{-1}) is the velocity of light.

Hooke's law for a simple harmonic oscillator model predicts a potential energy curve as a parabolic function of the interatomic distance. The potential energy is minimized at the equilibrium nuclear distance. Increasing interatomic distance leads to increased potential energy in a continuous manner. In a quantum mechanical approach (corpuscular theory), however, only certain energy levels are permitted. These energy levels are given by

$$E(n) = \left(n + \frac{1}{2}\right)h\nu,$$

where $n = 0,1,2\ldots$ is the vibrational quantum number. Transition between energy levels can only occur in discrete steps when sufficient energy E is provided, i.e.,

$$\Delta E = h\nu.$$

Transitions occur when $n \geq \pm 1$. The molecule will absorb the energy of the photon if it precisely matches the energy that is required for the transition between energy levels and when there is a change in the dipole moment associated with the vibration. The transitions in which $n = \pm 1$ are called fundamental vibrations and they are observed in MIR spectroscopy. The energy required to stimulate these transitions occurs at wavelengths between 2,500 and 25,000 nm (4,000–400 cm^{-1}).

In fact, vibrating bonds are anharmonic oscillators. When the interatomic distance becomes very small, atomic repulsion causes the potential energy to rise dramatically. As the interatomic distance increases, the bond will initially stretch and eventually break. This anharmonic behavior can be incorporated into the Schrödinger equation and leads to a new expression for permitted energy levels:

$$E(n) = \left(n + \frac{1}{2}\right)h\nu - \left(n + \frac{1}{2}\right)^2 x_e h\nu,$$

where x_e is a small and positive anharmonicity constant.

As a result of anharmonicity, energy levels become closer as n increases and transitions of the type $n = \pm 2$, $n = \pm 3$, or overtones are allowed. In addition, a combination band is produced when the photon excites simultaneously the vibration of two or more interatomic bonds that are sufficiently close to influence their respective vibrations. Combinations and overtones are seen at higher energy (lower wavelength) and occur in the NIR region (800–2,500 nm; 10,000–4,000 cm^{-1}). These bands

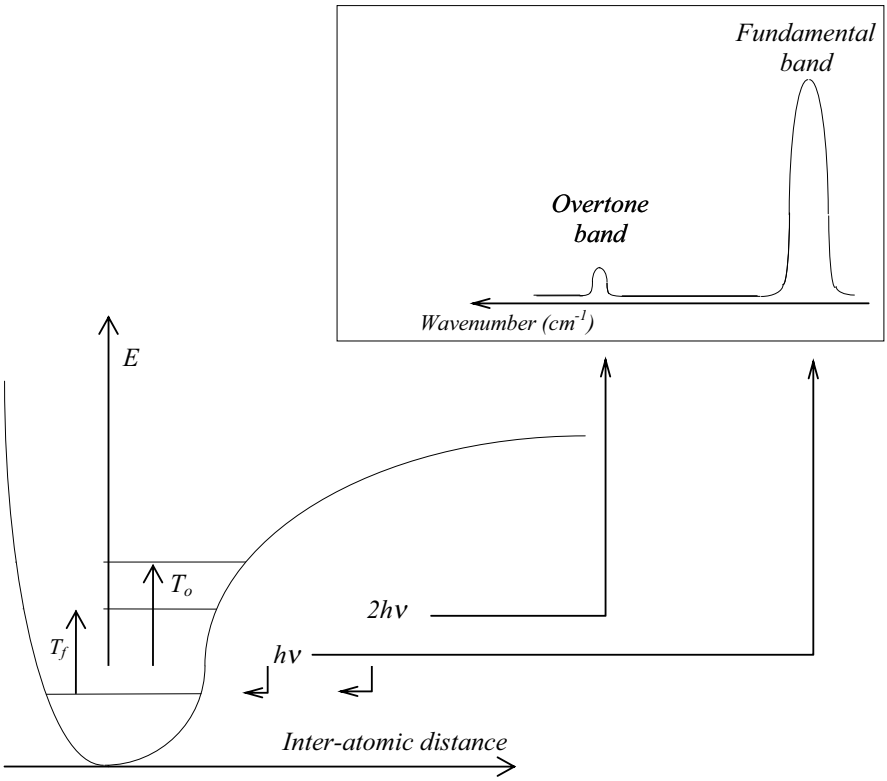

Fig. 10.1 Anharmonic oscillator and associated energetic transitions. Legend: E potential energy, T_f fundamental transition, T_o overtone transition

have lower intensity than fundamental bands. Figure 10.1 illustrates the anharmonic oscillator and the different associated energetic transitions (Barrow 1973; Williams and Norris 2001; Skoog et al. 1992).

10.2.2 Raman Spectroscopy

Raman spectroscopy involves a scattering process. When the electric field E interacts with a molecule, it exerts the same force on all electrons in the molecule and tends to displace them from their original position around the positively charged nuclei. The displacements result in an induced dipole moment π in the molecule that is proportional to the electric field:

$$\pi = \alpha E,$$

where α is the electric polarizability. Since π depends on α as well as E, the properties of the molecule can change π. In this context, α varies with time as a consequence of the vibrations of the molecule since the ease with which electrons may be displaced by the electric field depends on how tightly they are bound to the nuclei, which in turn depends on the interatomic distance.

When the electric field interacts with a vibrating molecule, the induced dipole moment has three components contributing to its time dependence. The first is a component vibrating with the frequency of the incident light. According to classical electromagnetic theory, an oscillating dipole radiates energy in the form of scattered light. Thus, the first component, light of the incident frequency (ν_{Ray}), will be scattered. This is the phenomenon of Rayleigh scattering. The second component is the one vibrating at a frequency that is the sum of the frequencies of the incident light and the molecular vibration. The scattered light arising from this second component is known as anti-Stokes Raman scattering ($\nu_{R(aSt)}$). The third component is the vibration at a frequency given by that of the incident light minus the molecular vibrations. This is called Stokes scattering ($\nu_{R(St)}$) (Grasseli and Bulkin 1991; Diem 1993; Schrader 1996).

Figure 10.2 shows an energy diagram of the Raman scattering effect and illustrates a schematic and simplified Raman spectrum. To simplify the presentation, only two electronic states (the ground and the first excited) and three vibrational states of each of them are shown. The intensity of the anti-Stokes Raman scattering bands of frequency < *R(aSt)* is lower than the intensity of Stokes Raman scattering bands of frequency < *R(St)* in view of the difference in population of the ground excited electronic states in a set of molecules at room temperature (Baranska et al. 1987). The Raman spectra studied and presented later on in this chapter concern only Raman Stokes scattering bands.

10.2.3 Fluorescence Spectroscopy

Fluorescence spectroscopy is a type of electromagnetic spectroscopy in which the fluorophore groups included in the samples are excited using a beam of light. Usually ultraviolet light is used and the emission of light of a lower energy is observed; typically, but not necessarily, the emission is in the visible range of the electromagnetic spectrum. In particular, conventional fluorescence spectroscopy provides an emission spectrum for a fixed excitation wavelength or an excitation spectrum for a fixed emission wavelength. The emission spectra are obtained by recording the signal of an emission monochromator at different wavelengths (λ_{em}) for a constant excitation wavelength (λ_{ex}), usually at a wavelength of high absorption. On the other hand, the excitation spectra are obtained by recording the signal from the excitation monochromator at different wavelengths (λ_{ex}), maintaining a constant emission wavelength (λ_{em}). The spectra provide information for both qualitative and quantitative analyses about fluorophore groups present in the sample. However, the applications of fluorescence spectroscopy in the characterization of edible oils are scarce because the fluorescence

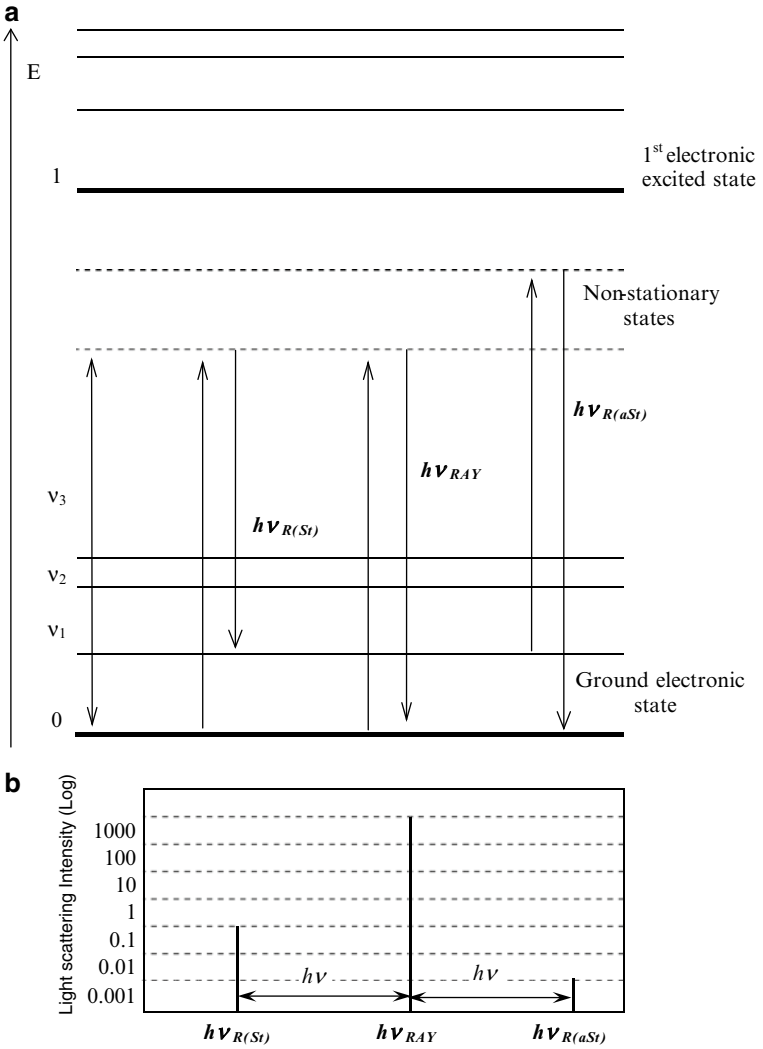

Fig. 10.2 (**a**) Raman scattering effect occurring during illumination of sample with monochromatic light. (**b**) Part of resulting Raman spectrum. Legend: *E* electronic state, *I* light-scattering intensity (log scale), ν_{Ray} frequency of Rayleigh line, $\nu_{R(aSt)}$ frequency of anti-Stokes Raman line, $\nu_{R(St)}$ frequency of Stokes Raman line (Adapted from Baranska et al. 1987)

characteristics of fluorophores are affected by the matrix. Although molecular fluorescence spectroscopy is a highly sensitive technique, a severe overlap of excitation and emission makes the spectra difficult to interpret (Patra and Mishra 2002). The fluorescence spectra can also be affected by the attenuation of the absorption intensity due to the absorption of the excitation wavelength (primary inner effect) and the emission wavelength (secondary inner effect) (Sikorska et al. 2004). These phenomena are more evident when working with right angle (RA) instruments (Lakowicz 1999). In RA

instruments, the collection of the fluorescence beam is collected at a right angle to the incident light. In other words, the emission is measured at 90° in relation to the excitation beam. In contrast, in modern front face (FF) instruments, the fluorescence beam is collected at an approximately 22–30° angle relative to the incident beam. This geometry minimizes the inner filter effects compared to RA instruments.

10.2.4 Band Position and Intensity

In vibrational spectroscopy, the probability of excitation for a particular vibration is determined by the so-called selection rules, which can be derived from the application of group theory to atomic vibrations in the molecules belonging to different classes of symmetry. Some factors tend to modify the band positions (i.e., vibration frequencies). The most important factors are the interatomic distances, the spatial arrangement groups, the Fermi resonance, the physical state of the sample, the polarity of the environment, the formation of hydrogen bonds, and the inductive, mesomeric, and field effects of neighboring groups. In this way, the difference in the force constant, for example, explain that the stretching frequency of double bonds is higher than those of single bonds (Baranska et al. 1987; Grasseli and Bulkin 1991; Diem 1993).

Infrared and Raman spectroscopy involve vibrational energy levels of the sample molecules that are related primarily to stretching or bending deformations of the molecular bonds. However, two main differences should be underlined between IR and Raman spectra. First, IR peaks tend to be broad and it is difficult to find a peak that is completely free of the influence of adjacent peaks or external parameters. On the other hand, a Raman spectrum tends to be composed of a series of isolated bands, and water and CO_2 have weak Raman scattering properties and, consequently, produce less interference in Raman scattering spectroscopy. Another difference is that polar groups (such as C=O and O-H) have strong IR absorption bands, whereas nonpolar groups (such as C=C and C-C) show intense Raman scattering bands. These two branches of vibrational spectroscopy in fact yield complementary information about molecular vibration, each one contributing to a spectral fingerprint of the molecules (Li-Chan 1994).

From a chemical point of view, Raman scattering arises from the change in polarizability or shape of the electron distribution in the molecule as it vibrates; in contrast, IR absorption requires a change in the intrinsic dipole moment with the molecular vibration (Grasseli and Bulkin 1991). More accurately, the Raman band intensity is proportional to the expression

$$\left(\partial\alpha / \partial Q\right)^2,$$

where α is the polarizability and Q the normal coordinate of the group of atoms of interest. The IR band intensity is proportional to the expression

$$\left(\partial\pi / \partial Q\right)^2,$$

where π is the induced dipole moment of the molecule. Thus, it might be expected that the same molecule may give IR and Raman bands with differing intensities and band shapes (Baranska et al. 1987).

Concerning fluorescence spectroscopy, to study the band position and intensity it is necessary to consider the following issues:

1. The excitation wavelength used to obtain the emissions spectra should be strongly absorbed by the fluorescent compounds; therefore it is recommended to obtain the full absorption spectrum of the sample and then select the most appropriate wavelength based on the maximum absorption intensities. It is important to use an excitation wavelength that is strongly absorbed because the emission fluorescence intensity is proportional to the absorption intensity.
2. Not all of the emission spectrum obtained with the selected excitation wavelength corresponds to the fluorescent compounds present in the sample. According to Stokes's law of fluorescence states, the wavelength of fluorescence radiation is greater than the exciting radiation. Consequently, the emission wavelengths should be at least five or ten units larger than the excitation wavelength. For example, for an excitation wavelength (λ_{ex}) at 350 nm, the bands that appear in the emission spectrum at wavelengths below 360 nm do not correspond to fluorescent compounds.
3. Other additional considerations that could lead to error are associated with overtones. Thus, it is important to note that the overtone area is located at twice the wavelength of excitation in the emission spectrum. In the interpretation of the spectra it is also convenient to omit the region of the spectrum that is located too far from the excitation wavelength (Fig. 10.3).
4. Primary and secondary inner filter effects are other considerations that should be taken into account in the traditional RA techniques (Lakowicz 1999). The inner filter effects imply the attenuation of the emission intensity due to the absorption of the incident excitation light and emitted light (Sikorska et al. 2004). These effects are avoided by working with diluted samples – in the case of oils, 1 % is enough. This solution also prevents saturation in the spectrum. Nevertheless, the spectra obtained from diluted samples are not always comparable to those obtained with original undiluted samples. This difference in the spectra is due to the original environment of the samples, which dramatically changes when they are diluted, and this could have a significant effect given that fluorescence properties are extremely sensitive to matrix changes (Strasburg and Ludescher 1995). To overcome this problem and examine native samples directly, the FF technique is more appropriate.

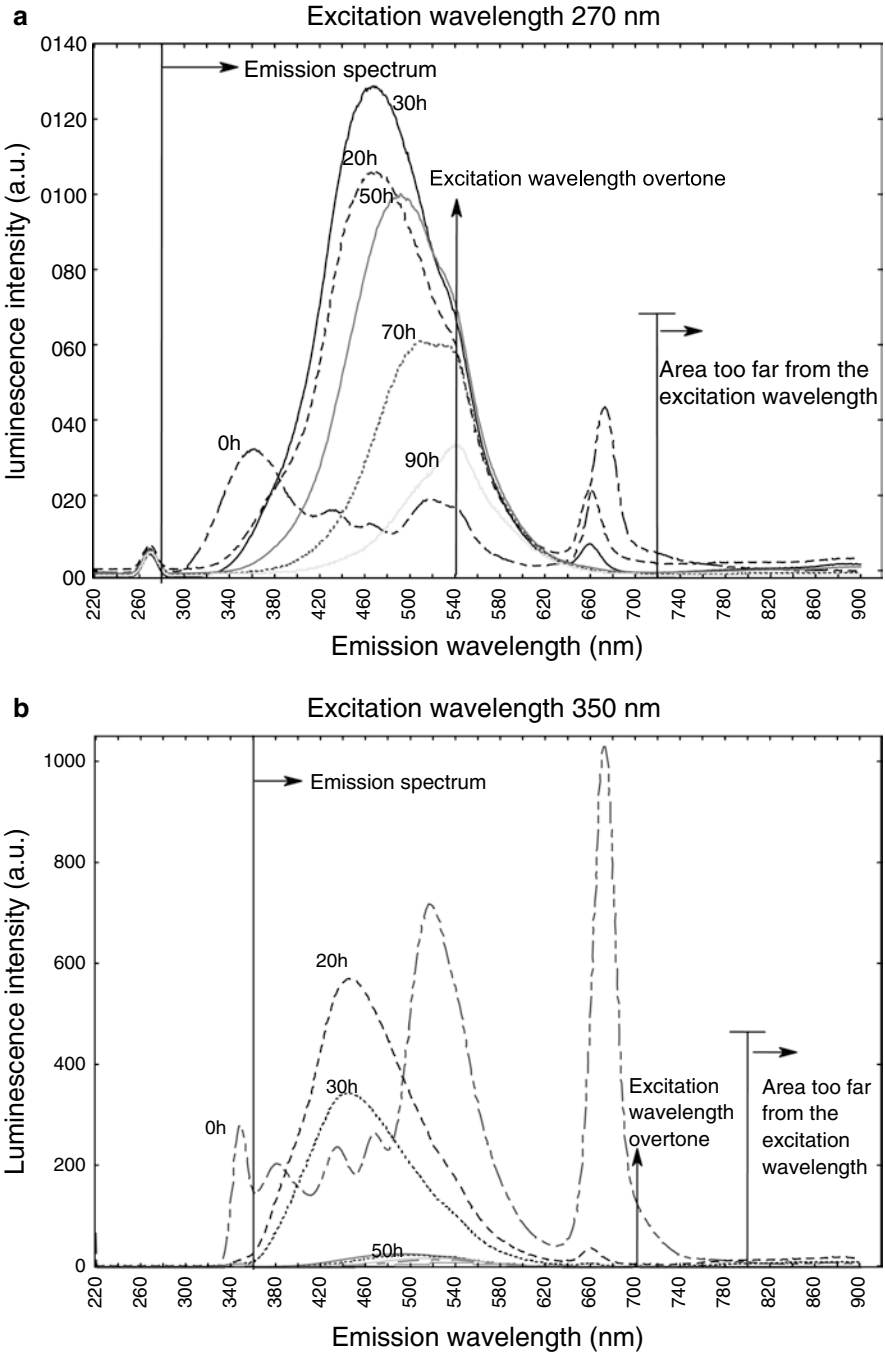

Fig. 10.3 Fluorescence emission spectra of virgin olive oils differing in their thermoxidation times (hours) collected under two different excitation wavelength: $\lambda_{exc} = 270$ nm (**a**) and $\lambda_{exc} = 350$ nm (**b**)

10.3 Instrumentation

Two of the main reasons for the development of new applications of spectroscopic techniques are the simplicity of the equipment and the sample presentation. Samples can be examined in their gaseous, liquid, or solid states. Enormous progress has been made, particularly over the two last decades, on the instrumental front (Diem 1993; Sharma and Schulman 1999; Li-Chan et al. 2010a).

Spectrometers can be classified according to the radiation source used, either thermal or nonthermal. Thermal sources (e.g., quartz-halogen or tungsten-halogen lamps) consist of a radiant filament that produces thermal radiation covering a narrow or wide range of frequencies in the vibrational spectral range. Nonthermal sources (e.g., light-emitting diodes, laser diodes, or lasers) emit narrower bands of radiation than those emitted by thermal sources. Another classification of spectrometers is based on the wavelength selection strategy: discrete or continuous wavelength selection. Discrete wavelength instruments, using filters or light-emitting diodes, make it possible to collect the absorbance at specific wavelengths and are not widely used. Continuous-spectrum instruments are based on grating monochromator, acousto-optical tunable filter, photodiode array, Fourier transform (FT) interferometer technologies, or microelectromechanical systems (MEMS) (Osborne et al. 1993; Williams and Norris 2001; Blanco and Villaroya 2002).

10.3.1 Near-Infrared (NIR) Spectroscopy

NIR instruments have been widely used for nondestructive rapid analysis in several important industries since the early 1970s. In the animal feed, grain, chemical, pharmaceutical, and food industries, NIR spectroscopy is used in offline, online, and inline modes. Several optical approaches have been used in NIR instruments, including filters, holographic gratings, acousto-optically tunable filters, light-emitting diodes, and the internal and external fitting of optic fibers (Scotter 1997; Osborne et al. 1993; Williams and Norris 2001).

Four configurations of spectral collection exist: transmission, transflection, diffuse reflection, and interactance. This has been addressed in detail by Wilson and Goodfellow (1994). In oil analysis, transmission and transflection modes are traditionally used and correspond to specific sample-handling designs. An important feature of NIR spectroscopy is that the shorter NIR wavelengths can penetrate deeply into the sample; thus, it is possible to obtain spectral data from a thick sample (i.e., 1–5 mm). In addition, classic crystal and quartz materials are free of absorbance in the NIR region.

A transmission cell is used to obtain spectra of liquids and slurries. To make a transmission measurement, the sample accessory is placed between the source and

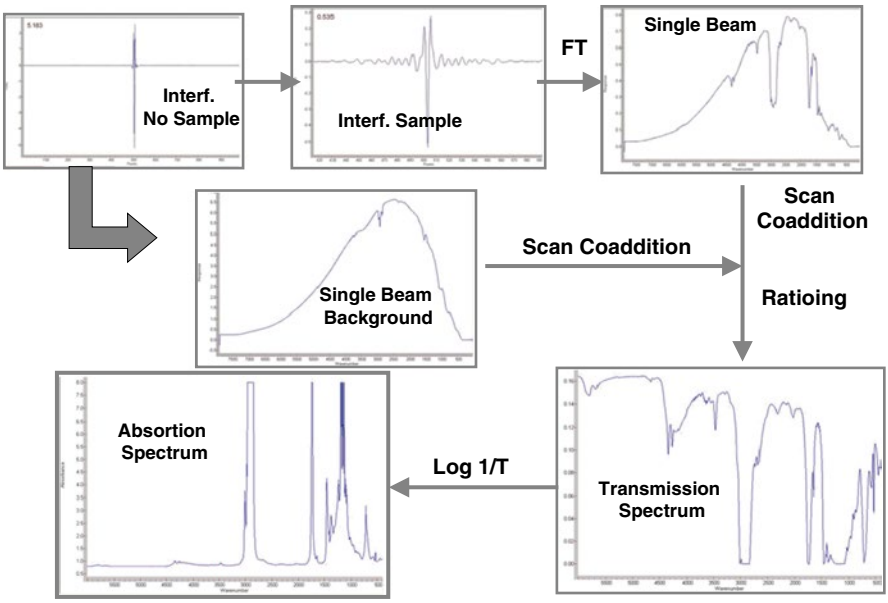

Fig. 10.4 Processing of signals in infrared spectroscopy from interferogram to absorption spectrum

the detector. The sample is introduced into the cell specially designed to have a constant sample thickness. Transmission cells are usually constituted by two crystal windows separated by spacers of different thicknesses, quartz cuvette of fixed thickness (e.g., 1 or 5 mm), or by disposable vials of fixed width (Williams and Norris 2001).

Transflection cells are designed for making transmittance measurements with instruments that are designed only to collect reflectance spectra (i.e., instruments where the source and the detectors are on the same side). A classic transflection cell is an aluminum cup covered with a slide glass (crystal or quartz) and having a gold plate as reflector. The energy traverses the sample once, is then reflected on the gold reflector, and traverses back to the sample before reaching the detector.

10.3.2 Mid-Infrared Spectroscopy

Until recently, MIR spectroscopy has been of limited use for the study of food materials due to a number of drawbacks. Food samples are often opaque and highly scattering. Furthermore, they often contain high concentrations of water, which absorbs strongly in the MIR region. Food materials, therefore, are not very

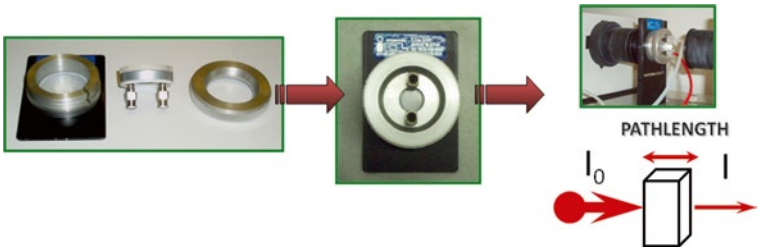

Fig. 10.5 Pictures of a typical transmission cell, demounted, assembled, and set up in FT-IR spectrometer

amenable to classic transmission techniques and sampling methods such as pellets or mulls (Wilson 1990). A second factor limiting the use of MIR spectroscopy with food samples has been that classic instrumental methods suffered from a lack of speed and from a low energy level of the sample due to the use of monochromators. However, the development of new sampling methods together with FT instruments have now made it possible to routinely analyze food samples by MIR spectroscopy (Wilson 1990; van de Voort and Ismail 1991).

The use of a Michelson interferometer allows much more energy to reach the sample, provides good wavelength reproducibility, and allows spectra to be collected in a very short time. Figure 10.4 shows the basic processing from the interferogram registered by a Michelson interferometer to transmission and absorption spectra. Apart from these combined advantages, it is worth noting that handle sampling is a major issue and is conditioned by the viscosity of the sample. FT MIR spectroscopy has made viable sample presentation techniques for edible oils, thus overcoming some of their analytical problems with MIR spectroscopy. The most important MIR sample presentation techniques applicable to oil analysis are transmission liquid cell and attenuated total reflectance (ATR) crystal, which are described in detail by Wilson and Goodfellow (1994). Both methods require a minimum of sample preparation.

10.3.2.1 Transmission Cells

Transmission cells allow for FT-IR analysis in transmission mode. In this mode the sample is located in the optical path of the IR beam (I_0). Figure 10.5 shows a typical transmission cell.

Liquid samples, such as virgin olive oil, are normally injected into the cell to form a thin-film squeezed between two windows. There are three main types of transmission cell, all employing metal frame plates, windows to enable light to enter and leave the sample, and spacers that define the optical path length (OPL). Thus, sealed cells employ permanently bonded spacers of a fixed thickness. This first type of cell is suitable for quantitative analyses, where an invariable OPL is required. The

Table 10.1 Main characteristics of window materials used for transmission cells in FT-IR spectroscopy

Window material	Working range (cm^{-1})	Refractive index	Advantages/disadvantages
NaCl	40,000–600	1.5	Low cost
			Highly hygroscopic; slightly soluble in alcohol; breaks easily
KBr	43,500–400	1.5	Low cost; good resistance
			Hygroscopic; soluble in alcohol and slightly in ether
KCl	33,000–400	1.5	Low cost
			Hygroscopic
CaF_2	77,000–900	1.4	Insoluble in water; resists most acids and bases; high hardness (suitable for high-pressure works)
			Expensive
BaF_2	66,666–800	1.5	Insoluble in water
			Soluble in acids and NH_4Cl; sensitive to mechanical shock
CsBr	42,000–250	1.7	Extended IR range
			Soluble in water and acids
CsI	42,000–200	1.7	Easier to handle than CsBr
			Hygroscopic; does not cleave; easily scratched
AgCl	25,000–434	2.0	Insoluble in water; inexpensive
			Darkens under UV radiation; corrosive to metals
KRS-5[a]	20,000–285	2.37	Insoluble in acids; does not cleave
			Slightly water soluble
ZnSe	10,000–555	2.20	Insoluble in water and weak acids and bases
			Expensive; brittle; must be handled with care
ZnS	10,000–714	1.5	Insoluble in water and weak acids
			Expensive; slightly soluble in acids (HNO_3, H_2SO_4, KOH)

[a]a mixed thallium bromide-thallium iodide

second kind, the demountable cells, may be dismantled to facilitate cleaning and enable the use of spacers with different thicknesses and, hence, different OPLs. Finally, piston cells enable the window separation to vary continuously over a range of OPLs. In any case, the OPL variations can be controlled by adding an internal standard with a known and distinct absorption (Ismail et al. 2006).

Liquid cells enable reasonable quantification of solute concentrations. Practical difficulties include the maintenance of a constant (repeatable) OPL and good window parallelism (to avoid wedging errors). The cell windows should be constructed from a material that is transparent to the MIR beam and, additionally, does not react with the samples. Thus, windows are commonly made of polished salt crystals (Table 10.1) that transmit IR radiation. Other materials with covalent bonds (e.g., glass) lack this property and, in consequence, cannot be used as window material. On the other hand,

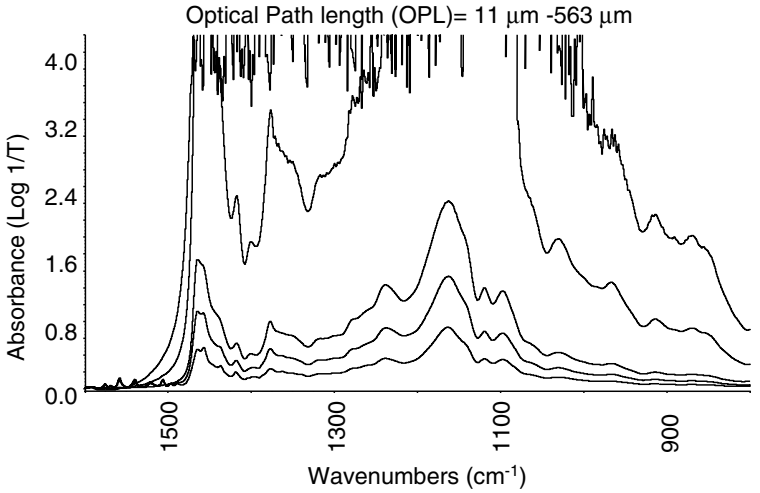

Fig. 10.6 Spectra of oils collected at different path lengths with KCl cell using spacers of several thicknesses (0.015–0.5 mm). Note: Spectra with larger OPL are off-scale

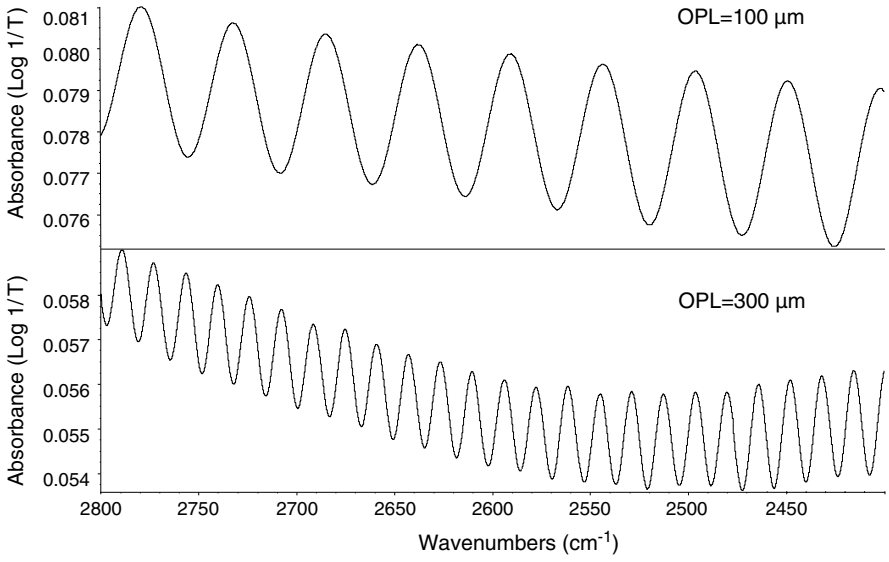

Fig. 10.7 Spectra collected from two empty transmission cells with optic path lengths (*OPL*) of 100 and 300 μm

given the limited energy provided by the IR source, strong absorbance by the solvent or nontarget chemicals may dominate the absorbance spectrum, obscuring weaker absorbance bands. Therefore, in some cases it is necessary to use very short OPLs (below 10 μm), which are difficult to produce and measure reliably.

One key aspect when operating with transmission cells in quantitative analysis is to know precisely the OPL to allow a correct calibration. The intensity of the IR spectral bands is determined by the OPL, which ultimately means the amount of sample between the two windows (Fig. 10.6). Then, an accurate quantitative analysis implies working under a constant and known OPL.

The procedure for determining the OPL is particularly important in demountable cells, and it should be carried out after cell assembly and prior to acquiring the spectra to make sure that no significant OPL change has resulted from the manipulation of the cell. One procedure consists in acquiring a spectrum with an empty cell. The spectrum (Fig. 10.7) recorded from the empty cell is characterized by a sinusoidal line with fringes (peaks) and valleys. The OPL is calculated by counting these interference fringes between two wavenumbers and applying the following equation:

$$OPL = \frac{n}{2(\overline{\nu}_2 + \overline{\nu}_1)},$$

where n is the number of peak-to-peak fringes, and

$\overline{\nu}_1$, $\overline{\nu}_2$ are the wavenumbers of the considered range.

Depending on the intensity of the IR band under study, the desirable OPL may lie below 100 μm. This short path length entails a difficult sample handling in the case of viscous liquids such as edible oils. For that reason, some new accessories have been designed for this particular case to ease sample loading (in the absence of bubbles) and cell cleaning. Thus, van de Voort (1994) developed a temperature-controlled transmission flow cell accessory that allows for the routine use of the FT-MIR technique in the quality controls of fats and oils. The instrument is composed of the basic FT-MIR spectrometer, a computer that controls the instrument, a temperature controller, the sample-handling accessory inlet, and control valves. All components of the sample accessory are heated (usually to 80 ± 0.2 °C) so that the sample can easily flow in the lines or the cell. The system includes a bypass line to flush out the bulk of the previous sample, which avoids having large samples pass through the cell and minimizes the cross contamination. In so doing, it is not necessary to clean the accessory between each spectral acquisition. In summary, an oil sample is heated in the test tube block, presented at the input line, and aspirated into the cell using the three-way valve.

Another approach to facilitate sampling of viscous oils is based on the concept of spectral reconstitution (SR) (van de Voort et al. 2007a). SR involves dilution with a less viscous liquid. The spectra of diluted samples are then converted into good facsimiles of the spectra of the neat oils, without a priori knowledge of the precise dilution factor. The dilution factor is calculated from an internal IR spectral marker

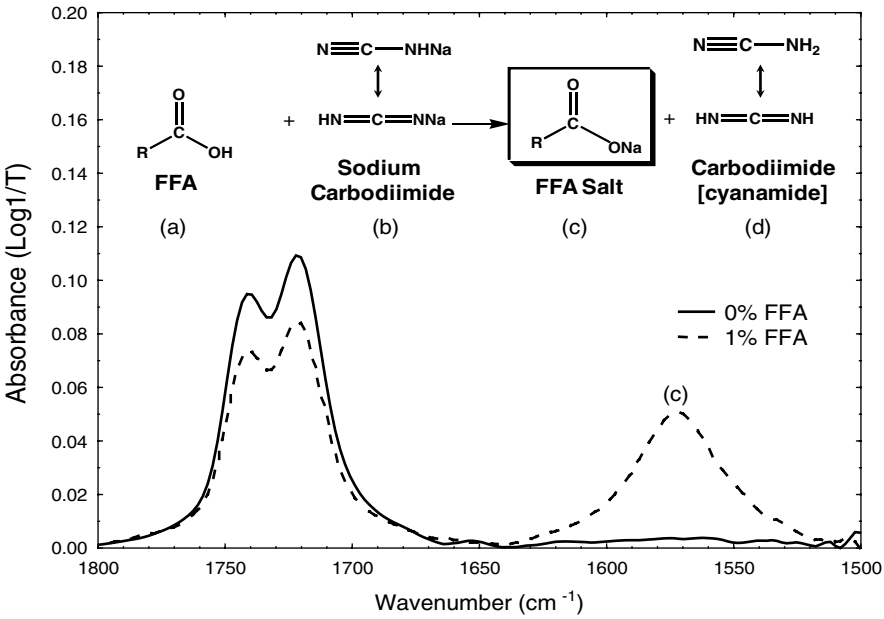

Fig. 10.8 Stoichiometric reaction associated with infrared analysis for free fatty acid (*FFA*) content and corresponding spectral changes taking place

that is added to the less viscous liquid and that does not interfere with the bands of the sample. The relation of the spectral bands of the marker in the less viscous liquid and the diluted samples gives information about the exact dilution factor (van de Voort et al. 2008). This procedure eliminates the need for a peristaltic pump, reduces sample volumes (from approximately 100 mL to approximately 5 mL), increases the number of samples per hour (up to 120 samples/h), and eliminates the need for solvent rinses, thereby drastically reducing disposal volumes.

The analysis of viscous samples with transmission cells can also be facilitated by the method of signal transduction-dilution. This method has been used mainly to measure acidity in mineral and edible oils (Li et al. 2009). In this procedure the chemical component to be characterized (e.g., acidity) is extracted with an oil-immiscible solvent (e.g., methanol) with a reagent (e.g., hydrogen cyanamide, NaHNC≡N) that reacts with the chemical component; this results in a measurable band. Figure 10.8 shows this stoichiometric reaction and the spectral changes that allow an accurate measurement of free fatty acid percentage. This procedure has been adapted to be performed in automated (Yu et al. 2009) and portable instruments (Li et al. 2008). The automated instrument (COAT, Thermal-Lube, Pointe-Claire, QC, Canada), also used with SR, includes a demountable IR cell, pumps, and valves to aspirate the samples, an autosampler for automated analysis, and a specific software (UMPIRE) that automatically analyzes different chemical features

Table 10.2 Commercial FT-IR sample cards and properties

Commercial name	Manufacturer/distributor	Film materials	Pathlength (μm)
3M IR cards[a]	3M	PE[b], PTFE[c]	10 and 100
PTFE and PTIR cards	International Crystal Laboratories	PE[b], PTFE[c]	Unknown
Real Crystal IR cards	International Crystal Laboratories	NaCl, KBr, KCl	Unknown
DOT.IR cards	PSI Performance Systematix	PTFE	Unknown
ST-IR cards	Thermo Scientific[d]	PE, PTFE	~10

[a]Discontinued
[b]Polyethylene
[c]Polytetrafluoroethylene
[d]Initially commercialized by Thermo Nicolet

and performs the mathematical operation necessary for SR (van de Voort et al. 2007b). On the other hand, the portable instrument (InfraSpec VFA-IR spectrometer, Wilks Enterprise, South Norwalk, CT) is a low-resolution IR spectrometer with no moving parts and an electronically modulated (pulsed) source combined with a linear variable filter mounted on a detector array (VFA). This instrument has also been used coupled with an attenuated total reflection accessory in addition to transmission cells.

Although transmission cells provide a wide range of possible applications, some adaptation and new designs have been presented for a better performance in particular cases. One of these modifications involves the hyphenation of a FT-IR spectrometer and another technique. Transmission flow cells are easier to hyphenate to other techniques in comparison with other FT-IR accessories. Thus, several systems have been mounted to connect a separative technique (e.g., HPLC or GC) to a transmission cell and a FT-IR detector (Vonach et al. 1997; Ahro et al. 2002; Kuligowski et al. 2010). Another modification in the cell is the inclusion of a heater to study the oxidative behavior of edible oils (Ismail et al. 2006), also used in NIR spectroscopy (Gonzaga and Pasquini 2006). The oxidation of edible oils has also been used in disposable cards, whose design is somewhat inspired by classic transmission cells.

10.3.2.2 Disposable IR Cards

Table 10.2 shows a summary of commercial FT-IR cards. The disposable IR cards were developed in the 1990s by 3M for the analysis of liquids or spreadable fats. The cards are made up of a cardboard holder containing a circular IR-transmitting window made of a microporous substrate (polytetrafluoroethylene substrate for 4,000–1,300 cm^{-1} or polyethylene substrate for 1,600–400 cm^{-1} MIR analysis), although some manufacturers are commercializing cards of other materials. The sample is adsorbed on the mycrocrystaline pores of the film material, resulting in an

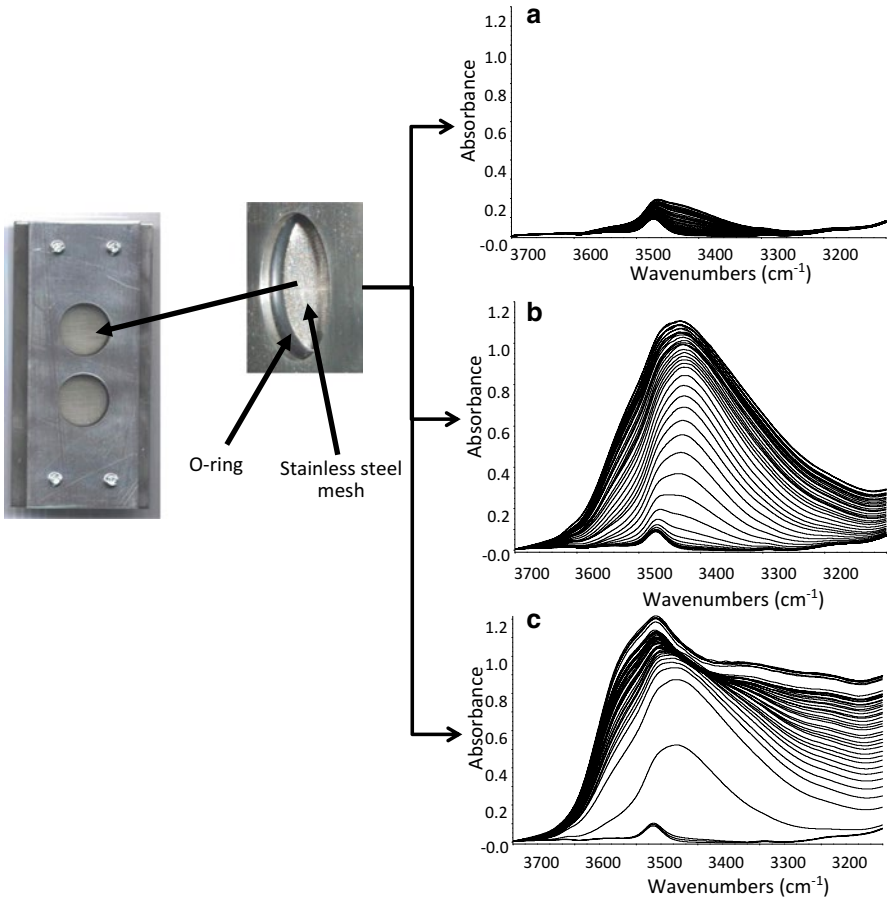

Fig. 10.9 Design of stainless steel mesh cell for infrared spectroscopy and spectra on OH stretching region (3,700–3,100 cm^{-1}) for canola oil in a mesh cell over a period of 42 days kept in the dark (**a**), exposed to room light (**b**), and heated at 50 °C (**c**) (*Source*: García-Gonzàlez and van de Voort (2009), with permission of Applied Spectroscopy)

effective path length of approximately 100 μm. The substrate bands of the microporous material can be subtracted from the sample spectra. A nonporous ring around the aperture prevents the sample from being absorbed by the cardholder. These cards were successfully applied to determine *trans* fatty acid content and the peroxide value (PV) of edible oils (Ma et al. 1998, 1999). Another type of IR cards (Type 2 STIR-PIR cards, Thermo-Nicolet) allows even shorter path lengths but lacks the nonporous ring around the aperture, and in consequence there is not consistency over time.

An improved version of these IR cards is the IR mesh cell (García-González and van de Voort 2009) (Fig. 10.9). Although it can be used for general applications, this

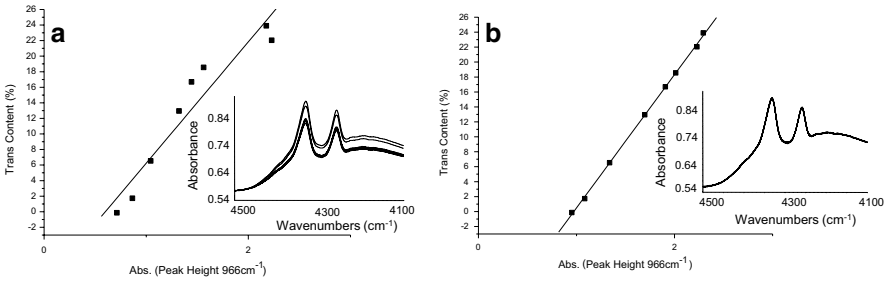

Fig. 10.10 Regression lines relating *trans* fatty acid content and peak height of 966 cm^{-1} band. (**a**) Data without normalization; (**b**) data normalized using CH overtone band at 4,334 cm^{-1} (shown in each panel as an inset) (*Source*: García-Gonzàlez and van de Voort (2009), with permission of Applied Spectroscopy)

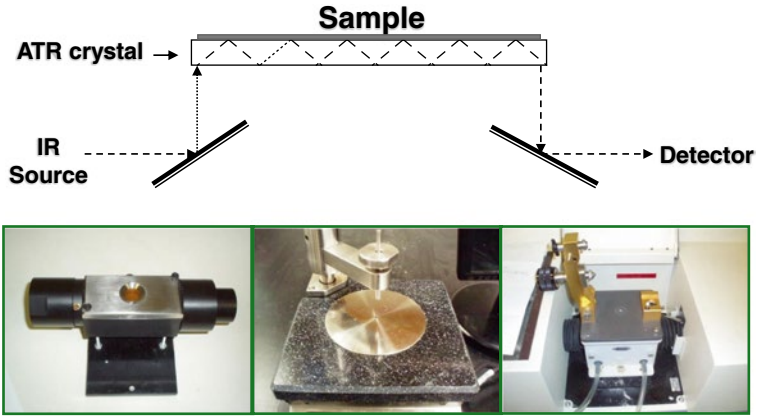

Fig. 10.11 Scheme of working principle of attenuated total reflectance (*ATR*) and three pictures of typical ATR accessories

cell is particularly adequate for running oxidation at moderate temperatures in a wide variety of conditions. The design of this new cell enables one to obtain a fairly consistent path length during the entire time of the experiment. This cell is endowed with a mesh that entraps the oil sample by means of its inherent surface tension. The high surface area provided by the mesh facilitates the rapid oxidation of the oil by air at ambient or slightly elevated temperatures with no need of extreme temperature conditions. These mesh cells are not disposable and can be easily cleaned and reused. Although the effective path length is fairly consistent over the course of the experiment, small changes in the sample thickness can been corrected using the CH combination band region (4,500–4,100 cm^{-1}) as a reference band (García-González and van de Voort 2009). This band provides information on the CH double bonds and, thus, on the amount of sample and the OPL. This normalization procedure makes it possible to obtain reproducible spectra despite the small changes in the OPL

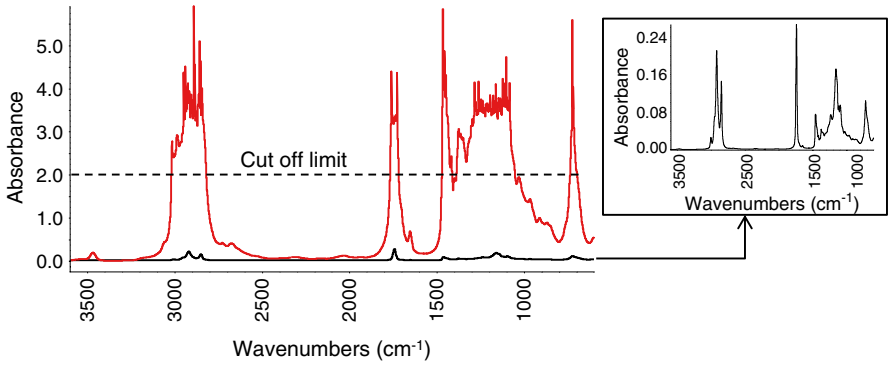

Fig. 10.12 Spectra of an oil collected with an ATR accessory (*black line*) and a transmission cell (*red line*)

over time or between different mesh cells. Thus, Fig. 10.10 shows the significant improvement in *trans* fatty acid calibration when the spectra have undergone normalization by the CH combination band.

10.3.2.3 Attenuated Total Reflectance (ATR)

Methods based on the ATR principle are available in a diverse range of configurations and optical designs. They typically require minimal sample presentation and are particularly suited to study highly absorbing samples such as edible oils. The spectral information arises from the interaction between the sample and the evanescent wave produced in an internal reflectance element (IRE). Infrared light is sent to the crystal at such an angle that it becomes internally reflected. Figure 10.11 shows a scheme of the working principle as well as some examples of ATR accessories, either multibounce or single-bounce (depending on the number of beam reflections within the ATR crystal).

Depending on the geometry and length of the crystal, the light will undergo multiple reflections before emerging from the crystal. At each reflection an evanescent wave is established that decays exponentially into the medium in contact with the crystal. If this medium is absorbing, then there will be a transfer of energy from inside the crystal to the surrounding medium and the emerging beam will be attenuated. ATR does not rely on the sample, which constitutes the surrounding medium, which is transparent or transmitting in the conventional sense (Harrick 1967).

ATR allows opaque or highly scattering samples to be used; the only proviso is that the sample must make intimate contact with the ATR crystal. This condition is fully completed with oil samples. The effective penetration (OPL) at any reflection is very short, typically a few microns, so that ATR can be used to overcome the strong absorption of materials. Thus, unlike the spectra obtained with transmission cells, ATR spectra have no cutoff limit or saturation problem and is suitable to study the whole spectra including the most intense bands (Fig. 10.12). A short effective

path length is obtained with no restrictions on the sample thickness, so the sample is simply poured onto the ATR crystal. The optical paths are very reproducible from one sample to another. ATR then allows easy sample measurement, which is one reason that there has been an upsurge in interest in the MIR region (Wilson 1990). ATR crystals should be constructed from a material with a high refractive index, which is highly transmitting, inert, robust, easily cleaned, and resilient to abrasion and corrosion (including dissolution). Classically horizontal ZnSe, Ge, Si, or diamond crystals with 1, 6, or 12 internal reflections are used in oil analysis (van de Voort 1994a; Baeten et al. 2005; Abbas et al. 2009). Some disadvantages of ATR analysis are the low sensitivity because of the short effective path length (weak bands need to be studied with transmission cells), the significant effect that contaminations on the crystal might have on the collected spectra, and the need for temperature control (the depth of penetration of the IR beam depends on temperature) (Ismail et al. 2006).

10.3.3 Raman Spectroscopy

In the past, the application of Raman spectroscopy in food science was considered to be of very limited use because of fluorescence interference, photodecomposition, wavelength calibration, lack of precise frequency base from scan to scan, and the difficulty of attaining high-resolution spectra with the classic dispersive Raman spectrometer (Chase 1987). However, major instrumental advances have contributed to the widespread use of Raman spectroscopy in recent years (Gerrard and Birnie 1992) and its application in food science (Ozaki et al. 1992; Keller et al. 1993; Li-Chan 1996, 2010a).

First was the demonstration, by Hirschfeld and Chase in 1986, that Raman spectra could be obtained with a FT spectrometer equipped with a Nd:YAG laser (NIR monochromatic light excitation), a Rayleigh rejection filter, and a germanium detector. Later on, the development of compact and reliable diode lasers improved the quality of the commercially available systems. A third contribution to these developments was the use of low-noise, multichannel coupled charge device (CCD) detectors. By coupling the appropriate laser and a CCD detector to a spectrograph, it is now possible to measure Raman spectra in a few seconds without exciting fluorescence. Commercial FT-Raman spectrometers offer a good signal-to-noise ratio, a high IR-light throughput, rapid analysis, and the accuracy of wavelength calibration (Levin and Lewis 1990; Diem 1993; Schrader 1996; Li-Chan et al. 2010a).

FT-Raman spectroscopy is arguably the most versatile and easy-to-use nondestructive analytical procedure developed. In fact, glass and water have a very weak Raman spectrum, making the technique even easier to use. Samples can be measured directly in the bottle in the case of an oil. In addition, a spherical cell, such as a nuclear magnetic resonance (NMR) tube, allows Raman scattering information to be collected easily and rapidly (Schrader 1996). On the other hand, if samples to be investigated cannot be transported to the spectrometer, then optical fibers can be

used (as in NIR spectroscopy). In the range of FT-Raman spectroscopy, quartz fibers have very high transmittance (Lewis et al. 1988; Hendra et al. 1997).

10.3.4 Fluorescence Spectroscopy

Fluorescence spectroscopy, like other vibrational spectroscopic techniques, is characterized by its simplicity of sample presentation. To obtain a fluorescence spectrum, it is necessary to excite a sample with an energy-specific excitation wavelength (λ_{ex}), which comes from an excitation source, passes through a filter or monochromator, and strikes the sample. Then a fluorescent light is emitted in all directions. Some of this fluorescent light passes through a second filter or monochromator, dividing the light into different emission wavelengths (λ_{em}), which reach a detector.

There are two general types of instruments: filter fluorometers, which use filters to isolate the incident light and flourescent light, and the most common spectrofluorometers, which use diffraction grating monochromators to isolate the incident light. Both types of instrument are composed of excitation sources, normally a xenon lamp, filter or monochromator in excitation, filter or monochormator in emission, and a detector. The detector is usually placed at 90° to the incident light beam to minimize the risk of transmitted or reflected incident light reaching the detector. The detectors can be classified as single-channel or multichannel. The difference between them is based on the number of wavelengths that they can detect at a time. Thus, the single-channel detector can only detect the intensity of one wavelength at a time. In contrast, the multichannel type detects the intensity at all wavelengths simultaneously.

Various light sources may be used as excitation sources, including lasers, photodiodes, and lamps such as xenon arcs and mercury-vapor lamps. Of these, only the xenon arc lamp has a continuous emission spectrum, with nearly constant intensity in the range of 300–800 nm and a sufficient irradiance for measurements down to just above 200 nm.

The most common accessory used to analyze the fluorescence spectrum of liquid samples, and vegetable oils in particular, are quartz cuvettes with different paths, internal widths, and volumes.

In addition to the conventional collection of emission spectra with a single excitation wavelength, some fluorometers can be adapted to conduct analyses under two particular modes that provide some advantages over the conventional mode. These particular ways of measuring are commonly known as excitation-emission florescence spectroscopy (EEFS) and synchronic fluorescence spectroscopy (SFS).

10.3.4.1 Excitation-Emission Fluorescence Spectroscopy (EEFS)

EEFS consists in measuring the emission spectra at different excitation wavelengths (λ_{ex}). The result of this measurement is a three-dimensional (3D) excitation-emission matrix (EEM). Compared to conventional fluorescence spectroscopy, this technique improves the selectivity of the method. Its main advantage is that it enables obtaining simultaneous information about the different fluorophores present in a sample. Furthermore, EEFS is useful for selecting the most convenient excitation wavelengths to study specific fluorescent compounds in complex matrices by conventional fluorescence spectroscopy. The measurements under this mode also have some disadvantages. The spectroscopic parameters must be optimized beforehand to avoid Rayleigh scattering caused as a result of the overlap between the ranges of wavelengths of excitation and emission. As a drawback, this mode consumes a longer analysis time to obtain a matrix (EEM), approximately 10 min depending on the spectral ranges used. The statistical data treatment is also more sophisticated or requires a preliminary decomposition of the information EEM in two-dimensional arrays. For this purpose, parallel factor analysis (PARAFAC) is an appropriate way to decompose and interpret 3D data matrices (Tena et al. 2012).

10.3.4.2 Synchronous Fluorescence Spectroscopy (SFS)

This technique consists in scanning the signal of two monochromators, the excitation and emission, simultaneously, keeping a constant interval of wavelengths ($\Delta\lambda$) between excitation (λ_{ex}) and emission (λ_{em}) wavelengths. Three types of SFS procedures can be distinguished depending on the scan rate: (1) constant-wavelength SFS, where the interval wavelength ($\Delta\lambda$) between λ_{ex} and λ_{em} is kept constant; this is the most widely used SFS procedure; (2) constant-energy SFS, where a frequency difference ($\Delta\upsilon$) is kept constant; (3) variable-angle SFS, where the excitation and emission wavelengths may be varied simultaneously but at different rates. These last two types are more difficult to implement, mostly because commercial fluorimeters are not endowed with the necessary software for such scans. Thus, a regular fluorimeter typically only allows a constant-wavelength SFS. The selection of $\Delta\lambda$ depends on which fluorophore compounds comprise the analytical targets of the study. Most of the reviewed literature on SFS indicates that 3D rendering helps in obtaining a better characterization of multifluorophore systems. The resulting 3D surfaces are obtained when the ZZ' axis is represented – the different wavelength intervals ($\Delta\lambda$) used in the course of the experiments – versus the XX' axis, which represents the range of synchronous wavelengths scanned. This graph is used to determine which $\Delta\lambda$ is the most appropriate for obtaining more information about particular spectral bands.

One advantage of total SFS is the narrowing of the bands, which simplifies the spectrum by minimizing the spectral overlap. This narrowing of bands depends on the selected wavelength interval ($\Delta\lambda$). The high selectivity of the total synchronous fluorescence spectra makes this technique suitable for the qualitative analysis of complex samples. The main disadvantages of this mode are the difficulty of selecting an appropriate $\Delta\lambda$ in the case of multicomponent samples and the requirement of specific instrumentation and software to take full advantage of the technique (monochromator plus driving software).

10.3.5 Online Analysis

In the food industry, monitoring of the process is a major issue in order to optimize it and to assure the quality of the end products. To this end, at-line or online analytical methods can be applied. With at-line methods, samples are taken from the process line and analyzed close to it or in a laboratory. At-line methods are time-consuming and do not allow one to obtain the required information in due time in order to act rapidly (or even instantaneous) on the process. Online methods, where the instrument is directly installed in the process line, is more appropriate for process monitoring. NIR, MIR, and Raman spectroscopy are techniques that are suitable for providing real-time measurements that can be integrated into an industrial process. Recent developments have been observed mainly in the setup of adequate sensors and software allowing the collecting of spectral information and to use it to pilot food processes. Online NIR spectroscopy has several advantages, such as speed of measurement, well-developed equipment and devices, absence of a need for sample preparation as well as analysis of simultaneous parameters. The main disadvantage is the need for robust calibrations and model transfer between instruments (Kondepati and Heise 2008). The online applications of NIR in food systems have recently increased significantly. Huang et al. (2008) published a review on NIR online analysis of foods such as meat, fruit, grain, dairy products, and beverages. Online MIR spectroscopy is less frequently used in the food industry but has several advantages over NIR spectroscopy such as high sensitivity, ability to distinguish between very similar structures, and good calibration transfer between instruments. Online MIR application suffers mainly from the strong absorption of water and the high cost (e.g., fiber optics suitable for MIR analysis are more expensive and less adapted for online control than those suitable for NIR analysis). Few studies on the use of MIR for online applications, such as monitoring a fermentation reaction, have been reported (Bellon-Maurel et al. 1994; Fayolle et al. 2000). Unlike MIR online spectroscopy, online Raman spectroscopy has few applications

in the food industry. However, it is commonly used in the pharmaceutical processing industry.

Few papers dealing with online use of NIR spectroscopy for the control of olive oil, olive pomace, and olive paste have been published. One of the first preliminary studies of the application of online NIR spectroscopic methods in this field was published by Hermoso et al. (1999). In this paper, the NIR technique was used to measure the oil content and humidity in olive pomace at the decanter. The study provided determination coefficients of 0.91 and 0.6 between NIR spectroscopy and the reference values of oil content and humidity obtained by NMR and the drying-oven method, respectively. Jiménez-Márquez et al. (2005) applied NIR transmittance spectroscopy to characterize virgin olive oils. Partial least-squares (PLS) models were developed for acidity value, bitter taste, and fatty acid composition. Gallardo-González et al. (2005) used NIR to determine in real time the moisture and fat contents of olive pastes and the resulting olive wastes generated in the two-phase oil extraction process. Coefficients of determination of 0.90 for humidity and 0.91 for oil content in olive paste samples were obtained.

More recently, some authors (Cayuela et al. 2009; Cayuela and Pérez-Camino 2010) predicted olive fruit and virgin olive oil parameters by directly measuring the fruit using NIR. The analyzed parameters were free acidity in olive oil, oil yield from physical extraction, oil content referring to fresh weight, oil content referring to dry matter and fruit moisture. The results indicated a very good predictive potential of the methodology and served to encourage improvement in the obtained models through the enlargement of calibration databases and models.

10.4 Data Acquisition

The data acquisition procedure in IR or Raman spectrometry is not tedious and can be done by nonskilled technicians. Basically, the principal steps are as follows: preliminary work for data acquisition (e.g., cool the detector with N_2 in Raman spectroscopy, heat the sample accessory in NIR or MIR spectroscopy), instrument performance verification, stabilization, and data collection. These steps are, for the most part, described in the technical manual supplied with the instrument. The performance of the instrument is generally checked by various automatic functions that are included in the program designed to control the spectrometer. However, before each experiment, it is appropriate to collect and store the spectrum of a defined standard (e.g., oil or chemical product defined as standard). In so doing, the spectral quality and the stability of the spectrometer can be verified each day.

The stabilization procedure is essential for acquiring a high-quality spectrum, i.e., a spectrum with a good signal-to-noise ratio. The manual of the instrument will contain the reference value normally reached by the spectrometer. To perform this work, the more convenient way is by successively collecting the spectral data of the

same sample. It is important to do this collection under conditions that will be used in practice. The acquisition of a series of spectra before the analytical step allows, according to the analytical conditions, the stabilization of the instrumental components (e.g., source, detector). The analytical conditions include the number of scans to coadd and the resolution of the spectrum. The best way to define these parameters is to carry out a repeatability study, changing one of the parameters at a time. In comparison to simple univariate analysis, little progress has been made so far in the quantification of variability in multivariate analysis. Hence, it is judicious to complete the statistical results from the univariate analysis (SD and CV) with those from a multivariate procedure such as cluster analysis. Cluster analysis develops a mathematical model evaluating the similarities and dissimilarities between multivariate data (Massart and Kaufman 1983). A convenient agglomerative procedure and linkage distance in the analysis of spectroscopic data are Ward's method and the city-block (Manhattan) distance, respectively (Chap. 12). A low value of the linkage distance indicates a high similarity, i.e., a good repeatability.

When the instrument performance has been checked and the stability of the instrument achieved, the data collection procedure can be carried out. This step includes the reference spectrum acquisition, the sample spectrum acquisition, and sample-handling cleaning. The reference spectrum consists of the spectrum of the empty sample accessory or the spectrum of a reference compound (e.g., ceramic plate in NIR spectroscopy). This step permits the removal of absorbances due to the instrument and sample handling used from the sample. Depending on the technique and the sample accessory used, the reference spectrum should be collected once a day (e.g., NIR) or before each spectral data acquisition (e.g., ATR/FT-MIR). After the reference acquisition, the sample is introduced in the sample accessory and its spectrum is collected. Before the following data acquisition, the sample must be removed and the accessory cleaned (this is not the case with automatic sampling methods, as discussed in Sect. 10.3.2.). Then, the cleaned sample handling should be spectrally checked to ensure that no residue from the previous sample remains.

10.5 Interpretation of Oil Spectra

The most frequently discussed drawback of spectroscopic techniques is the difficulty of chemically interpreting the spectral data. Separative techniques like chromatography generate information (chromatograms) mainly containing well-resolved and separate peaks, i.e., discrete information. Infrared and Raman spectroscopic techniques generate continuous information (spectra) rich in both isolated and overlapping bands. While in chromatography each peak is, in general, characteristic of a precise compound, in spectroscopy, the bands are the result of the vibration of one or more chemical bonds (e.g., C-H, C=C) present in all the compounds constituting

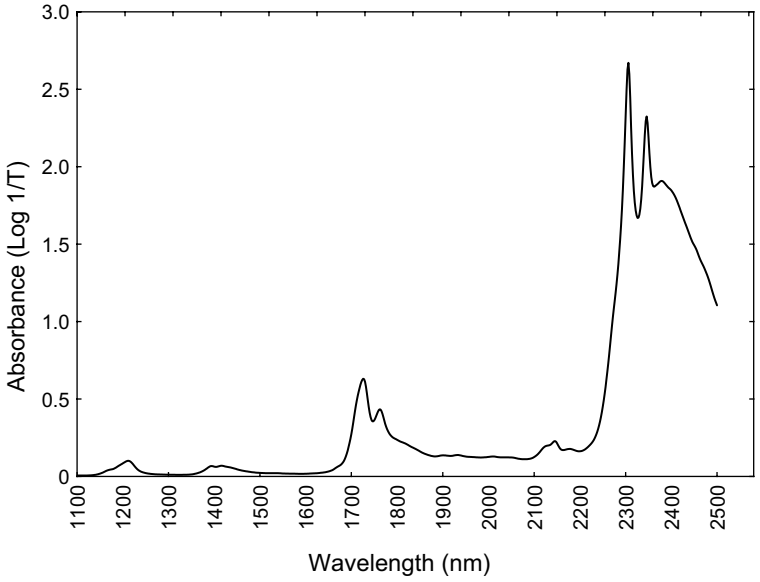

Fig. 10.13 Near-infrared spectrum of a virgin olive oil

the sample. Each band in the spectrum of a mixture contains the sum of the information of various molecules.

To make up for the unreadable information on the IR and Raman spectra, it is important to study the spectral features of pure chemical products. Edible oils mainly contain triacylglycerols (TAGs) whose types and proportion vary according to their source. Hence, the study of pure compounds such as TAGs (or fatty acid methyl esters) allows the band assignment of the principal absorption (NIR, MIR) or scattered (Raman) bands observed in the spectra. Various papers have presented the spectral features of pure chemical products (Holman and Edmondson 1956; Bailey and Horvat 1972; Sadeghi-Jorabchi et al. 1991; Sato et al. 1991; van de Voort et al. 1994b; Hourant et al. 2000; Baeten et al. 2001; Stefanov et al. 2010). In addition, various companies offer spectral libraries containing the characteristic spectra of the compounds concerned.

The analysis of various kinds of samples from different animal and vegetable sources permits the interpretation of the most noteworthy bands. The correlation at each frequency between the absorption (or scattering) intensity and chemical compounds (or indices) can be calculated using the fatty acid profile determined by gas chromatography. These correlation graphs help the analyst to underline the spectral features of each oil source and guide the subsequent data analysis.

To present the main characteristics of NIR, MIR, and Raman spectra, the relevant frequencies of pure chemical compounds will be presented and discussed later on. For

Table 10.3 Assignment of most noteworthy near-infrared absorption bands of a virgin olive oil spectrum

Wavelength (nm)	Molecule	Group	Vibration
1,090–1,180	$-CH_2$	C-H	Second overtone
1,100–1,200	$-CH_3$	C-H	Second overtone
1,150–1,260	-CH=CH-	C-H	First overtone
1,350–1,430	$-CH_2$	C-H	Combination
1,360–1,420	$-CH_3$	C-H	Combination
1,390–1,450	H_2O	O-H	First overtone
1,650–1,780	$-CH_2$	C-H	First overtone
	$-CH_3$	C-H	First overtone
	-CH=CH-	C-H	First overtone
1,880–1,930	H_2O	O-H	Combination
2,010–2,020	-CH=CH-	C-H	Combination
2,100–2,200	-CH=CH-	C-H	Combination
2,240–2,360	$-CH_3$	C-H	Combination
2,290–2,470	$-CH_2$	C-H	Combination

Table 10.4 Relevant near-infrared wavelengths (nm) of several lipids and bands that are correlated with some chemical indices (R>0.90)

	Spectral region		
Lipids	Second overtone	First overtone	Combination
Tricaprin (C10:0)		1,726, 1,800	2,128
Triolein (*cis* C18:1)		1,725	2,143
Trilinolein (*cis* C18:2)		1,665, 1,717	2,143
Trilinoelaidin (*trans* C18:2)		1,725, 1,800	2,131
Trilinolenin (*cis* C18:3)		1,665, 1,712	2,143
MUFA		1,724, 1,766	2,358
PUFA	1,162, 1,212[a]	1,660, 1,698, 1,730[a]	2,136, 2,176, 2,224, 2,310[a], 2,348[a], 2,434[a]
IV	1,164	1,664, 1,714, 1,740[a], 1,784[a]	2,144, 2,178, 2,340[a], 2,444[a]

UFA unsaturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids, *SFA* saturated fatty acids, *IV* iodine value
[a]negative correlation coefficient

each technique, the principal correlated frequencies with the total amount of unsaturated fatty acids (UFA=C16:1+C18:1+C18:2+C18:3), monounsaturated fatty acids (MUFA=C16:1+C18:1), polyunsaturated fatty acids (PUFA=C18:2+C18:3), saturated fatty acids (SFA=C6+C8+C10+C14+C16+C18), and iodine value (IV=1*C 16:1+1*C18:1+2*C18:2+3*C18:3) are displayed in the next paragraph.

Table 10.5 Assignment of most noteworthy mid-infrared bands of a virgin olive oil spectrum

Wavenumber (cm^{-1})	Molecule	Group	Vibration
3,007	*cis* -CH=CH-	C-H	ν
2,955	$-CH_3$	C-H	ν
2,924	$-CH_2$	C-H	ν
2,855	-CH2 and $-CH_3$	C-H	ν
1,746	-C=O	C=O	ν
1,653	*cis* -CH=CH-	C=C	ν
1,462	$-CH_2$	C-H	δ
1,377	$-CH_3$	C-H	δ
1,236	$-CH_2$	C-H	δ
1,300–800	Carbon skeleton	C-C	δ
1,200–1,000	-CO-O-	C-O	δ
990–960	*trans* -CH=CH-	C-H	δ
723	$-CH_2$	C-H	δ

ν stretching, δ deformation

10.5.1 Near-Infrared Spectra

NIR spectra show various overlapping peaks. As seen in the theory section, these bands are the result of overtones (first and second) and a combination of fundamental, largely hydrogenic, vibrations that occur in the MIR region. Various books and papers describe the assignment of the major NIR absorption bands (Holman and Edmondson 1956; Goddu 1957; Fenton and Crisler 1959; Williams and Norris 2001; Panford and deMan 1990; Sato et al. 1991; Sato 1994). Figure 10.13 and Table 10.3 display, respectively, the NIR spectrum (1,100–2,500 nm) obtained with a transmission cell and the assignment of the most noteworthy absorption bands of a virgin olive oil.

All studies that have used the NIR region of the electromagnetic spectra have shown that oil spectra contain information about the degree of unsaturation (IV) (Holman and Edmondson 1956), the total unsaturation (Goddu 1957), the carbon number (Wetzel 1983), and the composition of the unsaturated fraction (Sato et al. 1991). In addition, NIR spectra show specific information about *cis* isomers, while *trans* isomers have no noteworthy bands.

Sato et al. (1991) showed that mainly two regions of the NIR spectra have particular features (Table 10.4). First, an absorption intensity near 1,720 nm is characteristic of the first overtone of the C-H vibration of various chemical groups ($-CH_3$, $-CH_2$, =CH-) and varies according to analyzed TAGs. In fact, as the degree of unsaturation increases, the maximum point observed in the spectra of triolein at 1,725 nm shifts to 1,717 nm and 1,712 nm in spectra of trilinolein and trilinolenin, respectively. Second, the absorption band in the area of 2,143 nm, characteristic of the C-H vibration of *cis*-unsaturation, is more intense in polyunsaturated than in monounsaturated fatty acid spectra. Saturated and *trans* fatty acids show weak peaks and

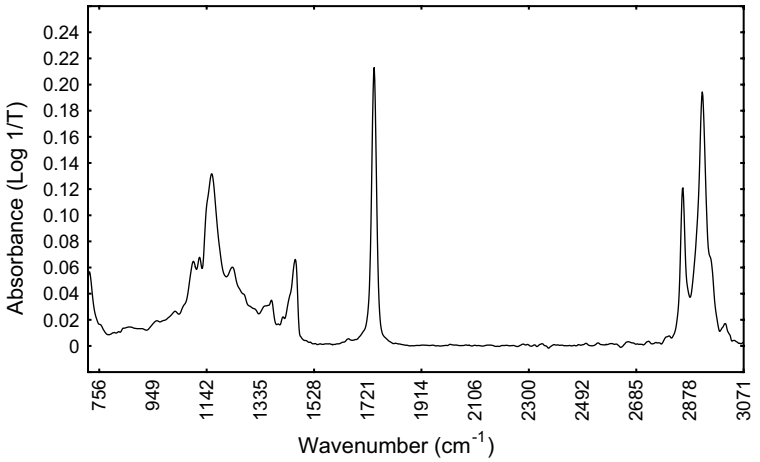

Fig. 10.14 FT-mid-infrared spectrum of a virgin olive oil

with maxima in the vicinity of 2,128 and 2,131 nm. Wavelengths in the region of 1,800 nm seem to be characteristic of saturated fatty acids.

A study of 104 samples from 18 different sources (animal and vegetable) showed that the spectral features of oils and fats agree with their fatty acid composition as determined by gas chromatography (Hourant 1995; Hourant et al. 2000). Oils with a high amount of polyunsaturated fatty acids have a maximum absorption band at lower wavelengths in the vicinity of 1,720 nm. Moreover, they have higher absorbance intensity, in the vicinity of 1,720 and 2,140 nm, than oils rich in monounsaturated fatty acids. Sunflower, walnut, and soybean oils present a maximum intensity near 1,720 nm, corn and rapeseed oils near 1,722 nm, and peanut, high oleic sunflower, and olive oils in the vicinity of 1,724 nm. The spectral regions 1,100–1,300 and 2,050–2,230 nm also show spectral features characteristic of these vegetable species. Table 10.4 regroups the wavelengths showing a high coefficient of correlation (greater than 0.90) between the absorption intensities and different chemical indices.

10.5.2 Mid-Infrared Spectra

A MIR spectrum of vegetable oil contains well-resolved peaks (3,100–1,700 cm^{-1}) and overlapping peaks (fingerprint region, 1,500–700 cm^{-1}) whose assignment is more difficult (Socrates 1994). Figure 10.14 displays the MIR spectrum of virgin olive oil, while Table 10.5 shows its most noteworthy bands (Fig. 10.14).

Based on the information contained in the MIR spectra, a series of methods has been developed to quantify the *trans* content (AOCS 1988; Sleeter and Matlock 1989; Ulberth and Haider 1992; van de Voort et al. 1995; Mossoba et al. 1996;

Table 10.6 Relevant mid-infrared wavenumbers (cm^{-1}) of several lipids and bands that are correlated with some chemical indices (R>0.90)

	Spectral region	
Lipids	= C-H stretching	Fingerprint region
Tristearic (C18:0)	–	–
Triolein (*cis* C18:1)	3,005	913
Trielaidin (*trans* C18:1)	3,025	966
Trilinolein (*cis* C18:2)	3,010	913
Trilinoelaidin (*trans* C18:2)	3,025	968
Trilinolenin (*cis* C18:3)	3,012	913
MUFA	3,011, 2,964	1,425, 1,396, 1,273, 1,134, 1,101, 914
PUFA	2,924, 2,854	1,464, 1,408, 1,313, 1,118
IV	3,011, 2,965, 2,922[a], 2,853[a]	1,429, 1,395, 1,267, 1,132, 1,117[a], 1,098, 922

UFA unsaturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids, *SFA* saturated fatty acids, *IV* iodine value
[a]negative correlation coefficient

Ratnayake and Pelletier 1996), the *cis* content (van de Voort et al. 1995), the peroxide content (van de Voort et al. 1994b), the aldehyde content in thermally stressed oils (Dubois et al. 1996), and the free fatty acid content (Ismail et al. 1993). MIR spectroscopy was also used in the determination of indices such as the anisidine value (Dubois et al. 1996), iodine value (Afran and Newberry 1991; Muniategui et al. 1992; van de Voort et al. 1992), saponification number (van de Voort et al. 1992), and the solid fat index (van de Voort et al. 1996).

The investigation of pure fatty acids underlines the fact that spectral features change with the degree of unsaturation (van de Voort et al. 1995). The C-H stretching vibration of $-CH_2$ and $-CH_3$ groups (2,950–2,800 cm^{-1}),the C=O stretching vibration of carbonyl groups (1,745 cm^{-1}), and the C-H bending vibration of $-CH_2$ and $-CH_3$ groups (1,400–1,200 cm^{-1}) have absorption band intensities that change with the degree of unsaturation of the lipid matter. Moreover, the peak centered near 3,005 cm^{-1} (C-H stretching vibration of *cis* -CH=CH-) in the spectrum of triolein shifts to higher frequency in the trilinolein (3,010 cm^{-1}) and trilinolenin (3,012 cm^{-1}) spectra as the degree of unsaturation rises (Table 10.6). On the other hand, *trans* fatty acids show a peak centered near 3,025 cm^{-1}.

The fingerprint region of pure fatty acids is rich in features indicative of the degree of unsaturation, the type of unsaturation (mono- or polyunsaturated), or the content of *cis* and *trans* isomers. In a range from 1,125 to 1,095 cm^{-1} (characteristic of C-O and C-C stretching vibration), the peak intensities and the shape of the spectra vary with the unsaturation of fatty acids.

A study of 64 samples from 13 sources revealed that certain absorption bands of oil spectra vary with their fatty composition (Hourant 1995). The weak peak near 3,010 cm^{-1} has a higher intensity as the major fatty acids in the sample are monounsaturated or polyunsaturated. Moreover, samples rich in C18:1 (e.g., olive oil) have higher absorbance near 2,953 and 2,922 cm^{-1} than those rich in C18:2.

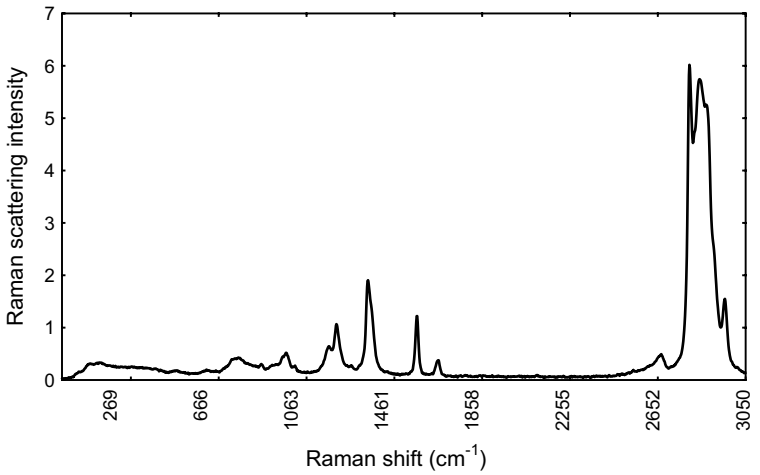

Fig. 10.15 FT-Raman spectrum of a virgin olive oil

Table 10.7 Assignment of most noteworthy Raman scattering bands of a virgin olive oil spectrum

Raman shift (cm^{-1})	Molecule	Group	Vibration
3,007	RCH=CHR	=C-H	ν
2,926	$-CH_2$	C-H	ν
2,897	$-CH_3$	C-H	ν
2,855	$-CH_2$	C-H	ν
1,748	RC=OOR	C=O	ν
1,670	*trans* RCH=CHR	C=C	ν
1,655	*cis* RCH=CHR	C=C	ν
1,441	$-CH_2$	C-H	δ
1,306	$-CH_2$	C-H	δ
1,270	*cis* RCH=CHR	=C-H	δ
1,100–1,000	$-(CH_2)_n-$	C-C	ν
900–800	$-(CH_2)_n-$	C-C	ν

ν stretching, δ deformation

However, the most important spectral features appear in the fingerprint region. Two bands near 1,121 and 1,098 cm^{-1} show interesting spectral features. The absorbance intensity in the vicinity of 1,121 cm^{-1} shows a positive correlation with the amount of oleic acid, while the intensity near 1,098 cm^{-1} is correlated with the amount of linoleic acid (Aparicio and Baeten 1997). In addition, the peak centered at 913 cm^{-1} is not present (or is very weak) in high oleic sunflower and olive oil, while it is more intense in samples rich in polyunsaturated fatty acids. Table 10.6 shows the wavenumbers with a coefficient of correlation between the absorption

Table 10.8 Relevant Raman shifts (cm^{-1}) of several lipids and bands that are correlated with some chemical indices (R>0.90)

	Spectral region		
Lipids	= C-H stretching	C=C stretching	C-H bending
Methyl oleate (*cis* C18:1)	3,006	1,654	1,439, 1,267
Methyl elaidate (*trans* C18:1)	2,995	1,667	1,439
Methyl linoleate (*cis* C18:2)	3,011	1,657	1,440, 1,265
Methyl linolenate (*cis* C18:3)	3,013	1,657	1,441, 1,266
MUFA	2,890, 2,874[a], 2,845		
PUFA	3,021, 2,922, 2,884[a], 2,870, 2,855[a]	1,667, 1,642	1,256
IV	3,007, 2,991, 2,911, 2,882[a], 2,855[a]	1,657, 1,646	1,268

UFA unsaturated fatty acids, *MUFA* monounsaturated fatty acids, *PUFA* polyunsaturated fatty acids, *SFA* saturated fatty acids, *IV* iodine value
[a]negative correlation coefficient

intensities and different chemical indices higher than 0.90. The region near 3,010–2,950 cm^{-1} and the fingerprint region (1,500–700 cm^{-1}) show the highest correlation with the different indices in relation to the degree of unsaturation of the samples.

10.5.3 Raman Spectra

The spectra of edible fats and oils obtained by FT-Raman spectrometers contain well-resolved bands with various scattering intensities and shapes. The spectra show good signal-to-noise ratios and contain information from different vibrational bands (stretching and bending) of various chemical groups. Raman scattering arises from the change in the polarizability or shape of the electron distribution in the molecule as it vibrates, while, in contrast, IR absorption requires a change in the intrinsic dipole moment with the molecular vibration (Grasseli and Bulkin 1991). Hence, polar groups (such as C=O and O-H) have strong MIR absorption bands, whereas nonpolar groups (such as C=C) show intense Raman scattered bands. Because the main feature of unsaturated fatty acids is their content of double bonds and their configuration (*cis* or *trans*), FT-Raman spectra are of great value in the study of lipids. Raman spectroscopy has been used in the determination of the total amount of unsaturation (iodine value) and of the *cis/trans* isomer content of edible oils (Bailey and Horvat 1972; Sadeghi-Jorabchi et al. 1990, 1991). Figure 10.15 and Table 10.7 display respectively the FT-Raman spectrum and the assignment of the most noteworthy bands of a virgin olive oil.

Bailey and Horvat (1972) studied the spectral features in the area of 1,660 cm^{-1} (C=C stretching vibration) of triolein, trielaidin, trilinolein, and trilinolenin. In this region, *trans* isomers had a peak centered near 1,670 cm^{-1}, while *cis* isomers showed a peak in the vicinity of 1,660 cm^{-1}. Later on, Sadeghi-Jorabchi et al. (1991) studied and underlined other characteristics of pure methyl esters in their work on the quantification of *cis* and *trans* content by FT-Raman spectroscopy. They showed the particular features of fatty acids near 3,010 cm^{-1} (=C-H

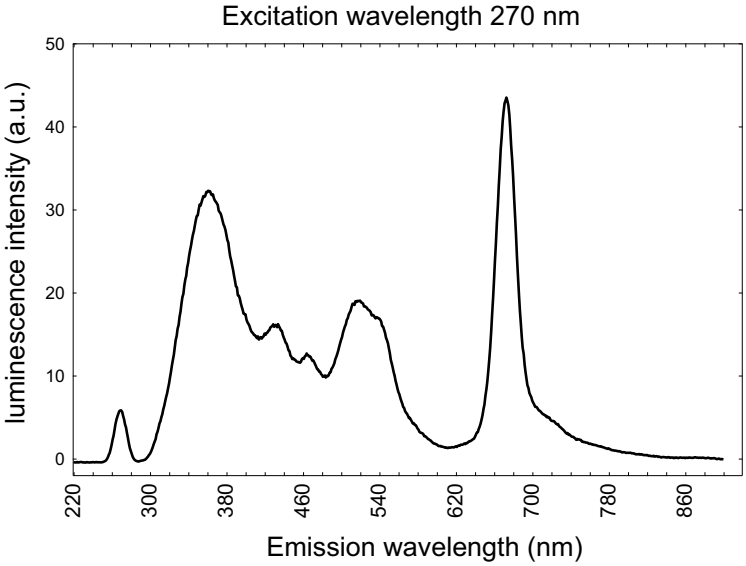

Fig. 10.16 Fluorescence spectra of a virgin olive oil

stretching vibration) and 1,270 cm^{-1}(=C-H bending vibration). In the area of 3,010 cm^{-1} a shift to a higher frequency and an increase in the scattering intensities occurs as the degree of unsaturation rises. Similar observations were reported at 1,660 and 1,270 cm^{-1}. Table 10.8 shows the main characteristics of various methyl esters.

The region near 3,010 cm^{-1} is particularly affected by the major fatty acid components (Baeten et al. 1998). In fact, samples relatively rich in polyunsaturated fatty acids (e.g., corn, sunflower, and sesame oils) had a more intense scattering band and a higher frequency maximum than samples rich in monounsaturated fatty acids (e.g., olive oil). This band is also important in the authentication of olive oil (Baeten et al. 1996). The usefulness of Raman shifts in the range 2,880–2,840 cm^{-1} (C-H stretching vibration of CH_2 and CH_3) for varietal discrimination has also been noted (Aparicio and Baeten 1998). In this region, samples rich in polyunsaturated fatty acids (e.g., rapeseed, sunflower, and walnut oils) have weaker scattering intensities than those that have a high content of monounsaturated fatty acids (e.g., olive oil).

The region of 1,660 and 1,265 cm^{-1} is also characteristic of the fatty acid profile of the fat or oil variety studied. Samples rich in polyunsaturated fatty acids such as walnut, sunflower, corn, and sesame oils have a maximum near 1,657 cm^{-1}, while olive and high oleic sunflower show a maximum near 1,655 cm^{-1}. The intensity at these Raman shifts rises with the degree of unsaturation. Near 1,259 cm^{-1}, the scattering intensities increase as the degree of unsaturation decreases. The fingerprint region (1,100–700 cm^{-1}) of pure methyl esters and of different oil varieties also have information (Sadeghi-Jorabchi et al. 1991). However, the poor signal-to-noise ratio

Table 10.9 Emission wavelength associated with fluorophores present in olive oil

Fluorescent compounds	λ_{em} (nm)	Reference
Pigment (chlorophylls and pheophytins)	692–765	Sayago et al. (2007)
α-, β-, and γ-tocopherols, phenols	275–400	Dupuy et al. (2005)
Chlorophyll a and b, pheophytin a and b	600–700	
Oxidized products from vitamin E	400–600	
Tocopherols and tocotrienols	300–350	Sikorska et al. (2005)
Chlorophylls and pheophytins	660–700	
Oxidized product	400	
Phenols	300–390	Zandomeneghi et al. (2005)
Chlorophylls and derivatives	640–800	
Tocopherols	328	Giungato et al. (2004)
Chlorophyll a	669	
Parinaric acid isomerization	406	
Vitamin E (oxidized products)	440, 475, 525	Guimet et al. (2004)
Chlorophylls	650 y 700	
Chlorophyll a	669	Galeano et al. (2003)
Chlorophyll b	653	
Pheophytin a	671	
Pheophytin b	658	
Hydroxyl radical	452,3	Tai et al. (2002)
K232 y K270	440–445	Kyriakidis and Skarkalis (2000)
Vitamin E derivatives	525	
Chlorophylls	681	

at these frequencies does not allow, at the moment, evaluation of the information. Table 10.8 regroups the wavenumbers showing a maximum coefficient of correlation between the absorption intensities and different chemical indices.

10.5.4 Fluorescence Spectra

Vegetable oils are commonly analyzed by fluorescence spectroscopy untreated or diluted at 1 % in hexano v/v. In particular, Fig. 10.16 displays the fluorescence spectrum of a virgin olive oil in its native form (nondiluted). The bands observed in this spectrum have been related to species that are shown in Table 10.9. In this table also appears the emission wavelength of these fluorophores. The spectral profile and the intensities of these bands dramatically vary with the oxidation degree of the samples, as was shown in Fig. 10.3. The bands of some fluorescent compounds, such as chlorophylls, are more intense than other fluorescent species, although the intensity greatly depends on the excitation wavelength. The magnitude of this peak with respect to others may cause problems when handling the whole data set. This

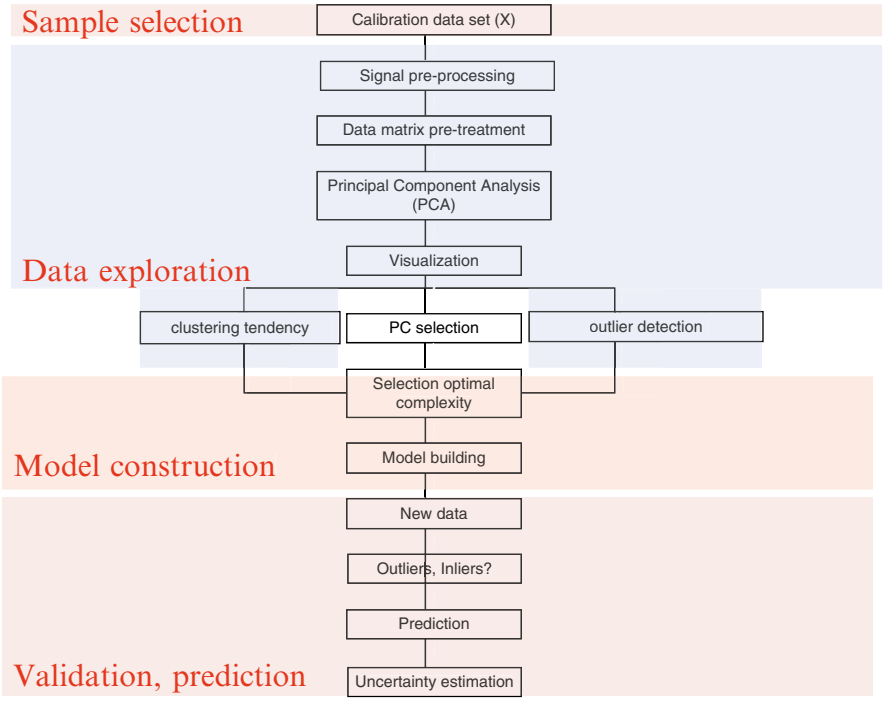

Fig. 10.17 Flow diagram showing principal steps involved in a spectral data treatment

problem can be avoided by studying narrower ranges of wavelengths instead of processing the information of the entire emission spectra.

10.6 Data Treatment

The main analytical problem with spectroscopic data is to extract the information in such a way that it can be used in quantitative analysis. IR and Raman spectra are usually the mean of various coadded spectra (normally between 100 and 200). The collection of a high number of coadded spectra is allowed by the rapidity of the acquisition of a single coadded spectrum in an FT instrument (around a few seconds). The spectra displayed throughout the present chapter are coadded spectra. These spectra are a rich source of multivariate data (more than 700 data points) where each frequency represents a variable. Various strategies have been proposed to investigate the spectral data set and to isolate areas, patterns, or latent variables correlated with the information concerned.

Figure 10.17 summarizes the classical steps for building a mathematical model (i.e., a quantitative or discriminant equation). The steps are the pretreatment of data,

outlier detection, calibration, and validation procedures including chemical, internal, and external validation.

The following sections briefly describe the data treatments and their respective objectives. For a thorough presentation of the ideas in this section, the reader may refer to Tabachnick and Fidell (1983), Williams and Norris (2001), and Martens and Naes (1989).

10.6.1 Pretreatment of Data

The signal obtained from a spectrometer contains information together with random noise. Noise can cause systematic errors in later predictions through the estimated calibration parameters. Thus, reducing noise or, in other words, improving the ratio of signal to noise is still an advantage.

Various pretreatments of data are available for different objectives, such as, for example, to improve the spectral quality (e.g., signal-to-noise ratio), to reduce the influence of external variation (e.g., variation produced by the sample-handling method), or to resolve the complexity of overlapping peaks (e.g., the combination bands or the fingerprint region of MIR spectroscopy). All depend on the objective sought, the technique investigated, the instrument and sample accessory used, the sample (neat or solution) studied, the type of mathematical model to be built, or the researcher's preferences.

Various algorithms are available to perform smoothing that basically concern how to reduce high-frequency ripple noise and, whenever possible, low-frequency noise. Thus, conceptually speaking, smoothing is simply a filtering process. From the large panoply of algorithms developed for electronic analog and computer systems from the 1960s to today, algorithms are available in the smoothing routines of the software packages developed for IR and Raman spectrometers. These routines usually contain algorithms such as moving average filters (Rabiner and Gold 1975), the least-squares polynomial smoothing developed by Savitsky and Golay (1964), and the classical Fourier smoothing methodology (Williams and Norris 2001; Martens and Naes 1989), among others. The running mean algorithm simply replaces the value at each point by the mean of the values in a wavelength (or wavenumber) interval surrounding it. The interval is centered at the given point, resulting in an odd number of data points per mean (Williams and Norris 2001). The Savitsky–Golay algorithm, the most familiar method of smoothing in analytical chemistry, is an indirect filter that fits the spectrum inside a wavelength (or wavenumber) interval with a polynomial by least-squares method. The parameters are the degree of the polynomial and the number of points to fit (Savitsky and Golay 1964). Fourier analysis makes an orthogonal transformation of the spectrum into a sum of sine and cosine spectral contributions (Aparicio et al. 1977) that allows certain frequencies to be kept (usually low frequencies) and removes those undesired frequencies that do contribute to noise (often the high-frequency ripple). The inverse FT is ultimately used for regenerating the spectrum.

Derivatives allow some compensation for the problems associated with overlapping peaks and baseline variations. Analysts generally use the first and second derivatives. This mathematical treatment calculates the tangent at each point of the raw spectral data. Each inflection point of the raw spectrum corresponds to a relative minimum or maximum of the first derivative spectrum while all maxima and minima on the raw spectrum are zero in the first derivative spectrum. The second derivative is advantageous for the resolution of overlapping peaks. Each minimum of the second derivative spectrum corresponds to a maximum of the raw spectrum, and obviously identical comments can be made regarding successive even derivatives (Williams and Norris 2001).

Normalization means changing a group of spectra so that unwanted sources of variability are suppressed. This helps the graphical understanding of the spectra and can reduce the complexity of the subsequent data treatment necessary to develop a calibration from spectroscopic data. The simplest example of this treatment is the subtraction of the spectral value at a single wavelength or wavenumber (the so-called reference wavelength or wavenumber) from all the spectral values; the result is a set of spectra with zero value at the reference wavelength or wavenumber. Normalization by closure is an alternative. This normalization consists in dividing the signal instrument responses at each wavelength or wavenumber by their sum (or mean) in each spectrum. Martens and Naes (1989) suggest this procedure when there is no variable that dominates the total sum of original instrument responses, but always after a graphical inspection of the ratios between some estimated values for independent variables.

Outliers are abnormal, erroneous, or irrelevant observations that can greatly influence mathematical model construction. A number of phenomena such as operator mistakes, noise spikes, instrument drifts, and inconsistent sample-handling position can affect a spectroscopic analysis (Williams and Antoniszyn 1987). Thus, both objects (cases) and variables can behave as outliers, and they are unavoidable in almost all statistical studies. They can only be removed or corrected. During calibration it is important to have them under control as they could decrease the prediction ability of the estimated calibration coefficients. The cross-validation curve can give clues about the presence of outliers in the calibration set, e.g., irregular deviations of the fitted curve of MSE versus the number of PLS factors (Martens and Naes 1989), although almost all multivariate statistical procedures have algorithms for outlier detection (Tabachnick and Fidell 1983). Other algorithms are based on leverage (a Mahalanobis distance that measures the position of independent variables relative to the rest) and residuals (difference between predicted and observed values in regression). Leverage is outlier sensitive, and a high leverage observation in a regression process means that the calibration set contains outliers. A plot of residuals (residuals against wavelength numbers) gives more than graphical information because an observation with large residuals indicates the presence of abnormal information (Cook and Weisberg 1982).

During prediction it is almost compulsory to have methods for detecting abnormalities in order to increase the certainty of the predicted results. The detection of these possibly abnormal observations can be based on data information such as the residual value and the prediction leverage (Martens and Naes 1989) or more classical methods based on the Mahalanobis distances (De Maesschalck et al. 2000) and on potential functions (Jouan-Rimbaud et al. 1999). Robust methods (Geurts et al. 1990) can also be applied such as resampling by the half-means (RHM) or the smallest half-volume method (SHV) (Egan and Morgan 1998; Pell 2000). However, most of these multivariate outlier detection techniques are often difficult to understand for nonspecialists and are not an easy matter due to the masking and swamping effects. The masking effect occurs when one outlier masks a second outlier. In this case, the second outlier can be considered an outlier only by itself, not in the presence of the first outlier. In the swamping effect, one outlier swamps a second observation because the latter can be considered an outlier only in the presence of the first one (Ben-Gal 2005). Most analytical chemists want to spend as little time as possible looking at the large variety of diagnostics for outlier detection. In consequence, simple methods are needed. For this reason, complete protocols for outlier detection have been developed with the maximum information that can be extracted from the data (Høy et al. 1998; Fernández Pierna et al. 2002). These protocols include not only the determination of classical measurements as Mahalanobis distance or the leverage value, but also the calculation of the uncertainty present in the outputs of the multivariate model, which is calculated as a function of the different sources of uncertainty present in the model (Fernández Pierna et al. 2003).

After analysis of the internal and external variables that can affect the mathematical model, the pretreatment of data should finish with a study of the repeatability and reproducibility of the method. The main element of repeatability is the standard deviation of a successive collection of spectra of the same sample under the most realistic experimental conditions. The repeatability study should include not only all the steps included in the data collection procedure (washing of the sample holder, sample removal, spectral acquisition), but also a study of the variability observed on different days. Reproducibility would imply a collaborative study about the comparison of spectral results of selected samples by diverse instruments at different laboratories. The results of the repeatability and reproducibility studies firmly determine the number of replicates of each case (sample) of the calibration and validation sets and the regions of the spectra that can be used in calibration.

10.6.2 Mathematical Model Construction

The purpose of IR and Raman instruments is to determine the concentration of chemical variables, such as *trans* content (i.e., quantitative analysis), or the assessment of

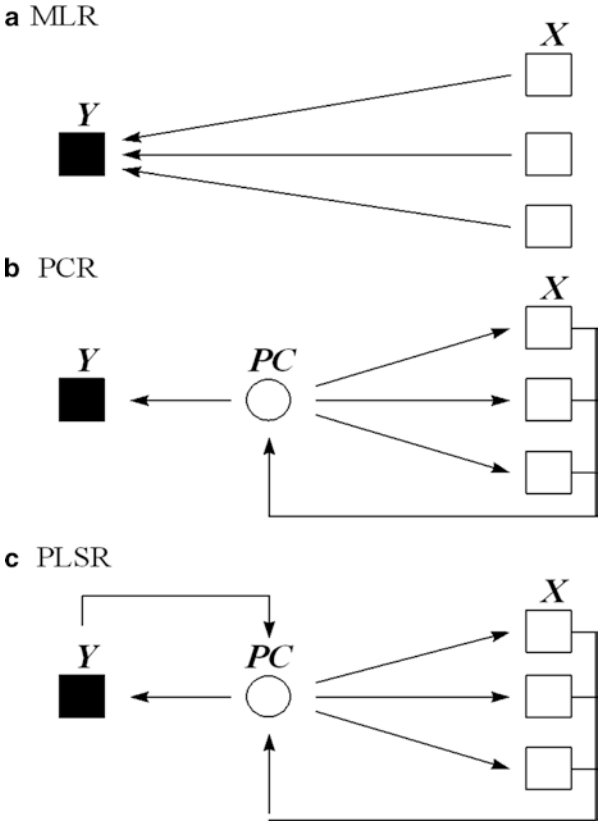

Fig. 10.18 Schematic presentation of (**a**) multivariate regression analysis (*MLR*), (**b**) principal component regression (*PCR*), and (**c**) partial least-squares regression (*PLSR*)

qualitative issues, such as authenticity or characterization (i.e., qualitative analysis). But to do this, the instrument must be calibrated for converting the IR or Raman optical signal to the desired quantitative or qualitative measurement. A model needs two processes, the calibration, or model design, and the validation, or model verification.

Calibration is usually carried out with chemical parameters (i.e., iodine value) quantified by nonspectroscopic techniques, e.g., chromatography. The dependent variable (e.g., iodine value) is then qualified as a direct measurement, while the independent variable (the spectrum) is described as an indirect measurement. However, it is the spectroscopic technique that responds directly to the problem description. For example, peptide bonds in proteins are directly represented in the spectrum, whereas the so-called direct method Kjeldahl analysis for proteins involves the measurement of total nitrogen, which requires several reaction steps and the application of a conversion factor to amine and protein measurement (Scotter 1997).

10.6.2.1 Calibration in Quantitative Analysis

Calibration means a formula (linear or nonlinear) establishing a relationship between the variation of the spectral data (independent variable) and the chemical reference data (dependent variable). The calibration is in fact a regression process with a strict pretreatment of data and rigorous analysis of the results.

Since two steps are necessary to achieve a mathematical model construction (calibration and validation), two sample sets are necessary, i.e., a set of *N* samples that would be used to construct the equation (calibration set) and a set of *M* samples that would allow studying the precision and the reliability of the equation (validation set). The number of samples in the validation set should be at least half of the calibration set. Moreover, the mean and the standard deviation of the two sample sets must be as close as possible, and the validation set must also be a subset of the calibration set covering the whole range of values. To assure these conditions and to have two homogeneous data sets, i.e., which should cover the experimental region uniformly, various methods have been developed and are described in the literature. The most common technique is the duplex method (Snee 1977), which is a modification of the Kennard and Stone technique (Kennard and Stone 1969). In this method, a sequential procedure is applied in order to split the data into two subsets. The method starts by selecting the two points that are furthest from each other and puts them both in a first set (training). Then, the next two points that are furthest from each other are put in a second set (testing), and the procedure is continued by alternately placing pairs of points in the first or second set.

Various calibration (or regression) procedures exist, with multiple linear regression (MLR), principal component regression (PCR), and PLS being the most commonly used in spectroscopy. Figure 10.18 shows a schematic design of these statistical procedures where the matrix Y_{ij} (or **y**) represents the values of the *j*-dependent variables (usually chemical analyses) of *N* ($i = 1 \ldots N$) calibration samples, while matrix X_{iw} (or **X**) represents the values of *w*-independent variables (spectral wavelengths or wavenumbers) of these *N* calibration samples. The simple regression equation can be written, in matrix convention, $\mathbf{y} = \mathbf{Xb} + \mathbf{f}$, while the objective of the calibration by least squares is to minimize the length $\mathbf{f} = \mathbf{y}\text{-}\mathbf{Xb}$ whose solution is equal to *Estimator*-$\mathbf{b} = (\mathbf{X}'\mathbf{X})^{-1}\ \mathbf{X}^{-1}\mathbf{y}$, where $\mathbf{X}'$ is the transpose matrix **X** and $\mathbf{X}^{-1}$ is the inverted matrix **X**.

The explanation of a dependent variable (e.g., iodine value) by only one wavenumber is rather difficult, and hence calibration needs to combine more than one wavenumber; this is multivariate calibration. Traditional MLR and stepwise multiple linear regression (SMLR) are expressed as

$$y = b_0 + \sum X_i b_i + \delta,$$

where *y* is the dependent variable, or analytical reference, $X_{i(i=1,n)}$ (the independent variables) are the spectral data (transformed or not) at the respective *n* wavelengths or *n* wavenumbers, and b_i ($i = 0,1,\ldots,N$) are the regression coefficients. To achieve higher regression values, the analyst might be tempted to increase the number of

spectral data in calibration; however, it is judicious to limit the number according to the sample number. Tabachnick and Fidell (1983) suggest that the number of samples should be ten times the number of independent variables. Anyway the MLR predictor has a deficient performance when there is collinearity in **X**, while SMLR gives a better prediction when an F-test is used for selecting variables because this algorithm enables removal of those X_i-variables that are most nonlinear in their response (Tabachnick and Fidell 1983).

Partial least-squares regression (PLSR) was designed to give a plausible solution to those studies where there are many collinear variables and a small calibration set, that is to say, where the number of variables is greater than cases (spectra), although some pitfalls have been described (Defernez and Kemsley 1996). PLSR can be applied for one single *y*-variable and several *y*-variables. In general PLSR-1 is more complex than PCR or PLSR-2 than canonical correlation based on simultaneous PCA of **X** and **Y** matrices. Martens and Naes (1989) state that calibration methods based on PLS regression can give a good understanding of the calibration data and a good approximation of many types of nonlinearities. Other alternative is ridge regression (Pfaffenberger and Dielman 1990), although it has not been widely used in spectroscopy despite the fact that it can be superior to PCR.

One of the most common applications of PCA is in those studies where X_i-variables are expected to be collinear. This is the case with spectral analysis (Cowe et al. 1985a, b), and PCA is able to express the main information in the variables of the raw calibration set by a lower number of variables, so-called principal components (Chap. 12). Once the analyst has decided how many principal components are necessary for retaining the essential information in **X** (i.e., applying cross validation), the rest of the process is similar to MLR, although the application of SMLR to PCA is strongly advised (Aparicio et al. 1992). At any rate, the analyst should select the best spectral data instead of the whole spectrum as the latter can contain large amounts of noise or superfluous information.

The described regression procedures assume that the relationship between the independent variables and the dependent variable is linear in nature. However, the nonlinear estimation leaves it up to the analyst to specify the nature of the relationship; for example, you may specify the dependent variable to be a logarithmic function of the independent variables, an exponential function, a function of some complex ratio of independent measures, etc. There are many noncategorical nonlinear estimations such as the quasi-Newton method (O'Neill 1971), piecewise nonlinear regression, Hooke–Jeeves method (Hooke and Jeeves 1961), simplex procedure (Fletcher and Reeves 1964), Hessian method, and others. Where all these other methods fail, the Rosenbrock pattern search method often succeeds. This method rotates the parameter space and aligns one axis with a ridge while all other axes remain orthogonal to this axis. If the loss function is unimodal and has detectable ridges pointing toward the minimum of the function, then this method will proceed with accuracy toward the minimum of the function. However, if all variables of interest are categorical in nature, or can be converted into categorical variables, the correspondence analysis module should also be considered.

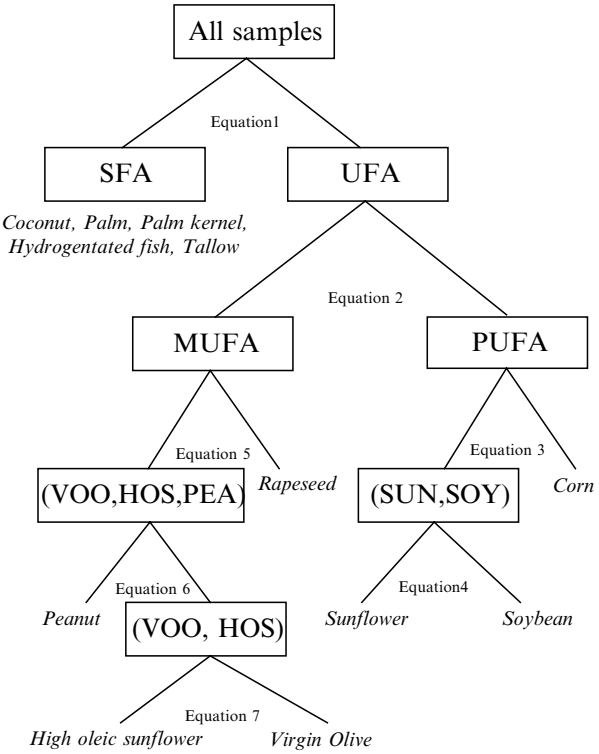

Fig. 10.19 Discrimination tree constructed using near-infrared and Raman spectral data. Legend: *SFA* samples rich in saturated fatty acids, *UFA* samples rich in unsaturated fatty acids, *MUFA* samples rich in monounsaturated fatty acids, *PUFA* samples rich in polyunsaturated fatty acids, *VOO* virgin olive oil, *HOS* high oleic sunflower, *PEA* peanut, *SUN* sunflower, *SOY* soybean

It is important to note that sometimes data are nonlinear. Deletion or appropriate weighting of nonlinear variables at the beginning of an analysis can decrease the nonlinearity problems. Also, in some cases an appropriate signal preprocessing can correct for the nonlinearity. These approaches can perhaps give better predictive ability than linear models with original variables or less complex models for the same predictive ability; however, alternatively, one may decide to adopt nonlinear models such as neural networks, support vector machines (SVM), or local regression approaches (De Maesschalck et al. 1999).

10.6.2.2 Qualitative Analysis: Classification Protocols

To construct a mathematical model for olive oil characterization or authentication, it is important to establish an intelligible, reproducible, valid, and predictive approach. Unfortunately, few authors propose a complete procedure to extract and use the information contained in IR or Raman spectra. The following sections are based on the results obtained with two protocols (Lai et al. 1994).

The first step of these two approaches is identical and concerns the division of the sample set into two subsets. As mentioned previously, the first subset

(calibration set) is used to construct the discrimination equation, while the second subset (validation set) permits the validation of the established model. The sample set studied must include all possible combinations of variables and the variation in all directions should be as large as possible but limited to the direction of interest (Naes and Isakson 1989).

In the procedure suggested by Lai et al. (1994), PCA is used because it constitutes an efficient data reduction method. As mentioned previously, a spectrum contains several hundred variables. But a multivariate statistical analysis requires that the case (sample) number exceed the variable number, and as a consequence, a reduction in the spectral variable number is necessary. Furthermore, the PCA procedure allows the removal of the apparent redundancy of the variables by transforming the original data into a set of principal component scores. When this is done, a rearrangement of the data takes place and the first few PC scores are sufficient to describe the information contained in the original variables. This procedure allows for data set simplification and the visualization of relationships within the data. After applying PCA, Lai et al. (1994) used discriminant analysis to construct a mathematical model on the basis of the scores. The squared Mahalanobis distances (SMD) are used to classify each case (sample) inside the predetermined groups. Later, the SMD from the established group means are calculated for each validation spectrum's PC scores and the new samples will be assigned to the nearest group mean. The percentage of correct classifications corresponds to the samples assigned to the correct group (i.e., species).

The approach presented by Aparicio and Baeten (1998) uses stepwise linear discriminant analysis (SLDA) to select frequencies and construct the mathematical models. SLDA is first applied to each part of the spectrum in such a way that the more relevant frequencies from each region are selected. After that, the SLDA procedure is applied to all the preselected variables and the discriminating equations are established on the basis of Mahalanobis distance and F-test (Tabachnick and Fidell 1983). The ellipses of the 95 % confidence region are calculated for each predetermined group during the calibration step (Aparicio and Baeten 1997). These ellipses allow an interpretation beyond the simple location of a validation sample and the calculation of the percentage of samples correctly classified during the validation procedure (Aparicio and Morales 1995). An alternative to these procedures could be to apply the Fisher test for removing variables without precise information and then apply PCA on the selected variables. The model can be used in an arborescent structure for distinguishing different types of fats and oils (Fig. 10.19).

Also, classical chemometric methods such as partial least squares discriminant analysis (PLSDA) (Martens and Naes 1989), and artificial neural networks (ANN) (Despagne and Massart 1998) are well-known and proven techniques for both qualitative and quantitative analysis of multivariate data. In the case of qualitative analysis, the SVM technique (Vapnik 2000) has been recently proposed and widely used in the literature (Burges 1998; Belousov et al. 2002; Fernández Pierna et al. 2004). The choice of SVM as classification method is justified by the great performance of these methods in all studies, which is mainly due to the uniqueness of the SVM solution for the problems of pattern recognition.

10.6.3 Validation Procedures

Analysts should pay attention to the validation procedures, which include the chemical, internal, and external validations (Fig. 10.17). Chemical validation is the interpretation and the elucidation (band assignment) of the frequencies used in the mathematical model. All selected spectral data (frequencies) should have a chemical or physical explanation in order to avoid regressions obtained by chance. To do this step successfully, the study of the spectral features (position and intensity of the bands) of pure chemical compounds and the correlation at each frequency between the intensities and chemical properties (e.g., determined by gas chromatography) is necessary.

Internal and external validations consist in the study of the efficiency and power of the mathematical models constructed. Internal validation is done with the samples involved in the construction of the equation (calibration step). Cross validation is a particular internal validation method (Martens and Naes 1989), although there are others such as leverage correction or Mallows *Cp* statistic (Chap. 12). An external validation is made by the observation of the quantification (quantitative analysis) or the classification (qualitative analysis) of new samples not used in the calibration procedure. The number and characteristics of these samples have been clearly established (Aparicio et al. 1992).

In order to perform a correct validation and to indicate the performance of the results, different standard expressions taken from basic statistics are applied. However, multivariate models are inherently complex, and as a result, theoretical advances with respect to the corresponding error analysis are relatively slow. For this reason, developing approximate expressions for sample-specific standard error of prediction when applying a multivariate model, mainly PLS, has received considerable attention in the chemometric-related literature in recent years (Faber 2000; Faber and Bro 2002). This calculation of uncertainty consists in the study of the uncertainty present in the outputs of the model. In most cases, this uncertainty is calculated as a function of the various sources of uncertainty present in the model (Fernández Pierna et al. 2003).

10.7 Potential of Infrared and Raman Spectroscopy

The potential offered by NIR, MIR, and Raman spectroscopy in the determination of various chemical compounds and chemical indices has been described, with more or less success, by various authors (Williams and Norris 2001; van de Voort 1994; Li-Chan 1996; Guillén and Cabo 1997). The following section briefly describes the methods used with olive oil, whereas their application in characterization is described in Chap. 12.

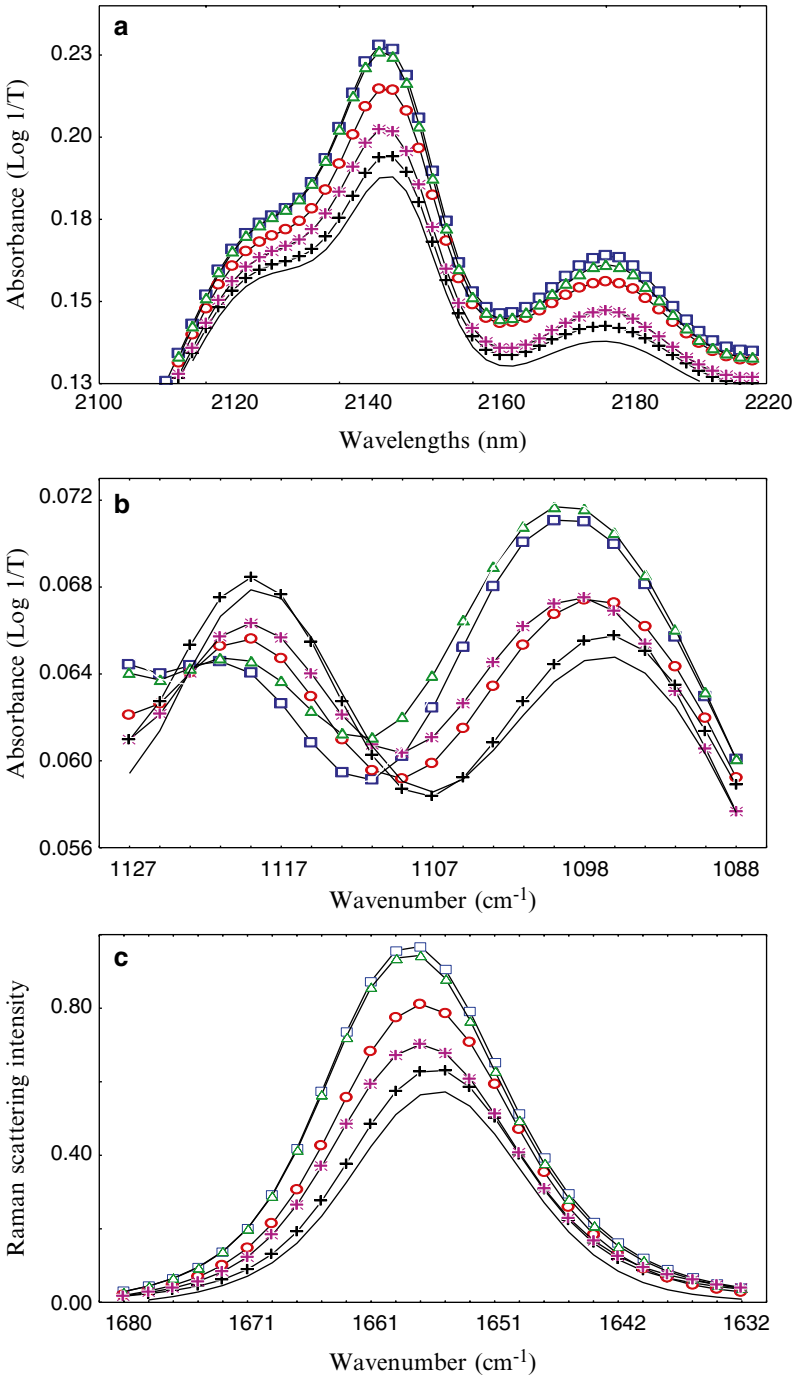

Fig. 10.20 Spectral regions of (**a**) near-infrared, (**b**) FT-mid-infrared, and (**c**) FT-raman spectra of the following edible oils: (⊟) sunflower oil, (-○-) rapeseed oil, (✳) peanut oil, (△) soybean oil, (✚) high oleic sunflower oil, and (—) virgin olive oil

10.7.1 Determination of Unsaturation Degree: Iodine Value

As was mentioned earlier, NIR, MIR, and Raman spectrum profiles are strongly influenced by the content and type of unsaturated groups. Figure 10.20 presents a spectral region of various edible oils and fats for each technique investigated. The regions shown are, respectively, the region 2,100–2,200 nm (=C-H vibration) of NIR spectra, the region 1,130–1,080 cm^{-1} (C-C and C-O-C vibration) of MIR spectra, and the region 1,680–1,650 cm^{-1} (C=C vibration) of Raman spectra.

Fenton and Crisler (1959) published a study showing the potential of NIR spectroscopy. They developed, using a series of chemical products, a rapid and reliable technique for the determination of the iodine value. The calibration equation was constructed with the information contained in the region 2,100–2,200 nm of the NIR spectrum. Some years earlier, Sinclair et al. (1952) had described the linear relationship between the number of *cis* double bonds of unsaturated fatty acid methyl esters and the ratio between the absorbance at 2,920 cm^{-1} (>CH_2 vibration) and the difference between the absorbances at 2,920 and 3,020 cm^{-1} (=C-H vibration). This study was later confirmed by the results achieved by other authors who proposed MIR spectroscopy as a technique to determine the degree of unsaturation (Chapman 1965). Later, Arnold and Hartung (1971), using the ratio of absorbances at 3,030 cm^{-1} (=C-H vibration) and 2,857 cm^{-1} (>CH2 vibration), showed the potential of a MIR instrument equipped with a transmission cell for iodine value determinations in fats and oils. Using the absorbance of wavenumbers from the same region (3,010 and 2,854 cm^{-1}), Afran and Newbery (1991) demonstrated the potential of an FT-MIR instrument coupled with an ATR accessory. The absorption intensities at 3,007 cm^{-1} (=C-H vibration) (Muniategui et al. 1992) and at 1,658 cm^{-1} (C=C vibration) (Bernard and Sims 1980) were also used to determine the total degree of unsaturation.

A FT-MIR/ATR instrument, together with a PLS procedure, was used by van de Voort et al. (1992) for determining the iodine value using TAGs as dependent variables. Spectral information from regions 3,200–2,600 cm^{-1} and 1,600–1,000 cm^{-1} was successfully used. Bailey and Horvat (1972) showed the high correlation between the iodine value and the ratio of the scattering intensities in the regions 1,691–1,626 cm^{-1} (C=C vibration) and 1,478–1,420 cm^{-1} (>CH_2 vibration) using Raman spectroscopy. Later, a Raman spectrometer equipped with a NIR excitation and interferometry technology was used by Sadeghi-Jorabchi et al. (1990) to study the possibilities offered by the new generation of such instruments in the determination of iodine value of oils and margarines. The quantitative program designed in this study used information from the scattering bands centered at 1,656 cm^{-1} (=C-H vibration) and at 1,444 cm^{-1}. The peaks in the vicinity of 3,010 cm^{-1} (=C-H stretching vibration) and 1,270 cm^{-1} (=C-H bending vibration) also showed a high correlation with the iodine value (Sadeghi-Jorabchi et al. 1991; Baeten et al. 1998).

10.7.2 Determination of Trans *and* Cis *Content*

Infrared methods for determining the *trans* isomer content of oils and fats are standardized (IUPAC 1992; AOCS 1988). These methods are based on the absorption band at 967 cm^{-1} (*trans* CH=CH vibration). However, Lanser and Emken (1988), using the peak area of the *trans* absorbance band at 966 cm^{-1}, estimated the *trans* unsaturation, which agreed with the results obtained by gas chromatography. Belton et al. (1988) used FT-MIR combined with ATR to develop a procedure for the estimation of isolated *trans* double bonds in oils and fats. Sleeter and Matlock (1989) developed a FT-MIR procedure for measuring the *trans* content of oils in a 100-μm KBr cell. Ulberth and Haider (1992) used *trans*-free methylated soybean oil mixed with methyl elaidate in combination with a FT-MIR spectral subtraction technique and PLS to assess low concentrations of isolated *trans* double bonds in hydrogenated fats such as margarine and shortenings. Then, van de Voort et al. (1995) designed a generalized, industrial sample-holder accessory for handling both fats and oils. It was incorporated into a FT-MIR spectrometer, and a method using PLS calibration was developed to determine the *cis* and *trans* contents of neat samples. Mossoba et al. (1996) also used attenuated total reflection spectroscopy to calculate the total *trans* content of hydrogenated oils by the information of the spectral region between 990 and 945 cm^{-1}.

Using Raman spectroscopy, Bailey and Horvat (1972) also determined the *cis/trans* isomer content of edible vegetable oils by measuring the intensities of C=C stretching fundamentals near 1,657 and 1,670 cm^{-1} that are associated with *cis* and *trans* configurations, respectively. As seen earlier, the use of FT-Raman spectroscopy has proved to be successful in the determination of total unsaturation of oils and margarines (Sadeghi-Jorabchi et al. 1990). Furthermore, Sadeghi-Jorabchi et al. (1991) have also used the FT-Raman scattering information from bands centered near 1,670, 1,656, and 1,444 cm^{-1} to estimate various levels of *cis* and *trans* isomers mixtures. A similar approach was used by Ozaki et al. (1992) to estimate the level of unsaturation of a wide range of fat-containing foodstuffs.

10.7.3 Determination of Saponification Number, Solid Fat Index, and Free Fatty Acids

Using the information obtained from a FT-MIR spectrometer equipped with an ATR accessory and the PLS methodology, van de Voort et al. (1992) proposed a method to determine the saponification number. They used the information contained in two MIR regions: 3,200–2,600 cm^{-1} and 1,850–1,000 cm^{-1}. Van de Voort et al. (1996) also showed the potential of MIR spectroscopy in the determination of the solid fat index. The calibration was done with selected parts of the spectrum: 3,015–3,005 cm^{-1}, 3,000–2,850 cm^{-1}, 1,750–1,740 cm^{-1}, 1,550–1,050 cm^{-1}, 980–960 cm^{-1}, and 750–730 cm^{-1} by a FT-MIR spectrometer equipped with a flow transmission

cell and PLS. Lanser et al. (1991) used peaks near 1,745 and 1,711 cm^{-1} to construct a model allowing the determination of the free fatty acid content in crude oils. The C=O carbonyl group of esters is present near 1,746 cm^{-1}, while the carboxylic group of free fatty acids has its characteristic peak at 1,711 cm^{-1}. Later, an FT-MIR instrument and ATR accessory were successfully used to determine the free fatty acid content in oils and fats (Ismail et al. 1993).

10.7.4 Monitoring the Oxidative Process, Measuring the Peroxide and Anisidine Values

The potential of FT-MIR instruments for the study of the complex changes that take place in a sample involved in an oxidation process has also been investigated (van de Voort et al. 1994a). The authors used oils oxidized under various conditions and recorded their MIR spectral changes. They identified the most noteworthy bands associated with common oxidation end products such as, for example, hexanal, decadienal, (E)-butyl hydroxide, demonstrating the usefulness of FT-MIR spectroscopy to detect oxidative changes.

A method based on FT-MIR spectroscopy was also proposed for the simultaneous monitoring of aldehyde formation and the determination of the anisidine value in thermally stressed oils (Dubois et al. 1996). The authors added aldehydes to an oil sample and thus built a calibration model by PLS.

10.8 Potential of Fluorescence Spectroscopy

Fluorescence spectroscopy is a rapid analytical technique with high sensitivity to determine the overall presence of series of compounds. The use of fluorescence to analyze olive oils was first proposed in 1925 by Frehse, who studied the possibility of detecting the presence of refined olive oil in virgin olive oil by examining the oils under a quartz lamp with a Wood filter; another early work showed good prospects for characterization of edible oils through fluorimetry techniques (Wolfbeis and Leiner 1984). However, this highly sensitive technique has been largely ignored for the characterization of edible oils. Only recently has progress been achieved in spectrofluorometers and several fluorescence techniques that have been introduced to facilitate the analysis of complex food. Thus, fluorescence spectroscopy has considerable potential to characterize virgin olive oils because of the large variety of fluorescent compounds (chlorophylls, pheophytins, tocopherols, vitamin E, and oxidized compounds) present in them (Sikorska et al. 2004; Guimet et al. 2004; Galano et al. 2003). On the other hand, there are remarkable differences between the fluorescence spectra of virgin olive oil and the other edible oils (Sikorska et al. 2005), which encourages the use of this technique for authentication purposes. The various categories of virgin olive oil also show particular emission spectra (Nicoletti 1990).

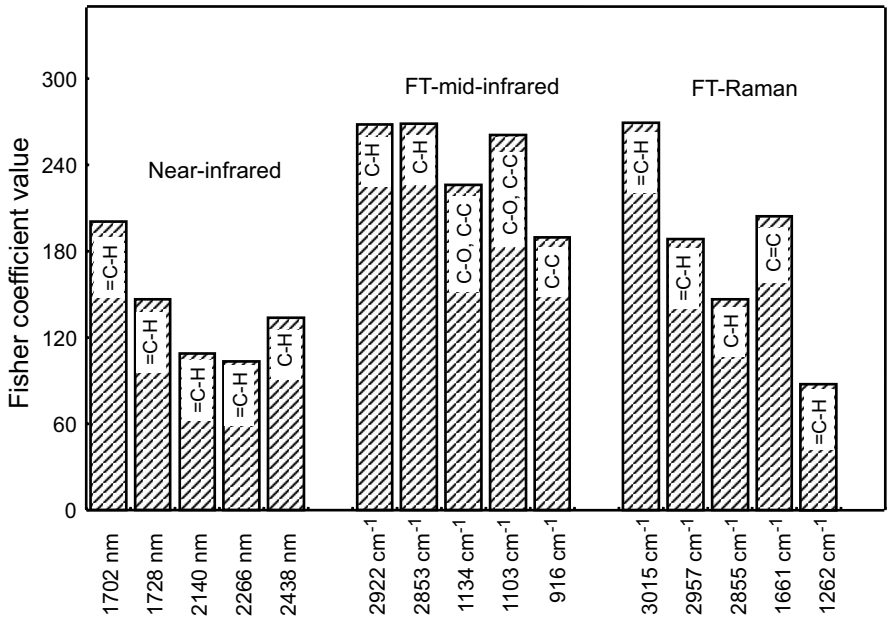

Fig. 10.21 The five most discriminant wavelengths (or wavenumbers) resulting from the study of near-infrared, FT-mid-infrared, and FT-Raman spectra of seven edible oil sources (corn, high oleic sunflower, peanut, rapeseed, soybean, sunflower, and virgin olive oils)

Some progress have been made in the development of new methods to detect adulteration, such as fraudulent mixtures of olive oil with hazelnut oil (Sayago et al. 2007), or to detect the oxidation degree of oils (Poulli et al. 2009a, b). The application of more advanced methods as EEFS and SFS makes the interpretation of the spectra more easy and informative than conventional spectroscopy.

Many fluorescent compounds present in virgin olive oil are involved in oxidation (e.g., phenols and vitamin E), and they evolve during different culinary practices such as frying. For that reason, fluorescence spectroscopy has recently been applied to evaluate the quality of thermoxidized oils (Tena et al. 2009, 2012). Other applications include the study of oil deterioration during long-term storage (Sikorska et al. 2008).

10.9 Conclusions

The previous sections have shown the potential of IR, Raman, and fluorescence spectroscopic techniques in oil analysis. NIR, MIR, and Raman spectra mainly contain information about unsaturated compounds. NIR spectroscopy can be used to determine the total level of unsaturation and the content of *cis* isomers. Excitation and

emission fluorescence spectra provide information about the minor compounds present in olive oil. The low cost and the possibility of coupling the NIR spectrometer to classical optical fibers provide a designed technique for implementation in continuous processes. MIR spectroscopy is classically used to determine the content of *trans* isomers, while information about *cis* isomers exists but is more limited. A MIR spectrometer seems to be an appropriate instrument for analytical laboratories. In fact, recent studies have demonstrated the great potential of MIR spectroscopy in the determination of classic chemical values and oil indices. The potential of this technique in the monitoring of oxidative processes is an additional advantage.

New developments in the instrumentation of Raman spectroscopy have promoted its importance for oil analysis. Raman spectra mainly contain information about *cis* and *trans* isomers. Due to the chemical origin of the bands, the information contained in the spectrum may be used to develop techniques for the determination of the total content of unsaturation, the type of unsaturation, and *cis/trans* isomer composition. In addition, a Raman spectrometer does not need a special sample-handling accessory and may be coupled to low-cost optical fibers.

In addition to the possibilities offered in quantitative analysis and in monitoring oxidative processes, IR and Raman spectroscopy show interesting perspectives in the characterization and adulteration detection of virgin olive oil. To compare the potential of NIR, MIR, and Raman spectroscopy in this domain, the spectra of 64 edible oils from seven varieties (corn, soybean, rapeseed, peanut, sunflower, high oleic sunflower, and virgin olive oils) were collected (Aparicio and Baeten 1998). The Fisher coefficient was used to underline the wavelengths and the wavenumbers having the highest power of varietal discrimination. Figure 10.21 displays, for each technique studied, the five most discriminant wavelengths or wavenumbers. NIR spectral data present Fisher coefficients lower than the data obtained in MIR and Raman spectroscopy. Four wavelengths underlined in NIR spectroscopy correspond to the C-H vibration of unsaturated groups. On the other hand, the wavenumbers extracted in MIR spectroscopy are characteristic of the C-H and C-C vibrations of the carbon skeleton and of the C-O of the ester groups. As in NIR spectroscopy, part of the Raman spectral data selected corresponds to the C-H vibration of unsaturated groups. The other wavenumbers are characteristics of C-H and C=C vibrations. Figure 10.10 clearly shows complementarity between vibrational spectroscopy (i.e., NIR and MIR spectroscopic techniques). This information can benefit from fluorescence spectroscopy, particularly in oxidation studies. Fluorescence spectroscopy is very versatile because it makes spectra acquisition possible in different modes (EEFS, SFS). These different modes provide several alternatives for a better interpretation of the spectra collected with conventional fluorescence spectroscopy to establish definitive and nonspeculative chemical assignments of the spectral bands like those in MIR, NIR, and Raman spectra.

References

Abbas O, Fernández Pierna JA, Codony R, von Holst C, Baeten V (2009) Assessment of the discrimination of animal fat by FT-Raman spectroscopy. J Mol Struct 924–926:294–300

Afran A, Newbery JE (1991) Analysis of the degree of unsaturation in edible oils by Fourier transforminfrared/attenuated total reflectance spectroscopy. Spectrosc-Int J 3:39–42

Ahro M, Hakala M, Kauppinen J, Kallio H (2002) Headspace FT-IR analysis of rapeseed oil oxidation. Appl Spectrosc 56:217–222

American Oil Chemists Society (AOCS) (1988) Officials methods and recommended practices of the American Oil Chemist's Society, method Cd 14–61. AOCS Press, Champaign

Aparicio R, Baeten V (1997) Possibilities offered by infrared and Raman spectroscopic techniques in virgin olive oil authentication. Olivae 69:38–43

Aparicio R, Baeten V (1998) Authentication of olive oil by FT-Raman. OCL 5:14–16

Aparicio R, Morales MT (1995) Sensory wheels: a statistical technique for comparing QDA panels – application to virgin olive oil. J Sci Food Agric 67:247–257

Aparicio R, Montaño J, Rodriguez-Izquierdo G (1977) Reactive power meter for nonsinusoidal systems. IEEE Trans Instru Meas 26:258–260

Aparicio R, Gutiérrez F, Rodríguez-Morales J (1992) Relationship between flavour descriptors and overall grading of analytical panels for virgin olive oil. J Sci Food Agric 58:555–562

Arnold RG, Hartung TE (1971) Infrared spectroscopy determination of degree of unsaturation of fats and oils. J Food Sci 36:166–168

Baeten V, Meurens M, Morales MT, Aparicio R (1996) Detection of virgin olive oil adulteration by Fourier transform Raman spectroscopy. J Agric Food Chem 44:2225–2230

Baeten V, Hourant P, Morales MT, Aparicio R (1998) Oil and fat classification by FT-Raman spectroscopy. J Agric Food Chem 46:2638–2646

Baeten V, Dardenne P, Aparicio R (2001) Interpretation of Fourier transform Raman spectra of the unsaponifiable matter in a selection of edible oils. J Agric Food Chem 49:5098–5107

Baeten V, Fernández Pierna JA, Dardenne P, Meurens M, García González DL et al (2005) Detection of the presence of hazelnut oil in olive oil by FT-raman and FT-MIR spectroscopy. J Agric Food Chem 53:6201–6206

Bailey GF, Horvat RJ (1972) Raman spectroscopic analysis of the cisltrans isomer composition of edible vegetable oils. J Am Oil Chem Soc 49:494–498

Banwell CN (1994) Fundamentals of molecular spectroscopy. McGraw-Hill, London

Baranska H, Labudzinska A, Terpinski J (1987) Laser Raman spectrometry: analytical applications. Ellis Horwood, Chichester

Barrow GM (1973) Physical chemistry. McGraw-Hill, New York

Bellon-Maurel V, Vigneau JL, Sévila F (1994) Infrared and near-infrared technology for the food industry and agricultural uses: on-line applications. Food Control 5:21–27

Belousov AI, Verzakov SA, von Frese JJ (2002) Applicational aspects of support vector machines. J Chemometr 16:482–489

Belton PS, Wilson RH, Sadeghi-Jorabchi H, Peers KE (1988) A rapid method for the determination of isolated trans double bonds in oils and fats using Fourier transform infrared spectroscopy combined with attenuated total reflectance. Lebensm Wiss Technol 21:153–157

Ben-Gal I (2005) Outlier detection. In: Maimon O, Rockach L (eds) Data mining and knowledge discovery handbook: a complete guide for practitioners and researchers. Kluwer Academic Publishers, New York, pp 131–146

Bernard JL, Sims LG (1980) IR spectroscopy for determination of total unsaturation. Res Dev 8:81–83

Blanco M, Villarroya I (2002) NIR spectroscopy: a rapid-response analytical tool. Trends Anal Chem 21:240–250

Burges CJC (1998) A tutorial on support vector machines for pattern recognition. Data Min Knowl Disc 2:121–167

Cayuela JA, Pérez Camino MC (2010) Prediction of quality of intact olives by near infrared spectroscopy. Eur J Lipid Sci Technol 112:1209–1217
Cayuela JA, García JM, Caliani N (2009) Predicción NIR de la humedad del fruto, acidez libre y contenido de aceite en aceitunas intactas. Grasas Aceites 60:194–202
Chapman D (1965) Infrared spectroscopy of lipids. J Am Oil Chem Soc 42:353–371
Chase B (1987) Fourier transform Raman spectroscopy. Anal Chem 59:881A–889A
Cook RD, Weisberg S (1982) Residuals and influence in regression. Chapman and Hall, London
Cowe IA, McNicol JW, Cuthbertson DC (1985a) A designed experiment for the examination of techniques used in the analysis of near infrared spectra. Part 1. Analysis of spectral structure. Analyst 110:1227–1232
Cowe IA, McNicol JW, Cuthbertson DC (1985b) A designed experiment for the examination of techniques used in the analysis of near infrared spectra. Part 2. Derivation and testing of regression models. Analyst 110:1233–1240
Defernez M, Kemsley K (1996) Potential pitfalls of chemometrics solutions to food authentication problems. In: Proceedings of food authenticity '96: Method for the measurement of food authenticity and adulteration. Institute of Food Research, Norwich
De Maesschalck R, Estienne F, Verdú-Andrés J, Candolfi A, Centner V et al (1999) The development of calibration models for spectroscopic data using principal component analysis. Internet J Chem 2:19
De Maesschalck R, Jouan-Rimbaud D, Massart DL (2000) The Mahalanobis distance. Chemometr Intell Lab 50:1–18
Despagne F, Massart DL (1998) Neural networks in multivariate calibration. Analyst 123:157R–178R
Diem M (1993) Introduction to modern vibrational spectroscopy. Wiley, New York
Dubois J, van de Voort FR, Sedman J, Ismail AA, Ramaswamy HR (1996) Quantitative Fourier transform infrared analysis for anisidine value and aldehydes in thermally stressed oils. J Am Oil Chem Soc 73:787–794
Dupuy N, Le Dréau Y, Ollivier D, Artaud J, Pinatel C, Kister J (2005) Origin of french virgin olive oil registered designation of origins predicted by chemometric analysis of synchronous excitation-emission fluorescence spectra. J Agric Food Chem 53:9361–9368
Egan WJ, Morgan SL (1998) Outlier detection in multivariate analytical chemical data. Anal Chem 70:2372–2379
Faber NM (2000) Response to comments on construction of confidence intervals in connection with partial least squares. J Chemometr 14:363–369
Faber NM, Bro R (2002) Standard error of prediction for multiway PLS – 1. Background and a simulation study. Chemometr Intell Lab 61:133–149
Fayolle PH, Picque D, Corrieu G (2000) On-line monitoring of fermentation processes by a new remote dispersive middle-infrared spectrometer. Food Control 11:291–296
Fenton AJ, Crisler RO (1959) Determination of cis unsaturation in oils by near-infrared spectroscopy. J Am Oil Chem Soc 36:620–623
Fernández Pierna JA, Wahl F, de Noord OE, Massart DL (2002) Methods for outlier detection in prediction. Chemometr Intell Lab 63:27–39
Fernández Pierna JA, Jin L, Wahl F, Faber NM, Massart DL (2003) Estimation of partial least squares regression (PLSR) prediction uncertainty when the reference values carry a sizeable measurement error. Chemometr Intell Lab 65:281–291
Fernández Pierna JA, Baeten V, Michotte Renier A, Cogdill RP, Dardenne P (2004) Combination of support vector machines (SVM) and near infrared (NIR) imaging spectroscopy for the detection of meat and bone meat (MBM) in compound feeds. J Chemometr 18:341–349
Fletcher R, Reeves M (1964) Function minimization by conjugate gradients. Comput J 7: 149–154
Galano T, Durán I, Correa CA, Roldán B, Rodriguez MI (2003) Simultaneous fluorometric determination of chlorophylls a and b and pheophytins a and b in olive oil by partial least squares calibration. J Agric Food Chem 51:6934–6940

Galeano T, Durán I, Correa CA, Roldán B, Rodriguez MI (2003) Simultaneous fluorometric determination of chlorophylls a and b and pheophytins a and b in olive oil by partial least squares calibration. J Agric Food Chem 51:6934–6940

Gallardo-González L, Osorio-Bueno E, Sánchez-Casas J (2005) Application of near infrared spectroscopy (NIRS) for the real-time determination of moisture and fat contents in olive pastes and wastes of oil extraction. Alimentación, Equipos y Tecnología 24:85–89

García-Gonzalez DL, van de Voort FR (2009) A novel wire mesh "cell" for studying lipid oxidative processes by fourier transform infrared spectroscopy. Appl Spectrosc 63:518–527

Gerrard DL, Birnie J (1992) Raman spectroscopy. Anal Chem 64:502R–513R

Geurts MD, Rinne HJ, Lawrence SH (1990) Alternative methods od dealing with outliers in forecasting sales with regression-based methods. In: Lawrence KD, Arthur JL (eds) Robust regression: analysis and application. Marcel Dekker, New York, pp 225–239

Giungato P, Aveni M, Rana R, Notarnicola L (2004) Modifications induced by extra virgin olive oil frying processes. Ind Aliment-Italy 43:369–375

Goddu RF (1957) Determination of unsaturation by near-infrared spectrophotometry. Anal Chem 29:1790–1794

Gonzaga FB, Pasquini C (2006) A new method for determination of the oxidative stability of edible oils at frying temperatures using near infrared emission spectroscopy. Anal Chim Acta 570:129–135

Grasseli JG, Bulkin BJ (1991) Analytical Raman spectroscopy. Wiley, New York

Guillén MD, Cabo N (1997) Infrared spectroscopy in the study of edible oils and fats. J Sci Food Agric 75:1–11

Guimet F, Ferré J, Boqué R, Rius FX (2004) Application of unfold principal component analysis and parallel factor analysis to the exploratory analysis of olive oils by means of excitation-emission matrix fluorescence spectroscopy. Anal Chim Acta 515:75–85

Harrick N (1967) Internal reflection spectroscopy. Wiley, New York

Hendra PJ (1997) Fourier transform Raman spectroscopy in pharmaceutical analysis and research. Int Lab 5:13A–13I

Hermoso M, Uceda M, García-Ortiz A, Jiménez A, Beltrán G (1999) Preliminary results of NIR "on line" measure of oil content and humidity in olive cakes from the two phases decanter. Acta Horticulturae 474:717–719

Herzberg G (1945) Molecular spectra and molecular structure. II. Infrared and Raman spectra of polyatomic molecules. Van Nostrand Reinhold, New York

Hirschfeld T, Chase B (1986) FT-Raman spectroscopy: development and justification. Appl Spectrosc 3:133–141

Holman RT, Edmondson PR (1956) Near infrared spectra of fatty acids and some related substances. Anal Chem 28:1533–1538

Hooke R, Jeeves FA (1961) Direct search solution of numerical and statistical problems. J Assoc Comput Mach 8:212–229

Hourant P (1995) Contrôle de qualité des matières grasses alimentaires par spectroscopie infrarouge. Catholic University of Louvain (UCL), Louvain-la-Neuve

Hourant P, Baeten V, Morales MT, Meurens M, Aparicio R (2000) Oil and fat classification by selected bands of near-infrared spectroscopy. Appl Spectrosc 54:1168–1174

Høy M, Steen K, Martens H (1998) Review of partial least squares regression prediction error in Unscrambler. Chemometr Intell Lab 44:123–133

Huang H, Yu H, Xu H, Ying Y (2008) Near infrared spectroscopy for on/in-line monitoring of quality in foods and beverages: a review. J Food Eng 87:303–313

International Union Pure and Applied Chemistry (IUPAC) (1992) Determination of content of isolated *trans*-unsaturated compounds by infrared spectrophotometry. In: Dieffenbacher A, Pocklington WD (eds) Standards methods for the analysis of oils, fats and derivatives, 7th edn. Blackwell Scientific Publication, Oxford, pp 99–102, Method 2.207

Ismail AA, van de Voort FR, Emo G, Sedman J (1993) Rapid quantitative determination of free fatty acids in fats and oils by Fourier transform infrared spectroscopy. J Am Oil Chem Soc 70:335–341

Ismail AA, Cocciardi RA, Alvarez P, Sedman J (2006) Infrared and Raman spectroscopy in food science. In: Hui YH (ed) Handbook of food science, technology and engineering. CRC Press, Boca Raton, pp 44.1–44.19

Jiménez-Márquez A, Molina-Díaz A, Pascual-Reguera MI (2005) Using optical NIR sensor for on-line virgin olive oils characterization. Sensors Actuat B 107:64–68

Jouan-Rimbaud D, Bouveresse E, Massart DL, de Noord OE (1999) Detection of prediction outliers and inliers in multivariate calibration. Anal Chim Acta 388:283–301

Keller S, Löchte T, Dippel B, Schrader B (1993) Quality control of food with near-infrared excited Raman spectroscopy. Fresenius J Anal Chem 346:863–867

Kennard RW, Stone LA (1969) Computer aided design of experiments. Technometrics 11:137–148

Kondepati VR, Heise HM (2008) The potential of mid- and near-infrared spectroscopy for reliable monitoring of bioprocesses. Curr Trends Biotechnol Pharm 2:117–132

Kuligowski J, Quintás G, de la Guardia M, Lendl B (2010) Analytical potential of mid-infrared detection in capillary electrophoresis and liquid chromatography: a review. Anal Chim Acta 679:31–42

Kyriakidis NB, Skarkalis P (2000) Fluorescence spectra measurement of olive oil and other vegetable oils. J AOAC Int 83:1435–1438

Lai YW, Kemsley EK, Wilson RH (1994) Potential of Fourier transform infrared spectroscopy for the authentication of vegetable oils. J Agric Food Chem 42:1154–1159

Lakowicz JR (1999) Principles of fluorescence spectroscopy, 2nd edn. Kluwer Academic Publishers, New York, pp 27–61

Lanser AC, Emken EA (1988) Comparison of FTIR and capillary gas chromatographic methods for quantitation of trans unsaturation in fatty acid methyl esters. J Am Oil Chem Soc 65:1483–1487

Lanser AC, List GR, Holloway RK, Mounts TL (1991) FTIR estimation of free fatty acid content in crude oils extracted from damaged soybeans. J Am Oil Chem Soc 68:448–449

Levin IW, Lewis EN (1990) Fourier transform Raman spectroscopy of biological materials. Anal Chem 62:1101A–1111A

Lewis EN, Kalasinsky VF, Levin IW (1988) Near-infrared Fourier transform Raman spectroscopy using fiber-optic assemblies. Anal Chem 60:2658–2661

Li Y, García-González DL, Yu X, van de Voort FR (2008) Determination of free fatty acids in edible oils with the use of a variable filter array IR spectrometer. J Am Oil Chem Soc 85:599–604

Li D, Sedman J, García-González DL, van De Voort FR (2009) Automated acid content determination in lubricants by FTIR spectroscopy as an alternative to acid number determination. J ASTM Int 6:1–12

Li-Chan ECY (1994) Developments in the detection of adulteration of olive oil. Trends Food Sci Technol 5:3–11

Li-Chan ECY (1996) The application of Raman spectroscopy in food science. Trends Food Sci Technol 7:361–370

Li-Chan ECY, Griffiths PR, Chalmers JM (2010a) Applications of vibrational spectroscopy in food science. Volume I: instrumentation and fundamental applications. Wiley, Chichester

Li-Chan ECY, Griffiths PR, Chalmers JM (2010b) Applications of vibrational spectroscopy in food science. Volume II: analysis of food, drink and related material. Wiley, Chichester

Ma K, van de Voort FR, Ismail AA, Sedman J (1998) Quantitative determination of hydroperoxides by FTIR spectroscopy using a disposable IR card. J Am Oil Chem Soc 75:1095–1101

Ma K, van de voort FR, Sedman J, Ismail AA (1999) *Trans* determination in fats and oils and margarine by FTIR spectroscopy using a disposable IR card. J Am Oil Chem Soc 76:1399–1404

Martens H, Naes T (1989) Multivariate calibration. Wiley, Chichester

Massart DL, Kaufman L (1983) The interpretation of analytical chemical data by the use of cluster analysis. Wiley Interscience, Chichester

Mossoba MM, Yurawecz MP, McDonald RE (1996) Rapid determination of the total *trans* content of neat hydrogenated oils by attenuated total reflection spectroscopy. J Am Oil Chem Soc 73:1003–1009

Muniategui S, Paseiro P, Simal J (1992) Medida del grado de insaturación de aceites y grasas comestibles por espectroscopia infrarroja y su relación con el índice de yodo. Grasas Aceites 43:1–5

Naes T, Isaksson T (1989) Selection of samples for calibration in near-infrared spectroscopy. Part I: general principles illustrated by example. Appl Spectrosc 43:328–335

Nicoletti GM (1990) La fluorescenza degli oli di oliva. Riv Ital Sost Grasse 67:389–396

O'Neill R (1971) Function minimization using a simplex procedure. Appl Stat-J Roy St 3:79–88

Osborne BG, Fearn T, Hindle PH (1993) Spectroscopy with application in food and beverage analysis. Longman Sci & Tech, Singapore, pp 1–77 and pp 120–141

Ozaki Y, Cho R, Ikegaya K, Muraishi S, Kawauchi K (1992) Potential of near-infrared Fourier transform Raman spectroscopy in food analysis. Appl Spectrosc 46:1503–1507

Panford JA, deMan JM (1990) Determination of oil content of seeds by NIR: influence of fatty acid composition on wavelength selection. J Am Oil Chem Soc 67:473–482

Patra D, Mishra AK (2002) Recent developments in multi-component synchronous fluorescence scan analysis. Trac-Trends Anal Chem 21:787–798

Pell RJ (2000) Multiple outlier detection for multivariate calibration using robust statistical techniques. Chemometr Intell Lab 52:87–104

Pfaffenberger RC, Dielman TE (1990) A comparison of regression estimators when both multicollinearity and outliers are present. In: Lawrence KD, Arthur JL (eds) Robust regression: analysis and applications. Marcel Dekker, New York, pp 243–270

Poulli KI, Chantzos NV, Mousdis GA, Georgiou CA (2009a) Synchronous fluorescence spectroscopy: tool for monitoring thermally stressed edible oils. J Agric Food Chem 57:8194–8201

Poulli KI, Mousdis GA, Georgiou CA (2009b) Monitoring olive oil oxidation under thermal and UV stress through synchronous fluorescence spectroscopy and classical assays. Food Chem 117:499–503

Rabiner LR, Gold B (1975) Theory and application of digital signal processing. Prentice-Hall, Englewood Cliffs

Ratnayake WMN, Pelletier G (1996) Methyl esters from a partially hydrogenated vegetable oil is a better infrared external standar than methyl elaidate for the measurement of total trans content. J Am Oil Chem Soc 73:1165–1169

Sadeghi-Jorabchi H, Hendra PJ, Wilson RH, Belton PS (1990) Determination of the total unsaturation in oils and margarines by Fourier transform Raman spectroscopy. J Am Oil Chem Soc 67:483–486

Sadeghi-Jorabchi H, Wilson RH, Belton PS, Edwards-Webb JD, Coxon DT (1991) Quantitative analysis of oils and fats by Fourier transform Raman spectroscopy. Spectrochim Acta A 47:1449–1458

Sato T (1994) Near-infrared spectroscopic analysis of deterioration indices of soybeans for process control in *oil* milling plant. J Am Oil Chem Soc 71:293–298

Sato T, Kawano S, Iwamoto M (1991) Near-infrared spectral patterns of fatty acid analysis from fats and oils. J Am Oil Chem Soc 68:827–833

Savitsky A, Golay MJE (1964) Smoothing and differentiation of data by simplified least square procedures. Anal Chem 36:1627–1630

Sayago A, García-González DL, Morales MT, Aparicio R (2007) Detection of the presence of refined hazelnut oil in refined olive oil by fluorescence spectroscopy. J Agric Food Chem 55:2068–2071

Schrader B (1996) Raman spectroscopy in the near-infrared – a most capable method of vibrational spectroscopy. Fresenius J Anal Chem 355:233–239

Scotter CNG (1997) Non-destructive spectroscopic techniques for the measurement of food quality. Trends Food Sci Technol 8:285–292

Sharma A, Schulman SG (1999) Mesurement of fluorescence spectra. In: Introduction to fluorescence spectroscopy. Wiley, New York, pp 69–99

Sikorska E, Romaniuk A, Khmelinskii IV, Herance R, Bourdelande JL, Sokorski M, Koziol J (2004) Characterization of edible oils using total luminescence spectroscopy. J Fluoresc 14:25–35

Sikorska E, Górecki T, Khmelinskii IV, Sokorski M, Koziol J (2005) Classification of edible oils using synchronous scanning fluorescence spectroscopy. Food Chem 89:217–225

Sikorska E, Khmelinskii IV, Sikorski M, Caponio F, Bilancia MT, Pasqualone A, Gomes T (2008) Fluorescence spectroscopy in monitoring of extra virgin olive oil during storage. Int J Food Sci Technol 43:52–61

Sinclair RG, McKay AF, Myers GS, Jones RN (1952) The infrared absorption spectra of saturated fatty acids and esters. J Am Oil Chem Soc 74:2578–2585

Skoog DA, West DM, Holler FJ (1992) Fundamentals of analytical chemistry. Saunders College Publ Int Edition, New York

Sleeter RT, Matlock MG (1989) Automated quantitative analysis of isolated (nonconjugated) *trans* isomers using Fourier transform infrared spectroscopy incorporating improvements in the procedure. J Am Oil Chem Soc 66:121–127

Snee RD (1977) Validation of regression models, methods and examples. Technometrics 19:415–428

Socrates G (1994) Infrared characteristic group frequencies: tables and charts. Wiley, New York

Stefanov I, Baeten V, Abbas O, Colman E, Vlaeminck B, De Baets B, Fievez V (2010) Analysis of milk odd- and branched-chain fatty acids using Fourier transform (FT)-Raman spectroscopy. J Agric Food Chem 58:10804–10811

Strasburg GM, Ludescher RD (1995) Theory and applications of fluorescence spectroscopy in food research. Trends Food Sci Technol 6:69–75

Tabachnick BG, Fidell LS (1983) Using multivariate statistics. Harper & Row, New York

Tai C, Gu X, Zou H, Guo Q (2002) A new simple and sensitive fluorometric method for the determination of hydroxyl radical and its application. Talanta 58:661–667

Tena N, García-González DL, Aparicio R (2009) Evaluation of virgin olive oil thermal deterioration by fluorescence spectroscopy. J Agric Food Chem 57:10505–10511

Tena N, Aparicio R, García-González DL (2012) Chemical changes of thermoxidized virgin olive oil determined by excitation-emission fluorescence spectroscopy (EEFS). Food Res Int 45:103–108

Ulberth F, Haider HJ (1992) Determination of low level *trans* unsaturation in fats by Fourier transform infrared spectroscopy. J Food Sci 57:1444–1447

Valeur B (2002) Molecular fluorescence: principles and applications. Wiley-VCH Verlag, Weinheim

van de Voort FR (1994) FTIR spectroscopy in edible oil analysis. Inform 5:1038–1042

van de Voort FR, Ismail AA (1991) Proximate analysis of foods by mid-FTIR spectroscopy. Trends Food Sci Technol 2:13–17

van de Voort FR, Sedman J, Emo G, Ismail AA (1992) Rapid and direct iodine value and saponification number determination of fats and oils by attenuated total reflectance/fourier transform infrared spectroscopy. J Am Oil Chem Soc 69:1118–1123

van de Voort FR, Ismail AA, Sedman J, Dubois J, Nicodemo T (1994a) The determination of peroxide value by Fourier transform infrared spectroscopy. J Am Oil Chem Soc 71:921–926

van de Voort FR, Ismail AA, Sedman J, Emo G (1994b) Monitoring the oxidation of edible oils by Fourier transform infrared spectroscopy. J Am Oil Chem Soc 71:243–253

van de Voort FR, Ismail AA, Sedman J (1995) A rapid automated method for the determination of cis and trans content of fats and oils by Fourier transform infrared spectroscopy. J Am Oil Chem Soc 72:873–880

van de Voort FR, Memon KP, Sedman J, Ismail AA (1996) Determination of solid fat index by FTIR spectroscopy. J Am Oil Chem Soc 73:411–416

van de Voort FR, Cocciardi R, Sedman J, Juneau S (2007a) An automated FTIR method for the routine quantitative determination of moisture in lubricants. Talanta 72:289–295

Chapter 11
Nuclear Magnetic Resonance: Methodologies and Applications

Photis Dais

Contents

11.1 Introduction

High-resolution nuclear magnetic resonance (NMR) spectroscopy has proven to be a powerful tool for the analysis of multicomponent systems such as olive oil (Sacchi et al. 1997; Vlahov 1999; Hidalgo and Zamora 2003; Mannina and Segre 2002;

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties*,
DOI 10.1007/978-1-4614-7777-8_11, © Springer Science+Business Media New York 2013

Mannina et al. 2003a; Dais and Spyros 2007; Brescia and Sacco 2008a, b; Mannina and Sobolev 2011). Recent advances in NMR methodology and instrumentation made this technique an alternative choice in olive oil analysis. With the exception of low detection limits (however, see below), the combined capabilities of multinuclear and multidimensional NMR methods made it feasible to obtain structural and quantitative information on a wide range of organic metabolites in plant extracts in a single experiment and, more importantly, with no (or minimal) sample pretreatment. These properties allow a substantial reduction in labor and time and the prospect of identification of unexpected or previously unknown components. NMR parameters, such as chemical shifts, spin multiplicities, coupling constants, and signal intensities, provide valuable pieces of structural information and ensure valid quantitative results.

Another facet of NMR spectroscopy involves relaxation time measurements and spin-echo methods in low magnetic fields. Low-resolution, time-domain NMR spectroscopy has found increasing use in the oil industry as a low-cost method of analysis of fats and oils (Gambhir 1992). New developments in relaxometry and diffusometry can be useful when the components of a mixture are chemically similar and have spectra that highly overlap (van Duynhoven et al. 2010). Discrimination of pure components in mixtures is based on differences in solution diffusivity. The implementation of pulsed-field gradients (PFGs) in NMR spectrometers advanced the analysis of mixtures in low- and high-field NMR. A brief note about a relatively old NMR technique based on deuterium NMR, Site-Specific Natural Isotope Fractionation Nuclear Magnetic Resonance (SNIF-NMR), will be mentioned briefly in relation to olive oil.

The aim of this chapter is to present the NMR methodologies that are being used in the analysis of olive oil and the progress they have made so far in olive oil quality control and authentication. Strategies will be suggested to overcome routine problems, for example, complex NMR data sets due to severe signal overlap in overcrowded ^{1}H NMR spectra, or ambiguities in quantitative analysis using ^{13}C NMR spectra. NMR instrumental and experimental hints to obtain high-quality spectra and useful information about sample preparation will be given as well. The potential of NMR spectroscopy for the analysis of olive oil will be presented, focusing on those components that can serve as potential markers of EVOO quality and authentication. A brief discussion will be made about the preprocessing of NMR data for chemometric analysis and about relevant applications to olive oil adulteration and classification studies according to geographical and varietal origin. The final section will be devoted to future trends and perspectives of the scientific aspects described in the chapter.

11.2 High-Resolution Nuclear Magnetic Resonance Spectroscopy

11.2.1 Multinuclear Methodologies

Three basic nuclear spins – ^{1}H, ^{13}C, and ^{31}P – have been used extensively for the analysis of olive oil, giving complementary and sometimes unique information about the identity of major and minor constituents of olive oil. In contrast to ^{1}H and

^{13}C nuclei, ^{31}P NMR spectroscopy is a distractive methodology, inasmuch as it requires derivatization of the olive oil components bearing hydroxyl and carboxyl groups (e.g., phenolic compounds) with a phosphorus reagent (see below). Recording high-resolution, one-dimensional (1D) ^{1}H, ^{13}C, and ^{31}P NMR spectra for qualitative analysis employing the one-pulse sequence is a trivial matter and the success of the analysis depends, among other things, on the available instrumentation and the skill of the NMR user.

In general, ^{1}H NMR spectra of mixtures are expected to be more complicated than ^{13}C and ^{31}P spectra. The presence of scalar coupling among neighboring protons and the much smaller chemical shift range of protons (approximately 15 ppm) results in overcrowded spectra with severe signal overlap. The much higher paramagnetic contribution to the shielding of ^{13}C and ^{31}P nuclei increases dramatically their chemical shift range, approximately 250 ppm for ^{13}C and approximately 1,000 ppm for ^{31}P; this capacity facilitates the wider distribution of signals. Moreover, ^{13}C and ^{31}P spectra are usually obtained under broadband proton decoupling to eliminate all couplings with protons of the molecule, resulting in single signals for nonequivalent carbon or phosphorus nuclei. This ensures further signal separation, while at the same time the intensity of the decoupled signals increases mainly due to a nuclear Overhauser enhancement (NOE) contribution. It is worth mentioning that proton decoupling greatly simplifies ^{13}C (^{31}P) spectra, but it removes valuable structural information inherent in the coupled spectra.

Apart from the wide range of ^{31}P chemical shifts, the 100 % natural abundance of the ^{31}P nucleus and its high sensitivity, which is only approximately 15 times less than that of the proton nucleus, make ^{31}P NMR experiments a reliable analytical tool to determine amounts on the order of micromoles, or lower, depending on the available instrumentation. These properties of the ^{31}P nucleus should be contrasted with the low natural abundance (1.1%) and sensitivity of the ^{13}C nucleus, which in addition is characterized by long relaxation times. Therefore, ^{31}P NMR spectroscopy can be considered an alternative methodology to ^{1}H and ^{13}C NMR in cases where severe overlap is observed in ^{1}H NMR spectra or quantitative ^{13}C NMR experiments require lengthy accumulations and long relaxation delays to achieve a satisfactory signal-to-noise ratio (see below). Since no constituent of olive oil (besides phospholipids) contains phosphorus nuclei, detection of minor components bearing hydroxyl or carboxyl groups, such as diacylglycerols and phenolic compounds, has been accomplished with prior replacement of the labile hydrogens of functional groups of olive oil constituents with the reagent 2-chloro-4,4,5,5-tetramethyldioxaphospholane (labeled 1), according to the reaction scheme shown in Fig. 11.1, and the use of ^{31}P chemical shifts of the phosphitylated compounds (labeled 2) to identify the labile centers (Spyros and Dais 2000). Compound 1 reacts rapidly (approximately 15 min) and quantitatively under mild conditions (within the NMR tube) with hydroxyl groups. As an example, Fig. 11.2 shows the 202.2 MHz ^{31}P NMR spectrum of a phosphitylated olive oil sample in a region where the signals of diacylglycerol isomers, total free sterols, and free fatty acids appear. The signal at 145.15 belongs to the internal standard cyclohexanol used for quantitative purposes.

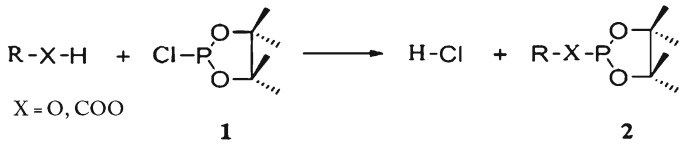

Fig. 11.1 Reaction of hydroxyl and carboxyl groups of olive oil constituents with phosphorus reagent 2-chloro-4,4,5,5-tetramethyldioxaphospholane (**1**)

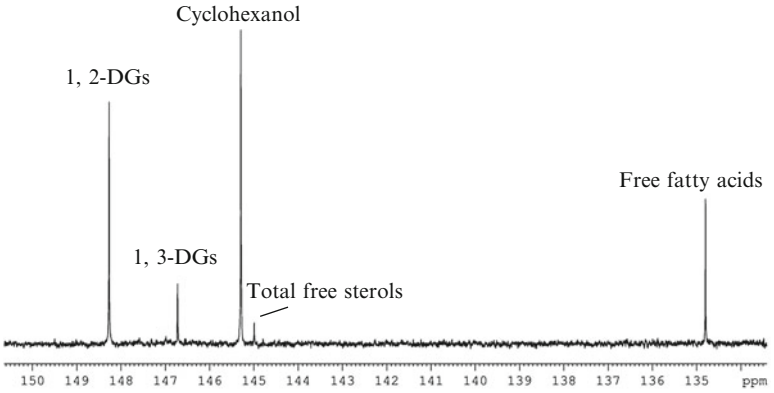

Fig. 11.2 202.2 MHz ^{31}P NMR spectrum of virgin olive oil. The region where the phosphitylated total sterols, diacylglycerols, and free fatty acids resonate is illustrated. The phosphitylated cyclohexanol is used as internal standard. *1,2-DAD* 1, 2- diacyglycerols, *1,3-DAG* 1, 3-diacylglycerols (*Source*: Vigli et al. (2003), with permission of American Chemical Society)

11.2.2 Multidimensional Nuclear Magnetic Resonance Methodologies for Spectral Assignments

Depending on the extent of overlap in 1D ^{1}H NMR spectra of mixtures, it is not always possible to extract spectroscopic parameters with certainty. This is exactly the case with the 500 MHz ^{1}H NMR spectrum of the polar part of olive oil extracted with a mixture of methanol:water (80/20 v/v) in DMSO-d_6 solvent (Fig. 11.3). This spectrum, which can be divided into three frequency regions, contains signals of a large number of phenolic compounds (Christophoridou and Dais 2009). The development of multidimensional NMR techniques offers the possibility to unravel hidden resonances, either through bond (scalar spin-spin coupling) or through space (dipolar coupling) connectivity. The basic concept of 2D NMR is the introduction of an additional time interval, the evolution period, between the preparation and detection intervals of the 1D pulse sequence (Fig. 11.4). Depending on factors that influence the transverse magnetization of the nuclear spins during the evolution time t_1 (e.g., Larmor precession or scalar coupling), different types of correlations are developed between homonuclear and heteronuclear spins. In some experiments

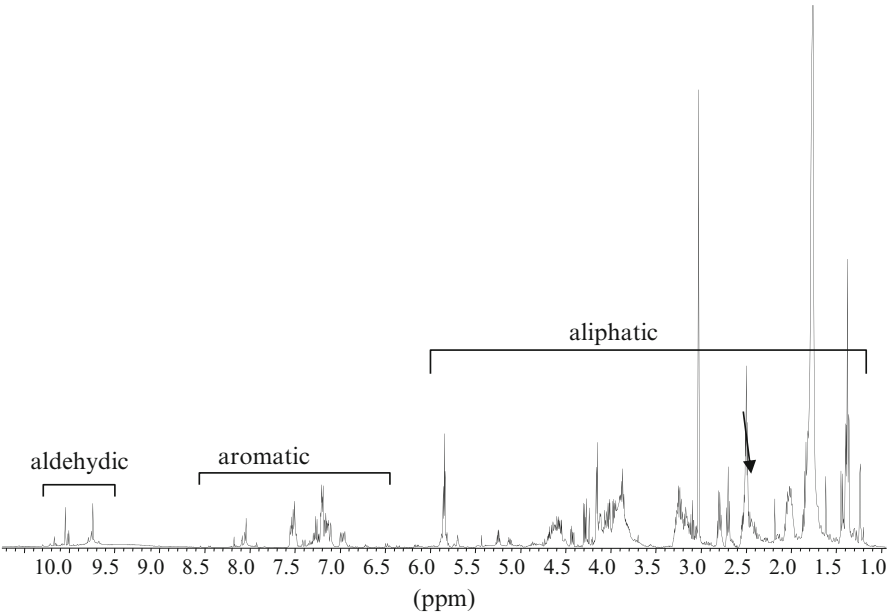

Fig. 11.3 500 MHz ^{1}H NMR spectrum of polar fraction of olive oil in DMSO-d_6 solvent. Three separate frequency regions for aldehydic, aromatic, and aliphatic protons of phenolic compounds were observed

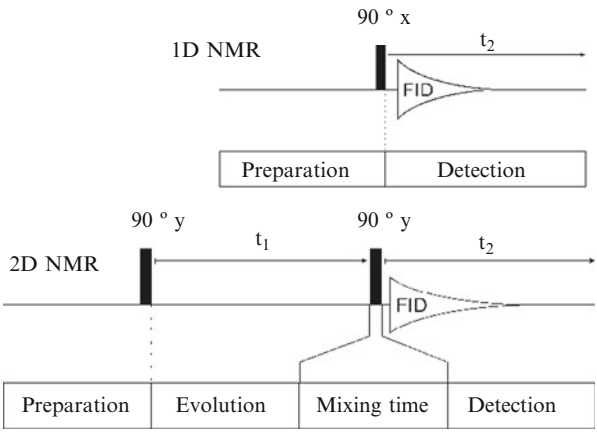

Fig. 11.4 Schematic representation of one-dimensional (1D) and two-dimensional (2D) pulse sequences with mixing time. FID = free induction decay

an additional time interval, a single pulse, a time period, or a combination of both is added between the evolution and the detection interval, the so-called mixing period (Fig. 11.4), to guarantee the transfer of magnetization from one type of spin to the other. The evolution time is kept variable. Actually, it is made stepwise longer (incremented) in analogy to the detection time t_2. For each t_1 increment, a separate

free induction decay (FID) is detected in t_2. Thus an NMR signal is obtained in the time domain, $S(t_1, t_2)$, which is a function of two time variables. Double Fourier transformation gives a 2D spectrum (stacked or contour plot), whose axes describe different spectroscopic parameters (e.g., chemical shifts, coupling constants, NOEs) evolved during the evolution period. Correlations of different spectroscopic parameters in homonuclear or hetronuclear 2D spectra are visualized by the so-called cross peaks. Moreover, the removal of unwanted signals by the selection of pertinent coherence pathways by means of pulsed field gradients make unnecessary the time-consuming and relatively inefficient phase-cycling procedure. NMR pulse sequences of different dimensionalities (2D, 3D, and 4D) have been developed in recent years and implemented in modern NMR spectrometers. However, no more than ten basic 2D NMR methods have found widespread application in food analysis. These methods are summarized in Table 11.1. Pulse sequences are arranged according to the type of correlated nuclei, the nature of the correlation, and pertinent applications. A short description and appropriate references for each type of the pulse sequence in Table 11.1 can be found in Berger and Braun (2004).

The gradient-selected 500 MHz COSY spectrum in Fig. 11.5 has greatly facilitated the profiling of phenolic compounds contained in the polar part of olive oil depicted in Fig. 11.3. This spectrum, recorded in less than 15 min, allowed the identification of most phenolic compounds and confirmed previous lengthy assignments based on model compounds (Christophoridou and Dais 2009). Figure 11.6 shows the 600 MHz gradient-selected TOCSY spectrum of an olive oil sample, which shows correlations between all protons of olive oil, even distant ones, as long as there are couplings between all intervening protons. Table 11.2 shows the identity of protons and the numbering system adopted by most researchers. The observed connectivities between the glycerol backbone protons and between the acyl-chain protons provides solid proof of previous assignments based on triacylglycerol model compounds. It is interesting to note that the very good resolution of the TOCSY spectrum permits the observation of the allylic protons (H6′) of the linolenyl chain, which are connected with the methyl protons (H9) of the same chain (δ 0.96). TOCSY and COSY experiments provide a particularly powerful combination, which could be sufficient to identify most metabolites present in mixtures.

The assignment of carbon resonances of glycerol and fatty acids is of crucial importance for the analytical characterization of olive oil. The 600 MHz ^{13}C NMR spectrum of olive oil in $CDCl_3$ illustrated in Fig. 11.7 shows a large number of signals spread over a wide range of chemical shifts. This made the spectrum appear complicated but nevertheless much more informative than the ^{1}H NMR spectrum, which extends to a narrow region of a few parts per million (Fig. 11.6). The resonances of the glycerol backbone carbons, as well as those of the fatty acids, were assigned in previous studies by several investigators. Most of these studies utilized triacylglycerol model compounds to assign carbon signals. However, this procedure raised doubts about the correctness of the assignments, especially for the interior carbons of acyl chains, and by the fact that carbon chemical shifts were found to be concentration dependent, particularly for those in the carbonyl region of the spectrum (Mannina et al. 2002). Two-dimensional NMR spectroscopy is capable of

Table 11.1 Two-dimensional (2D) NMR techniques used mostly in food science

Name	Acronym	Application	Comments
Homonuclear (H-H) techniques			
Correlation Spectroscopy	H,H-COSY		
(a) Normal		Signal assignment	Replaced by the more powerful TOCSY
(b) With delay		Detection of small couplings	Diagonal signals with mixed phases
Double quantum filtered correlation spectroscopy	DQF-COSY	Determination of *J* values	Phase sensitive; low-intensity diagonal signals
Total correlation spectroscopy	TOCSY	Signal assignment within a scalar-coupled proton system	Extent of magnetization transfer depends on length of spin-lock period
Nuclear Overhauser enhancement spectroscopy	NOESY	Distance information, 3D structure of large molecules, molecular dynamics information	Major disadvantage is its dependence on molar mass and viscosity, which can change its sign; this may cause its disappearance under certain conditions
Rotating frame Overhauser enhancement spectroscopy	ROESY	Separation of chemical exchange and NOE in small and medium-sized molecules; dynamic information	Sign is always positive; applicable to small and medium-sized molecules; shorter mixing times
Heteronuclear (H-X) techniques			
Heteronuclear correlation spectroscopy	HETCOR	Heteronuclear shift correlation through one-bond coupling	Used for carbon signal assignment; no field gradients required; short setup; good resolution at carbon dimension, but low sensitivity
Heteronuclear multiple quantum coherence	HMQC	Heteronuclear shift correlation through one-bond coupling	Two to four times more sensitive than HETCOR depending on molecular size; good resolution in proton dimension; broader cross peaks than HETCOR due to unsuppressed H-H couplings
Heteronuclear single quantum coherence	HSQC	Heteronuclear shift correlation through one-bond coupling	Better resolution than HMQC; no signal broadening by H-H coupling
Heteronuclear multiple bond coherence	HMBC	Heteronuclear shift correlation through two- and three-bond coupling	Suppression of unwanted one-bond coupling does not work equally well for all protons
Heteronuclear Overhauser effect spectroscopy	HOESY	Through space H-X correlations; distance information between quaternary carbons and nearby protons	Good resolution at X-nucleus dimension
Incredible natural abundance double quantum transfer experiment	2D-INADEQUATE	Structure elucidation; provides unequivocal C-C connectivity	Poor sensitivity

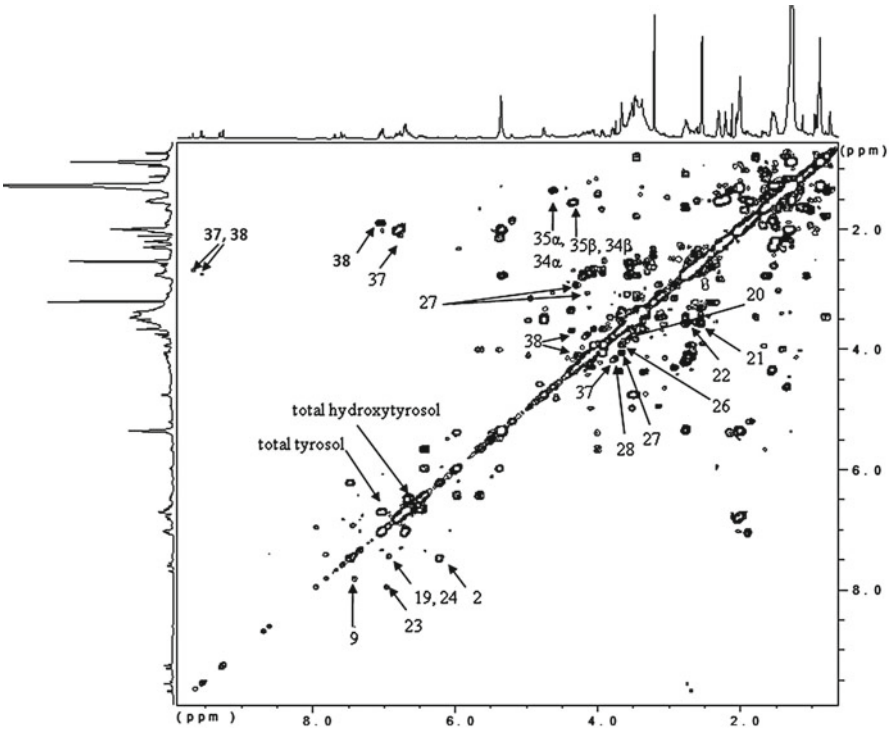

Fig. 11.5 500 MHz gradient-selected COSY spectrum of polar fraction of olive oil in DMSO-d_6 solvent. Codes correspond to phenolic compounds: (*2*) *p*-coumaric acid, (*9*) vanillic acid, (*19*) vanillin, (*20*) homovanillyl alcohol, (*21*) free tyrosol, (*22*) free hydroxytyrosol, (*23*) apigenin, (*24*) luteolin, (*26*) (+) pinoresinol, (*27*) (+) 1-acetoxypinoresinol, (*28*) syringaresinol, (*34α*) aldehydic form of oleuropein; isomer 5*S*, 8*R*, 9*S*, (*34β*) aldehydic form of oleuropein; isomer 5*S*, 8*S*, 9*S*, (*35α*) aldehydic form of ligstroside; isomer 5*S*, 8*R*, 9*S*, (*35β*) aldehydic form of ligstroside; isomer 5*S*, 8*S*, 9, (*37*) dialdehydic form of oleuropein lacking a carboxymethyl group, (*38*) dialdehyde form of ligstroside lacking a carboxymethyl group

confirming and, in some cases, correcting earlier assignments. The combination of the gradient-selected HSQC and HMBC experiments can be used to observe correlations between heteronuclei. The former experiment correlates the chemical shifts of carbons with those of the directly attached protons, whereas the latter experiment connects the carbon with protons two or three bonds away. A recent modification of the HSQC pulse sequence combines the usual C-H bond correlation with carbon multiplicity selection similar to that obtained using the DEPT-135 experiment. The edited gradient-selected HSQC spectrum (Fig. 11.8) shows correlations between the glyceridic protons and carbons of olive oil. Correlations phased negatively (unframed cross peaks) and positively (framed cross-peaks) represent methylene and methine protons, respectively. This spectrum is of major help for the assignment of the glyceridic carbons and several of the acyl-chain carbons. In addition, it achieves a partial separation of the olefinic proton signals through their correlation with the well-resolved olefinic carbon resonances in the region 127–131 ppm (Fig. 11.8).

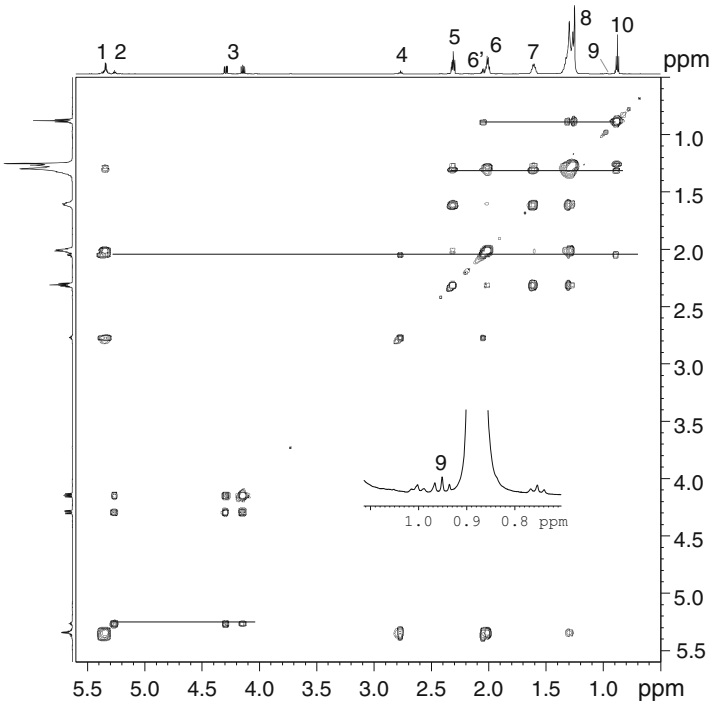

Fig. 11.6 600 MHz gradient-selected TOCSY spectrum of olive oil sample in $CDCl_3$ solvent. The numbering system for protons was explained in Table 11.2. The *inset* magnifies the triplet of the methyl protons of the alpha-linolenyl chain

Table 11.2 Chemical shifts and assignment of signals in ^{1}H NMR spectrum of virgin olive oil in chloroform-d solvent

Signal	δ (ppm)	Proton	Attribution
1	5.30	C*H*=C*H*	All unsaturated fatty acids
2	5.16	C*H*-OCOR	Triacylglycerols
3	4.19	CH_2-OCOR	Triacylglycerols
4	2.77	C*H*=CH-CH_2-CH=CH	Linoleyl and alpha-linolenyl chains
5	2.22	CH_2-COOH	All acyl chains
6	2.03	CH_2-CH=CH	Oleyl and linoleyl chains
6′	2.04	CH_2-CH=CH	Linolenyl chain
7	1.62	CH_2-CH_2-COOH	All acyl chains
8	1.21	$(CH_2)_n$	All acyl chains
9	0.96	CH=CH-CH$_2$-CH_3	Alpha-linolenyl chain
10	0.85	CH$_2$CH$_2$CH$_2$-CH_3	All acyl chains except alpha-linolenyl

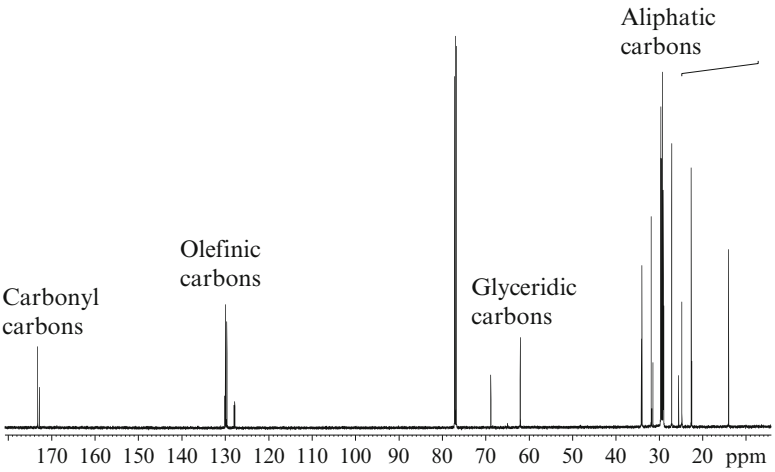

Fig. 11.7 500 MHz ^{13}C NMR spectrum of an olive oil sample in $CDCl_3$ solvent. Four separate frequency regions for the carbonyl, olefinic, glyceridic, and aliphatic carbon nuclei are observed (*Source*: Vlahov (1999), with permission of Elsevier)

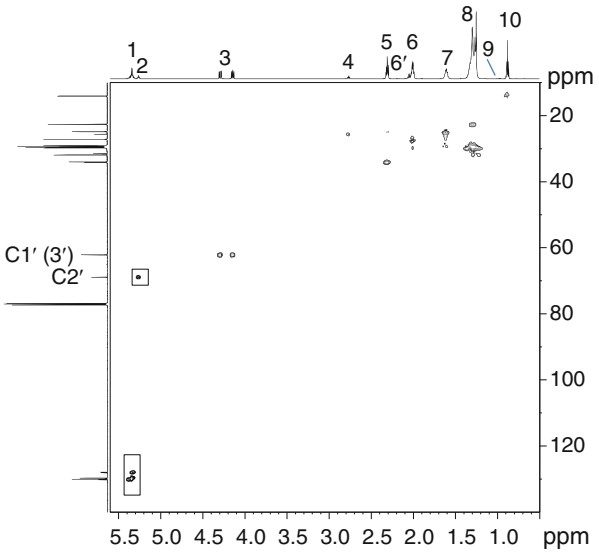

Fig. 11.8 600 MHz edited HSQC (E.HSQC) spectrum of olive oil sample in $CDCl_3$ solvent showing one-bond correlations between carbons and directly attached protons. Correlations phased negatively (*unframed*) and positively (*framed*) represent the methylene and methine protons, respectively

The HMBC spectrum of the olive oil sample in Fig. 11.9a illustrates cross peaks correlating the protons of the glycerol segment with the carboxyl carbons of the attached fatty acid chains. Earlier assignments of these carbon signals for vegetable oils were explained by taking into consideration the double bond inductive effect on the carbonyl groups (Vlahov 1999) or the use of model compounds (Mannina et al. 1999a).

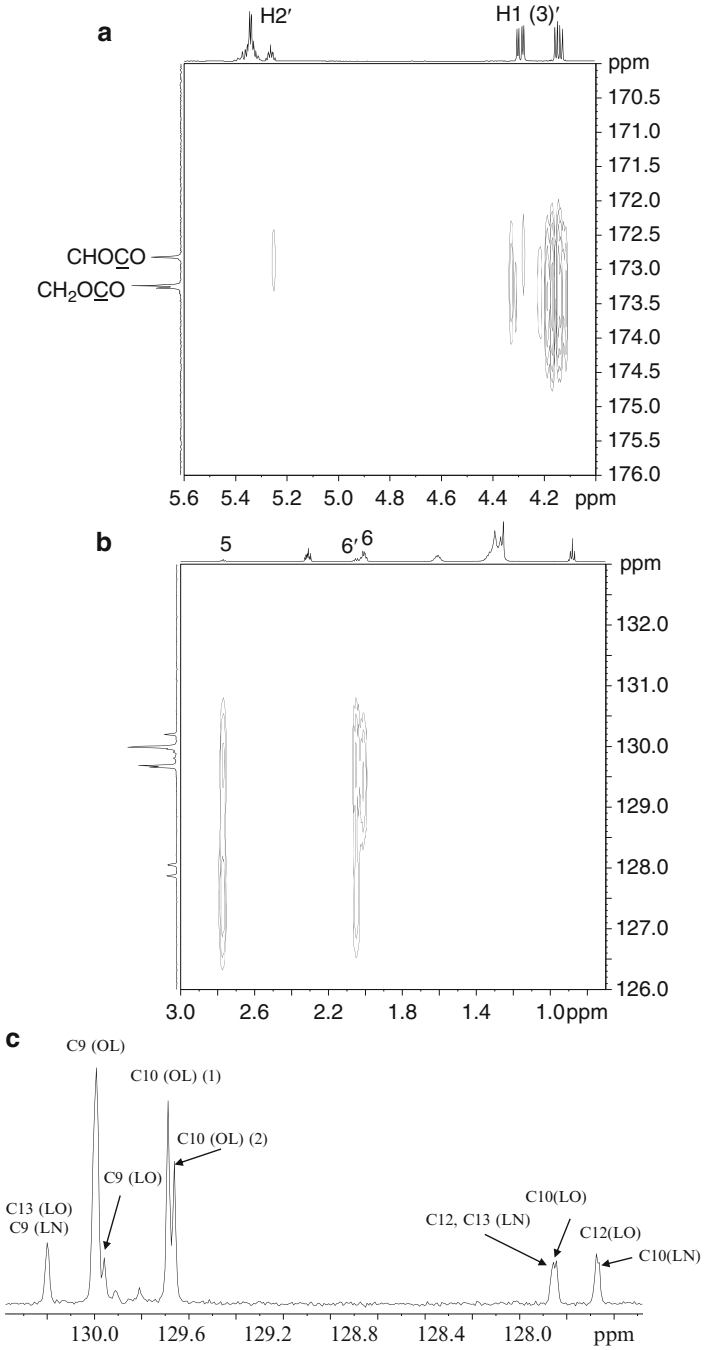

Fig. 11.9 Portions of 600 MHz gradient-selected HMBC spectrum of olive oil sample in $CDCl_3$ solvent showing connectivities (**a**) between glycerol backbone protons and corresponding carbonyl carbons; (**b**) between allylic and bis-allylic protons and olefinic carbons of the fatty chains; (**c**) detailed assignment of olefinic carbons. *Numbers 1 and 2 in parentheses* denote the position of the attached acyl chains to the glycerol moiety

Another portion of the same spectrum (Fig. 11.9b) depicts the connectivity between the olefinic carbons and the corresponding allylic and bis-allylic protons of oleyl, linoleyl, and linolenyl chains. The assignment of the olefinic carbons for an olive oil sample is shown in Fig. 11.9c. The positional distribution of the acyl chains on the glycerol backbone and the complete assignment of the ^{13}C NMR signals in tripalmitin were achieved using the hybrid pulse sequence HSQC-TOCSY (Simonova et al. 2003). This technique, which exploits the resolving power of two powerful pulse sequences, is based on the magnetization transfer between a carbon atom and all remote hydrogen atoms that belong to a common coupling pathway with the carbon atom's directly bonded hydrogen.

11.2.3 Quantitative Nuclear Magnetic Resonance

Quantitative analysis using ^{1}H NMR is not a difficult task since signal intensities are directly proportional to the number of protons in each functional group. Moreover, proton nuclei are characterized by relatively small spin lattice relaxation times (T_1) because they constitute the exterior of the molecule, and thus they relax effectively by intra- and intermolecular ^{1}H-^{1}H dipolar interactions. For this reason, no long relaxation delays are necessary to run quantitative ^{1}H NMR spectra. However, certain precautions should be taken into consideration regarding ^{13}C and ^{31}P nuclei. As mentioned earlier, ^{13}C is a very insensitive nucleus, and therefore more scans are required to obtain a satisfactory signal-to-noise ratio (S/N). In addition, spin–lattice relaxation times (T_1) of carbon nuclei, which are located in the interior of the molecule, are much longer than those of protons, and therefore a large delay time ($5\times T_1$) is required between successive pulse sequences to guarantee full relaxation of the excited nuclei (return of the nuclear magnetization to thermal equilibrium). A long relaxation delay is a prerequisite for quantitative analysis in order to obtain strong and reproducible signals amenable to accurate integration. A remedy for this problem could be the addition of paramagnetic agents, e.g., $Cr(acac)_3$, whose lone electrons interact strongly with nuclear spins, decreasing significantly the T_1 values. Another factor that plays an important role in ^{13}C quantitative analysis is the NOE. For protonated carbons, where ^{1}H-^{13}C dipolar interactions are the dominant relaxation mechanism, NOE is close to its highest value (approximately 1.987) and can be ignored. In contrast, nonprotonated carbons will give signals that are highly dependent on NOE. Consequently, these carbons should not be used as a basis for analytical measurements, unless depression of the NOE is accomplished using the so-called inverse gated decoupling technique. In this experiment, the decoupler of the spectrometer is on only during acquisition and off during the rest of the experiment, thus prohibiting the buildup of NOE while acquiring decoupled spectra. Inverse gated decoupling is vital for the ^{31}P nucleus, whose relaxation is stimulated by the chemical shift anisotropy mechanism in addition to ^{1}H-^{31}P dipole–dipole interactions. Needless to say, when taking up all the

aforementioned safety measures for quantitative analysis based on ^{13}C and ^{31}P nuclei, the duration of the experiment is prolonged significantly.

Another way to obtain quantitative ^{13}C NMR spectra for protonated carbons is the use of the distortionless enhancement by polarization transfer (DEPT) pulse sequence, which transfers the polarization (bulk magnetization) from the abundant ^{1}H nuclei to the dilute carbon ^{13}C nuclei (Vlahov 1997; Vlahov et al. 2001). The drawback of this technique is that resonances of quaternary carbons (e.g., carbonyl carbons of the fatty acyl chains) cannot be detected.

11.2.4 Hyphenated Nuclear Magnetic Resonance

The combination of liquid chromatography (LC) with NMR has proven to be a powerful and time-saving method for the separation and structural characterization of unknown compounds in complex mixtures (Exarchou et al. 2005). In addition, the low detection limits attainable by chromatographic techniques help to increase the sensitivity of NMR. This was further improved by introducing a postcolumn solid-phase extraction (SPE) unit (Corcoran et al. 2002), which allowed significant enrichment of the analyte concentration and, hence, the performance of 1D and 2D experiments, even for the less sensitive ^{13}C nucleus. The LC-SPE-NMR technique has been used in two instances: for the analysis of phenolic compounds in olive oil (Christophoridou et al. 2005) and the identification of phytochemicals in olive-leaf extracts (Goulas et al. 2009). Figure 11.10 illustrates selections of 600 MHz LC-SPE-NMR spectra of various phenolic metabolites resulting from the hydrolysis of oleuropein (Christophoridou et al. 2005). These spectra were recorded for HPLC fractions transferred to a peak-trapping unit equipped with solid-phase cartridges after UV detection and water addition for temporary storage, dried with nitrogen gas, and transferred to the NMR flow probe with CD_3CN. The spectrum in Fig. 11.10a is consistent with the dialdehydic form of oleuropein lacking a carboxymethyl group. Figure 11.10b is more interesting because it reveals for the first time the existence of two coeluted isomers of the aldehydic form of oleuropein in olive oil, namely 5*S*, 8*R*, 9*S* and 5*S*, 8*S*, 9*S* at C8 (the ring of elenolic acid linked to the hydroxytyrosyl moiety contains three chiral centers at C5, C8, and C9). Figure 11.10c illustrates the spectrum of the hemiacetal at C-3 of the dialdehydic form of oleuropein lacking a carbomethoxy group detected by Montedoro (Montedoro et al. 1992) in olive oil. Another example indicating the potential of this technique is shown in Fig. 11.11, which depicts the 600 MHz LC-SPE-TOCSY spectrum resulting from a fraction of the UV chromatogram corresponding to the dialdehydic form of ligstroside lacking the carbomethoxy group (Christophoridou et al. 2005). Overall, the hyphenated NMR allowed the detection of 27 phenolic compounds, including a large number of secoiridoid derivatives and several new phenolic components, which had not been reported previously as constituents in the polar part of olive oil.

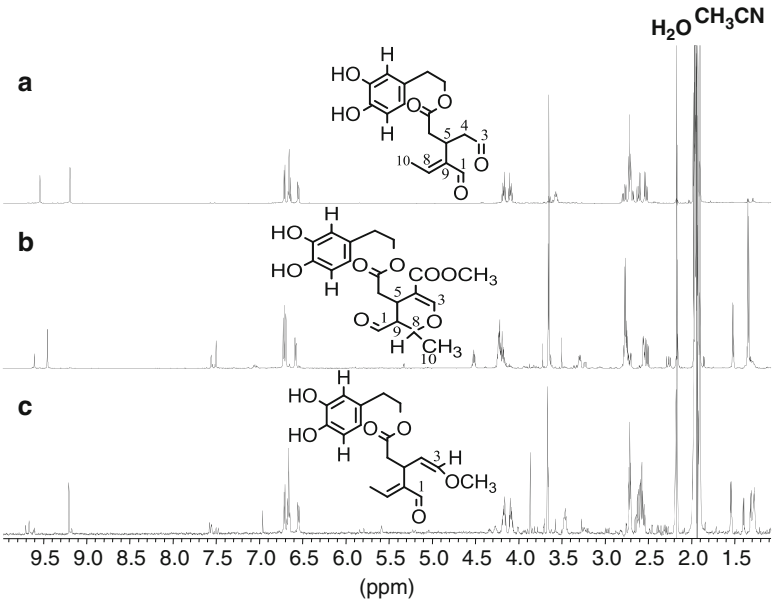

Fig. 11.10 600 MHz LC-SPE ^{1}H NMR spectra of oleuropein derivatives: (**a**) dialdehydic form of oleuropein lacking a carboxymethyl group; (**b**) two coeluted isomers of aldehydic form of oleuropein; (**c**) hemiacetal of dialdehydic form of oleuropein. The suppressed signals of H_2O and CH_3CN solvents give spikes at δ 1.95 and δ 2.18 (*Source*: Christophoridou et al. (2005), with permission of American Chemical Society)

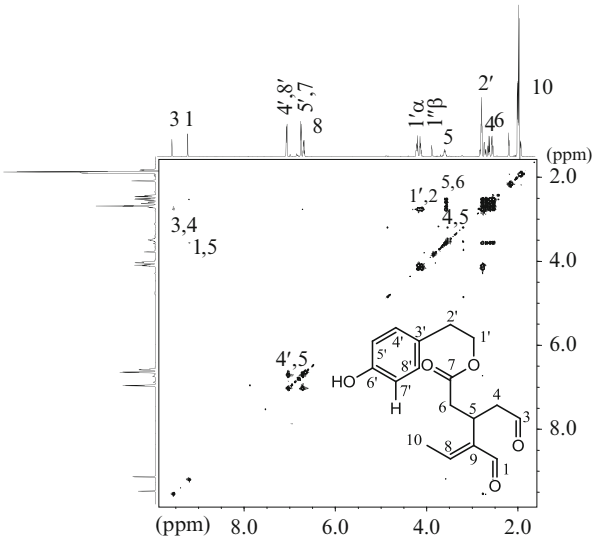

Fig. 11.11 600 MHz LC-SPE-NMR-TOCSY spectrum of dialdehydic form of ligstroside lacking a carboxymethyl group

11.2.5 Site-Specific Natural Isotope Fractionation Nuclear Magnetic Resonance

SNIF-NMR is a sophisticated NMR technique that exploits the nonrandom distribution of deuterium nuclei at specific sites of organic molecules physically present in food products (Martin and Martin 1990). The deuterium nonrandom distribution, which occurs in the course of physical, chemical, and biochemical transformations, is expected to be different for natural and synthetic molecules of the same kind, affording thus a means for their discrimination. A quantitative measure of the deuterium fractionation is the deuterium isotopic ratio $(^2H/^1H)_i$ for specific molecular sites *i*. Isotopic fractionation in plants occurs during photosynthesis following different pathways (e.g., Calvin or C3 plant photosynthesis and Hatch-Slack or C4 plant photosynthetic mode). Therefore, the isotopic ratio $(^2H/^1H)_i$ for some common compounds of plant extracts is expected to be different for different plants. This methodology has been used extensively to detect adulteration of wine, honey, and fruit juices (Martin and Martin 1995; Cotte et al. 2007). Application of SNIF-NMR to olive oil is rare, focusing mainly on investigating the intramolecular distribution of deuterium in fatty acids and triacylglycerols (Lui et al. 1995; Royer et al. 1999). Nevertheless, these preliminary studies have shown that the deuterium isotopic ratio may be used as an index for botanical characterization of olive oils and differentiation according to their regional origin. The main limitations of this technique are due to the rather poor sensitivity and chemical shift resolution of ^2H-NMR, which preclude observation of complex metabolites present in diluted media or available only at the submilligram level. Also, SNIF-NMR requires a rather tedious sample preparation.

11.3 Nuclear Magnetic Resonance Diffusometry and Relaxometry

Low-resolution pulsed NMR (time-domain NMR) has been used for many years for the determination of oil content and moisture in oilseeds and the solid fat content in fat blends (Gambhir 1992). Methodologies that have been employed for this type of analysis relied on free induction decay (FID) analysis and the spin-echo technique. The introduction of pulsed-field gradients (PFG) combined with spin-echo pulse sequences (Stilbs 1987) enabled the separation of the various components of mixtures on the basis of differences in their self-diffusion coefficients, the latter being dependent on the hydrodynamic size of each component. Discrimination according to hydrodynamic size was accomplished through differences in signal decay, which is the same for all proton resonances associated with a pure component. A typical PGF spin-echo sequence consists of two short magnetic field gradient pulses and a diffusion delay time, Δ, between pulses. The Hahn spin-echo sequence or the stimulated echo sequence can be used, depending on the range of diffusion time Δ to be

explored. In restrictive media, such as seeds, the self- diffusion coefficient, D, can be derived from the attenuation of the signal in a field gradient as a function of the parameters of the product $G\delta$ and the delay time Δ; G and δ are the amplitude and the duration of the magnetic field gradient pulses, respectively. The order of the magnitude of the measurable self-diffusion coefficient ranges from 10^{-5} to 10^{-10} m^2/s.

Recently, high-power PFG spin echo yielding a field strength of 350 G/cm was applied for the rapid screening of extra virgin olive oil (EVOO) adulteration with seed oils (Šmejkalová and Piccolo 2010). Application of a spin-echo pulse sequence allowed the determination of the diffusion coefficients of all vegetable oils for each signal in the low-resolution spectra. These parameters were used subsequently to classify the various vegetable oils and to detect adulteration of olive oil. An interesting application of this method was found in the analysis of liquid mixtures at high magnetic fields. This is the so-called diffusion-ordered spectroscopy (DOSY) NMR experiment, which was proposed more than 10 years ago (Morris and Johnson 1992). DOSY is a 2D NMR experiment, in which the signal decays exponentially according to the self-diffusion behavior of individual molecules. Because the diffusion behavior is related to properties of an individual molecule – size, shape, mass, and charge – as well as its surrounding environment (solution, temperature aggregation state), each component in a mixture can be separated based on its own diffusion coefficient. The value of DOSY is that it can be used as a noninvasive method to obtain both physical and chemical information. The easy and cheap implementation is another advantage of DOSY. It could be said that DOSY can be an alternative to LC-NMR. Figure 11.12 shows the 600 MHz ^{1}H DOSY spectrum of an olive oil sample in the region spanned by the glycerol backbone protons of di- and triacylglycerols. The horizontal (F1) axis encodes the proton chemical shifts, whereas the vertical (F2) axis describes self-diffusion coefficients. The cross peaks align themselves along horizontal lines, each corresponding to one sample component (molecule). The acylglycerols are separated by diffusion coefficient (the higher the molecular weight, the lower the diffusion coefficient).

11.4 Instrumentation-Practical Aspects

The major drawback of NMR as an analytical tool is its inferior sensitivity compared to other analytical methods (e.g., chromatography, mass spectrometry). Therefore, efforts have been made in recent years to augment NMR sensitivity. Fundamentally, there have been three principal ways in the pursuit of improved NMR sensitivity: the development of superconducting NMR solenoids capable of producing higher magnetic fields; improvement or invention of novel experimental methods, such as the inverse-detected heteronuclear shift correlation methods; and considerable efforts in the area of NMR probe development. Magnetic fields as high as 23.5 Tesla (1,000 MHz for the proton nucleus) are currently manufactured. However, a magnet of 14.1 Tesla (600 MHz) with a homogeneity of tens of Hertz and resolution as high as 0.05 Hz is adequate and satisfies almost all the research needs of food scientists.

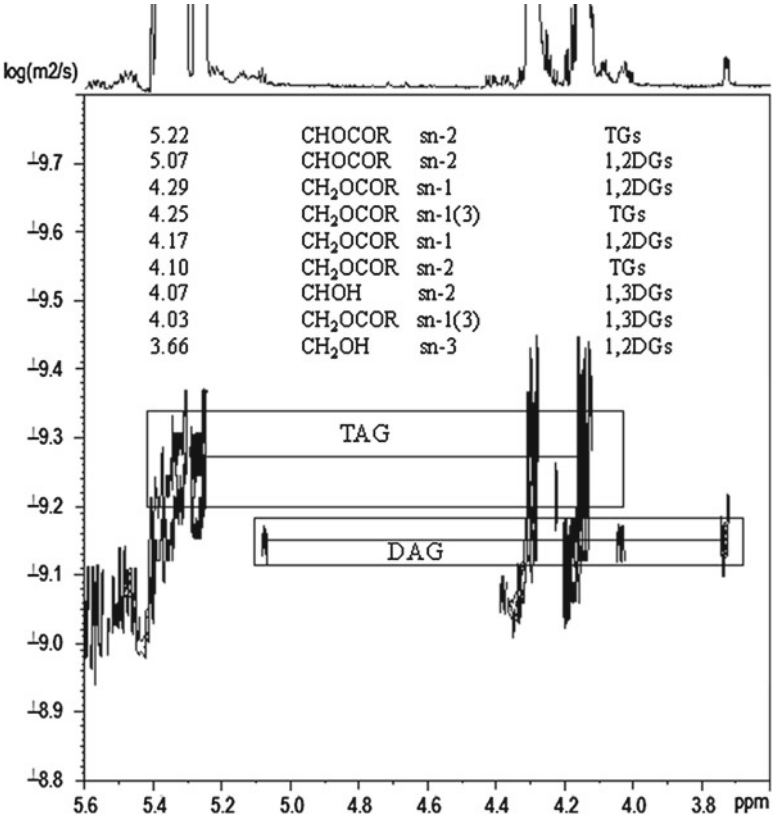

Fig. 11.12 600 MHz DOSY ^{1}H NMR spectrum of olive oil sample in region where di- and triacylglycerol resonances appear. The acylglycerols are separated by diffusion coefficient

Inserted into the magnet is the detector system or probe. The probe contains tunable radio-frequency coils for excitation of the nuclear spins and detection of the resultant signals as the induced magnetization decays away. There are several types of probes, which fulfill almost all needs for sample analysis in the liquid state (and solid state). For 1D and, particularly, 2D NMR experiments, including experiments requiring solvent suppression, broadband (BB) inverse probes with z-gradient facilitate observation/irradiation of all NMR nuclei in the range ^{31}P to ^{15}N in addition to ^{1}H and ^{19}F. The inner coil of these multipurpose probes is optimized for ^{1}H observation, while the outer coil is tunable over a frequency range that permits decoupling of nuclei ^{31}P and ^{15}N, depending on the probe configuration. The advantage of inverse experiments over direct ^{13}C, ^{15}N, or ^{31}P detection is that the ^{1}H nucleus with the highest gyromagnetic ratio is detected, yielding the highest possible sensitivity. Further sensitivity gain (three to four times) is achieved by cryogenically cooling (approximately 20 K) the radio-frequency coils and first-stage receiver electronics of the NMR probe reducing the thermal noise.

The new-generation NMR spectrometers include a very effective digital lock for excellent magnet stability and second-generation analog-to-digital converter (ADC) (converts the analog NMR signal, a voltage, to a binary number proportional to the magnitude of the signal that is stored in the computer memory for further processing) to obtain the highest possible digital spectral resolution and dynamic range. The dynamic range describes the ability of an ADC to sample a small signal in the presence of a large one and depends on the ADC resolution. The latter is expressed in bits; a 16 bit corresponds to a resolution of $2^{16} - 1$ and is able to represent values in a range of −32,767 to +32,767 ($2^{16} = 66{,}536$) with one bit reserved to represent the sign of the signal. This means that the smallest signal that can be detected has a maximum amplitude of 1/32,767. Signals smaller than this amplitude will be lost, whereas too strong signals, like those of solvents, can cause ADC overflow. Moreover, the word length of the computer of the spectrometer must exceed the ADC resolution; otherwise memory overflow will result, with a consequent loss of information. Modern spectrometers (600 MHz) are equipped with ADCs with 32- or even 64-bit resolution.

Depending on the experiment, different resolutions or sensitivities are required. Resolution is important for the integration of small signals, such as those in the carbonyl region of ^{13}C NMR spectra of olive oil. Since the digital resolution is proportional to the reciprocal of the acquisition time, its enhancement can be achieved either by increasing the memory size or by decreasing the spectral width. For older NMR spectrometers, the user should be cautious in adopting the latter remedy to avoid signal aliasing (where the signal becomes indistinguishable).

A large number of 1D NMR experiments, such as the measurement of relaxation times and NOEs, and all 2D NMR experiments involve several pulses separated by timed variable delays and are controlled by pulse programs written in a high-level language. A dynamic software package implemented in the computer of the spectrometer is indispensable in order to interpret these complicated pulse sequences. Moreover, the software should offer a well-designed and user-friendly interface for acquisition, processing, and analysis of the NMR data.

Screening of a large number of samples is of great interest for industrial applications. Various automation options, including autosamplers, robotic sample preparation systems, and probes with automated probe tuning, are available on the market. The automation procedure should be monitored and controlled with appropriate software.

The instrumentation for LC-NMR experiments should be conducted on a dedicated NMR spectrometer equipped with an ultra-shielded magnet to allow hyphenation with the nearby HPLC system. The detection system of HPLC is selected according to the programmed analysis. For instance, UV or diode array detection systems are suggested for the analysis of phenolic compounds. The outlet of the detector is connected to the flow NMR probe, whereas a computer-controlled valve-switching interface regulates the transfer of each fraction separated by HPLC to the NMR probe. The stop-flow or the on-flow process is used depending on the availability of a loop storage device that allows the collection of fractions. The LC-SPE-NMR system requires a SPE unit, whose utilization was described previously. Since the

volume of the liquid from the SPE unit to the flow probe is small (approximately 200 μL), the use of deuterated solvent is both practical and economical, thus making it possible to avoid stringent solvent suppression requirements. Furthermore, the fact that deuterated solvents are only needed for the final step means that chromatographic separation can be performed using the less expensive protonated solvents.

11.5 Sample Preparation

For ^{1}H NMR spectra recorded on a 600 MHz spectrometer, 1–2 mg of the oil are dissolved in 0.5 mL of chloroform-d and placed in 5 mm NMR tubes. A tiny amount (approximately 10 μL) of DMSO-d_6 may be added to facilitate dissolution of olive oil polar compounds (phenols, volatiles). Since proton exchange in DMSO is slow relative to the NMR scale, signals caused by hydroxyl protons emerge in the spectrum, complicating the analysis. In this respect, the hydroxyl hydrogens should be replaced by deuterium atoms (hydrogen-deuterium exchange) by shaking the sample with D_2O before dissolution in DMSO-d_6. For the less sensitive nuclei (e.g., ^{13}C) the amount of olive oil is increased to 5–10 mg. Regarding olive oil minor constituents – e.g., phenolic compounds and their hydrolysis products – the first step is the extraction of the desired metabolites. In general, the choice of solvent depends on the nature of metabolites and the extraction efficiency. For the extraction of phenolic compounds, a mixture of methanol:water 80:20 v/v was found to be most effective (Montedoro et al. 1992). Olive oil lipophilic components (e.g., triacylglycerols, diacylglycerols, sterols) are removed with nonpolar solvents such as hexane. The final extract is lyophilized to concentrate the phenolics and remove water.

Sample preparation for ^{31}P NMR analysis is somewhat lengthy since it requires prior derivatization by the phosphorus reagent 1 according to the reaction scheme in Fig. 11.1. Compounds are dissolved in a mixture of pyridine-chloroform-*d* that contains a small amount of the paramagnetic reagent $Cr(acac)_3$ [chromium (III) acetylacetonate] to diminish the phosphorus spin–lattice relaxation time. Pyridine is added to capture the released HCl during the phosphitylation reaction (Fig. 11.1).

11.6 Olive Oil Analysis

Analysis of olive oil using NMR spectroscopy has been described in several good review articles mentioned in the introductory section; the reader is advised to consult them for details. Table 11.3 summarizes olive oil constituents that have been determined by NMR methods. The second to last column of this table describes the pros and cons of the NMR methods relative to official or well-recognized analytical techniques. A comparison of the NMR methods with conventional analytical techniques in determining certain olive oil constituents was done recently (Dais et al. 2007).

Table 11.3 Olive oil constituents determined by NMR spectroscopy; advantages and disadvantages with official or other recognized methods

Constituent	Magnetic nucleus	Comparison with official or well-recognized methods	References
Fatty acid profile (*cis* and *trans*) (*positional distribution*)	^{1}H and ^{13}C	*Pro*: faster and simpler than official method (GC) *Con*: no determination of individual saturated fatty acids	1, 2
Triacylglycerols profile	^{13}C	*Pro*: much faster and simpler than enzymatic procedure of official method *Con*: requires high spectral resolution	2
Mono- and diacylglycerols	^{1}H and ^{31}P	*Pro*: faster than official method (GC) *Con*: no determination of acyl-chain profile	1, 3
Phenolic compounds profile	^{1}H and ^{31}P	*Pro*: faster and simpler than HPLC; identification at molecular level; new phenolic compounds have been discovered *Con*: requires derivatization for ^{31}P NMR	4, 5, 6
Total, free, and esterified sterols	^{1}H and ^{31}P	*Pro*: much faster than official method (GC) *Con:* no determination of individual sterols; requires strong magnetic fields (≥500 MHz)	7
Free glycerol	^{31}P	*Pro*: much faster than periodate oxidation method *Con*: requires derivatization	8
Phospholipids	^{31}P	*Pro*: faster and more accurate than TLC, GC-MS *Con*: requires derivatization	9
Free acidity	^{31}P	*Pro*: faster and more accurate than official methods of titration *Con*: requires derivatization	10
Water content	^{31}P	*Pro*: much faster and more accurate than Karl Fischer, dry-oven method and azeotropic distillation *Con*: requires derivatization	11
Iodine value	^{1}H and ^{13}C	*Pro*: much faster than official methods of titration and GC *Con*: less accurate than official method	10, 12
Squalene	^{1}H	*Pro*: much faster than TLC, GC, HPLC *Con*: complex spectra (observation of single signal in spectra), less accurate; requires strong magnetic fields (≥ 600 MHz)	13
Cycloarthenol	^{1}H	*Pro*: much faster than TLC, GC, GC-MS *Con*: Complex spectrum (observation of only two signals), less accurate, requires strong magnetic fields (≤600 MHz)	13
Chlorophyll	^{1}H	*Pro*: much faster detection of adulteration with defective chlorophyll (lacking Mg) *Con*: observation of a single signal (NH of porphyrin), requires strong magnetic fields (≤600 MHz)	13

(continued)

Table 11.3 (continued)

Constituent	Magnetic nucleus	Comparison with official or well-recognized methods	References
Volatiles	^{1}H	*Pro*: much faster than GC, GC-MS, LC-GC *Con*: detection of a small number of volatiles, requires strong magnetic fields (≥600 MHz)	1
Oxidation process	^{1}H and ^{13}C	*Pro*: monitoring at molecular level *Con*: less sensitive than optical and conventional methods (rancimat, oxidation analytical indices)	14, 15
Oxidation stability	^{13}C	*Pro*: determination at molecular level, better prediction than conventional methods *Con*: requires lengthy sample pretreatment (chromatographic fractionation)	16
Color determination	^{13}C	*Pro*: determination at molecular level *Con*: Lengthy sample pretreatment, lack of validation	17
Sensory quality	^{1}H	*Pro*: sensory attributes (odors and tastes) have been associated with characteristics signals in the spectrum *Cons*: lack of validation	13

References: 1, Sacchi et al. (1997); 2, Vlahov (1999); 3, Spyros and Dais (2000); 4, Chistophoridou et al. (2005); 5, Christophoridou and Dais (2006); 6, Christophoridou and Dais (2009); 7, Hatzakis et al. (2010a); 8, Hatzakis et al. (2010b); 9, Hatzakis et al. (2008); 10, Fronimaki et al. (2002); 11, Hatzakis and Dais (2008); 12, Miyake et al. (1998); 13, Mannina and Segre (2002); 14, Guillen and Ruiz (2006); 15, Medina et al. (1998); 16, Hidalgo et al. (2002); 17, Zamora et al. (2003)

11.6.1 ^{1}H NMR

This NMR methodology is the best one for the quantification of fatty acids, despite the fact that it is unable to determine individual fatty acids (unlike gas chromatography). Due to signal overlap, quantification of the sum of saturated fatty acids (SFAs), the monounsaturated oleic acid (MUFA), and the polyunsaturated linoleic and linolenic acids (PUFAs) can be obtained by means of mathematical equations using appropriate signal intensities as variables. This procedure exempts linolenic acid, which can be quantified from the signal of the methyl protons of the linolenyl chain at δ 0.96. The much higher sensitivity of the proton nucleus makes this technique suitable for detection and direct quantification of minor compounds of olive oil, such as free fatty acids, volatiles (alcohols and aldehydes), the two diacylglycerol isomers, total sterols, the hydrocarbon squalene, the tetracyclic alcohol cycloarthenol, and chlorophyll. Recent advances in ^{1}H NMR include the detection and quantification of phenolic compounds in the polar part of olive oil mentioned previously, and, in combination with ^{31}P NMR, it enables the quantification of total, free, and esterified sterols (Hatzakis et al. 2010a).

11.6.2 ^{13}C NMR

Almost all analyses performed by ^{1}H NMR can also be accomplished by ^{13}C NMR. Despite the sensitivity disadvantages, ^{13}C NMR is the preferred technique to obtain information about the positional distribution of the saturated, oleyl, linoleyl, and linolenyl chains on the glycerol moiety (Vlahov 1999; Mavromoustakos et al. 1997; Mannina et al. 1999a). This is achieved from the signal integrals in the carbonyl region of the spectrum as well as from those of the olefinic carbons. Moreover, the 5 ppm distance between the signals of the allylic carbons of *cis* and *trans* double bonds allows an easy and accurate quantification of *trans* fatty acids (Gao et al. 2009). Nevertheless, the insensitivity of the ^{13}C nucleus, the very small quantity of *trans* lipids contained in foods, and the need for screening a large number of samples render this NMR technology problematic in terms of instrument time and cost. *Cis-trans* isomers have been determined by the ^{1}H NMR technique as well using the allylic methylene protons adjacent to *cis* and *trans* double bonds (Sedman et al. 2010). However, the much smaller chemical shift difference (0.15 ppm) between the two signals requires a magnetic field ≥14.1 T (600 MHz) to obtain a good separation of *cis* and *trans* proton signals. Recently, fractionation of olive oil by means of column chromatography was proposed (Zamora et al. 2002) in order to increase the concentration of minor compounds, thereby boosting the potential of ^{13}C NMR analysis. As expected, the ^{13}C NMR spectrum of the oil fraction was more complex than that of intact oil due to the presence of additional signals of minor compounds. Statistical analysis of selected signal intensities in the spectrum of the oil fraction allowed prediction (with great accuracy) of the stability of 66 vegetable oil samples, including virgin and refined olive oil (Hidalgo et al. 2002), and the calculation of oil colors (Zamora et al. 2003).

11.6.3 ^{31}P NMR

This NMR method was introduced recently as a complementary analytical technique for the analysis of olive oil. ^{31}P NMR spectra of olive oil were used for the detection and quantification of phospholipids (Hatzakis et al. 2008) extracted from olive oil with a mixture of ethanol:water (2:1 v/v). The main phospholipids found in olive oil were phosphatidic acid, lyso-phosphatidic acid, and phosphatidylinositol. Direct phosphitylation of an olive oil sample was performed for the quantification of diacylglycerols, total free sterols, and free fatty acids (Fig. 11.2), as mentioned previously, whereas phenolic compounds were identified and quantified in the polar part of olive oil (Christophoridou and Dais 2006). The resonances of the phosphitylated aliphatic and aromatic hydroxyl groups of phenolic compounds are illustrated in Fig. 11.13. Fifteen polyphenols were detected and quantified, including simple phenols, lignans, and flavonols. The secoiridoid derivatives were determined in the form of total hydroxytyrosol and total tyrosol. The amounts of phenolic compounds determined by ^{13}P NMR agree very well with those obtained by applying ^{1}H NMR (Christophoridou and Dais 2009).

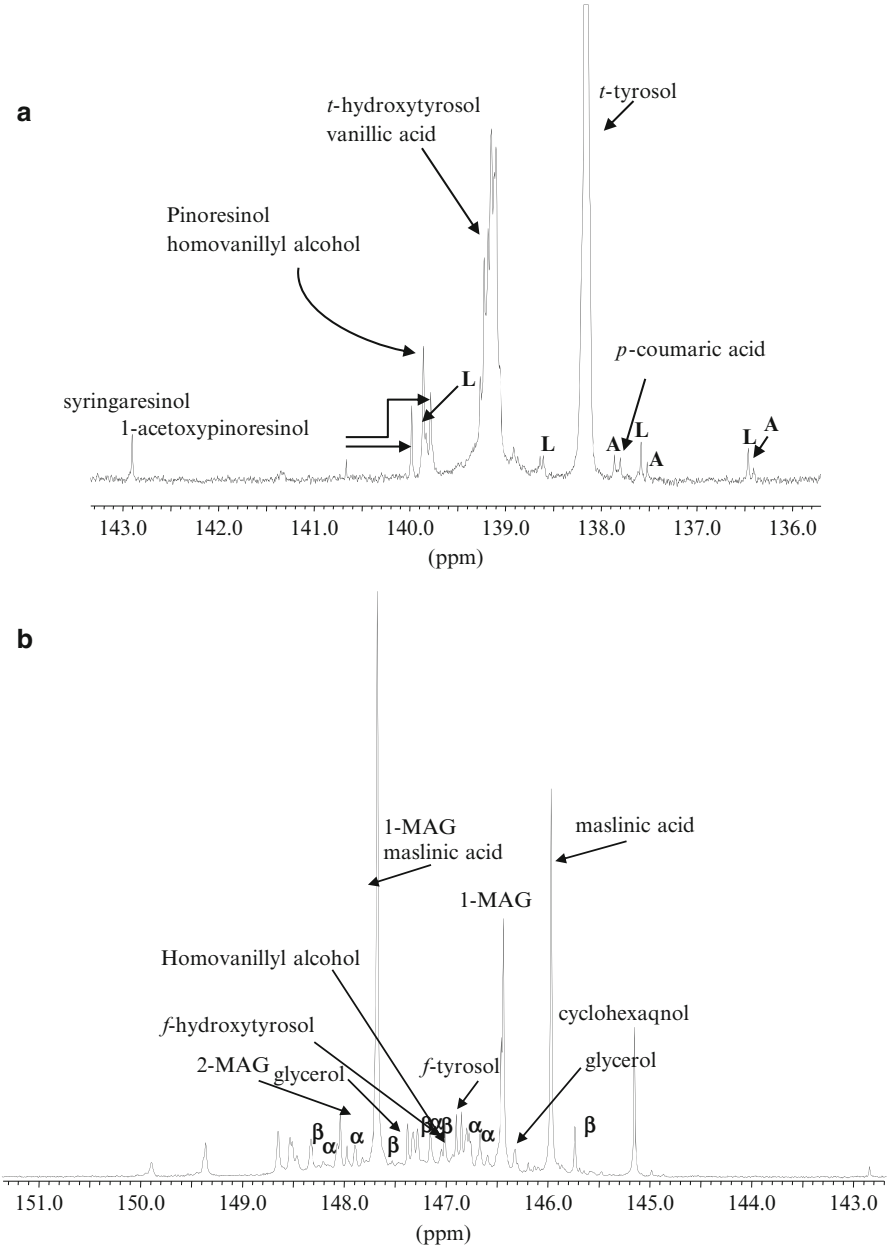

Fig. 11.13 202.2 MHz ^{31}P NMR spectrum of phosphitylated polar fraction of virgin olive oil sample in chloroform/pyridine solution: (**a**) aromatic region; (**b**) aliphatic region. *A* apigenin, *L* luteolin, *1-MAG* 1-monoacylglycerols, *2-MAG* 2-monoacylglycerols, *α* α-D-glucopyranose, *β* β-D-glucopyranose, *f- and t-* free and total, respectively (*Source*: Christophoridou and Dais (2006), with permission of American Chemical Society)

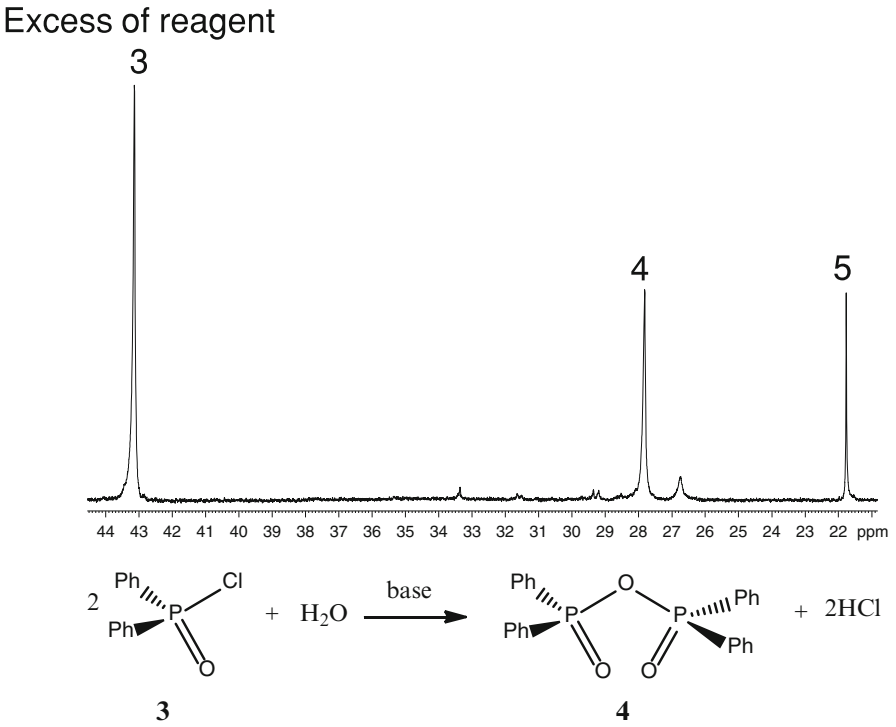

Fig. 11.14 ^{31}P NMR spectrum (at 202.2 MHz) of a phosphitylated sample of olive oil with reagent 3 in pyridine/chloroform solution. The phosphitylation reaction illustrated below the spectrum leads to the formation of the anhydride product 4. Methyltriphenylphosphonium iodide (5) is the internal standard. (*Source*: Hatzakis et al. (2008), with permission of American Chemical Society)

Another application of ^{31}P NMR in olive oil analysis is the determination of water content in olive oil (Hatzakis and Dais 2008). Water is transferred to olive oil during olive crushing and malaxation, and its content in olive oil has long been recognized as an important factor determining the quality of olive oil (IOC 2003). Small quantities of water in olive oil are responsible for the creation and persistence of the suspended and dispersed material that constitutes the so-called veiling of olive oil. This oil is not attractive to the consumer, although some investigators have claimed that water and small particles dispersed in the oil have some antioxidant effects (Lercker et al. 1994). At any rate, water inducing degradation of minor compounds during storage contributes to the perception of wine-vinegar and acid flavors, which deteriorates the organoleptic properties of olive oil. Phosphitylation of water molecules with compound 1 or, alternatively, with the reagent diphenylphosphinic chloride (3) was successful in determining the olive oil moisture with high accuracy (Hatzakis and Dais 2008). Reagent 3 reacts cleanly and instantaneously with water molecules under mild conditions with no side reactions, giving a sole product and, hence, a single signal in the ^{13}P NMR spectrum (Fig. 11.14).

11.7 Nuclear Magnetic Resonance Data Manipulation for Chemometric Analysis

Multivariate statistical analysis of NMR data can be performed on the basis of either selected ^{1}H or ^{13}C signal intensities of the oil samples or suitable chemical parameters determined by NMR. It could be said that the first method resembles the chemometric approach of metabonomics, whereas the second technique is similar to the quantitative method or targeting profiling of metabonomics. In the first method, the selected resonances should show large intensity variability and be independent of one another. In the second method, olive oil constituents are identified and quantified before statistical analysis. For the first method to work, it is critical to have a large number of spectra from many different samples collected and processed identically. The quantitative method does not require identical conditions for spectral collection. Moreover, the latter method allows unambiguous compound identification and precise quantification. Compound identities and concentrations permit explicit recognition of olive oil metabolites and, hence, intermediate interpretation of their influence on olive oil characteristics. However, the quantitative method is not as amenable to automation as the chemometric procedure.

Regardless of the method used, preprocessing and NMR data manipulation are crucial for subsequent multivariate analysis, as well as for the type of statistical method that can be utilized. Data manipulation can be performed in both the time and frequency domain after FT. Briefly, the following steps are necessary for NMR data manipulation and processing:

Time domain: zero-filling, linear prediction, or both; window function multiplication for resolution or sensitivity enhancement, Fourier transform.

Frequency domain: baseline offset correction, phasing, signal alignment, scaling (only for the chemometric method).

The next important step is the choice and application of the multivariate statistical method that can provide the highest possible discrimination of olive oils from different areas or cultivars, as well as olive oils from foreign oils in the context of adulteration studies. Statistical methods used in data analysis and validation of statistical models for prediction are discussed in details in Chap. 12.

11.8 Olive Oil Quality and Authentication

A proposed index to monitor the freshness of olive oil is the ratio of diacylglycerol isomers (Sacchi et al. 1997). The diminution of the 1,2-DAG concentration during olive oil storage owing to its isomerization to more stable 1,3-DAG is indicative of VOO aging. The ratio *D* (1,2-DAG over total DAG) has been used as

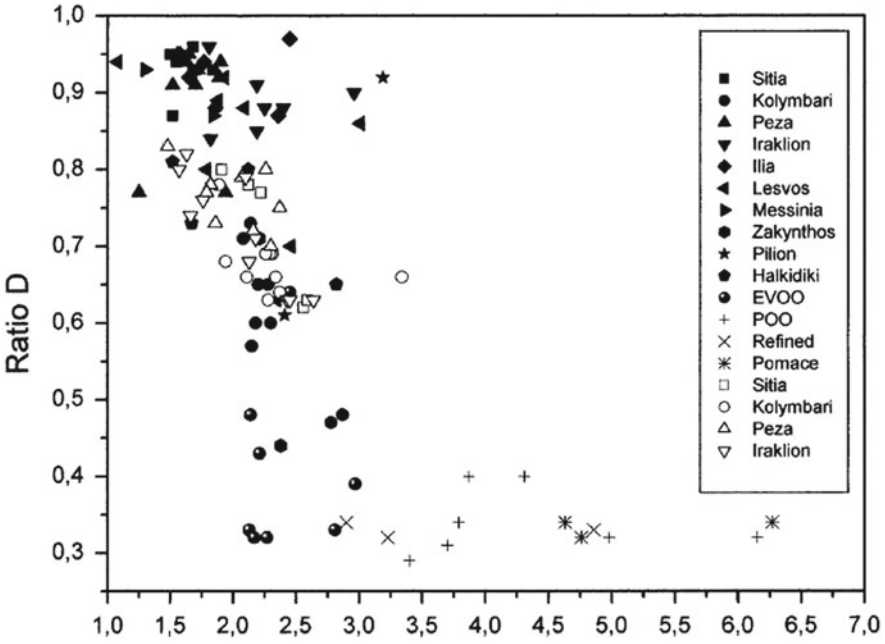

Fig. 11.15 Plot of ratio *D* versus total diacylglycerols for virgin olive oils of various regions of Greece, commercial extra virgin (*EVOO*), commercial pure olive oils (*POO*), refined olive oils, and pomace oils. For the four regions of Crete (Sitia, Kolymbari, Peza, and Iraklion) the *solid symbols* correspond to crops of the period 2000–2001, whereas the *empty symbols* correspond to crops of the period 1999–2000 (*Source*: Fronimaki et al. 2002, with permission of American Chemical Society)

a secondary quality index to map the quality of a large number of EVOO samples (Fronimaki et al. 2002). The plot of *D* values versus total DAG in Fig. 11.15 can be considered as a quality map for Greek EVOOs collected in two harvest periods. EVOO samples pointing toward the upper left corner of the diagram are considered fresh oils, whereas those characterized as old oils have low *D* values and, depending on the total DAG, are oriented toward the lower left or right corner of the diagram. Refined oils, olive-pomace oils, and mixtures of olive oil and refined olive oil known as pure olive oil or blended olive oil, which are not EVOOs, tend clearly to the lower right corner of the diagram. Further studies (Spyros et al. 2004) have succeeded in giving quantitative information about olive oil aging through a mathematical equation that connects the storage time (age) of EVOO with the ratio *D* and free acidity.

The usual approach to detecting EVOO adulteration is to compare the chemical composition of the suspect olive oil with limits for several of its constituents or physical constants imposed by the European Commission, the International Olive

Council (IOC), and other official food organizations. Any adulterant addition is expected to modify the concentration of these constituents, or at least it will indicate an anomaly in its chemical composition. Along these lines, NMR offers a number of clues for olive oil adulteration, either by simple observation of the NMR spectra or by quantitative analysis. For instance, the appearance of a resonance in the carbonyl region ascribed to saturated fatty acids at the *sn*-2 position of glycerol is considered fraudulent (EC 1991, 1995). Careful observation of the signals in particular regions of the ^{1}H NMR spectra of vegetable oils reveals slight differences in the chemical shifts of the saturated and unsaturated acids that allow discrimination of these oils and detection of likely adulteration (Guillen and Ruiz 2003). Another example is the detection of adulteration of EVOO by other oils based on the influence of the added oils on 12 ^{13}C signal intensities of EVOO samples (Mavromoustakos et al. 2000). Nevertheless, there exist three major problems associated with this procedure; inherent physical variation of olive oil characteristics influenced by extraneous factors is often observed. The cumulative rainfall during the summer period affected the composition of fatty acids and of phenolic compounds, whereas low temperatures during the olive harvest period influenced the contents of chlorophyll, carotenoid pigments, and α-tocopherol (Morelló et al. 2006). Another example is the concentration of waxes, which should not exceed 250 ppm for EVOO. However, the concentration may exceed this limit during olive oil storage due to an increase in esterified compounds, thus making uncertain the classification of this olive oil sample as genuine (Aparicio and Aparicio-Ruiz 2000). A second problem appears when a foreign oil has a very similar chemical composition to that of EVOO (e.g., hazelnut oil). Another dilemma is caused by the discrepancy observed in the physical concentration of some constituents of local EVOOs with those of official limits. This is attributed to specific climatic conditions prevailing in those regions affecting the ripening of the olive fruits at the time of harvesting. Such examples occur for olive oils originating from certain regions of Argentina and Australia (Ceci and Carelli 2007). Finally, slight differences in the NMR spectral parameters could become less reliable when a real adulteration problem is confronted.

New developments in the detection of fraud adopt chemometric methods. The general scheme of this methodology is as follows: the first step is the buildup of a reliable statistical (classification) model that discriminates EVOO from potential adulterants (seed oils or lower grade olive oils). This is usually done by applying multivariate statistical analysis either to signal intensities or to concentration values of certain olive oil components. The second step involves the preparation of a series of mixtures of EVOO with different adulterant concentrations and the use of the previous classification model to identify the mixtures considered as unknown samples. The adulterated EVOO samples lie between the group of EVOOs and the respective groups of seed oils or olive oils of lower quality. Table 11.4 shows a list of olive oil adulteration studies performed so far using NMR spectroscopy.

Table 11.4 Olive oil adulteration[a] detected by NMR

Biomarker	Adulterant	Experimental conditions	Nucleus/frequency	Detection limit	Data processing	References
UFA	SOY	DEPT sequence, $CDCl_3$ solution	^{13}C/75.5 MHz	7–20 % depending on FA	Standard	Vlahov (1997)
UFA	VEO	$CDCl_3$ solutions	^{1}H/300 MHz	5 %	DA	Mavromoustakos et al. (2000)
Volatiles and FA	HAZ	NMR for volatiles, GC for fatty acids	^{1}H/600 MHz	10 %	LDA	Mannina et al. (1999b)
^{1}H NMR[b]	SUN, HAZ	$CDCl_3$ solutions	^{1}H/500 MHz	10 %	ANOVA, LDA	Fauhl et al. (2000)
^{13}C NMR[b]	VEO	$CDCl_3$ solutions	^{1}H/300 MHz	5 %	SDA	Zamora et al. (2001)
DAG, FA	VEO	Phosphitylation	^{1}H/500 MHz, ^{31}P/202 MHz	5 %	ANOVA, LDA	Vigli et al. (2003)
^{1}H and ^{13}C NMR[b]	HAZ	$CDCl_3$ solutions	^{1}H/600 MHz	8 %	ANN	García-González et al. (2004)
DAG	ROO, LVOO	Phosphitylation, pyridine/$CDCl_3$ solutions	^{31}P/202 MHz	5 %	ANOVA, LDA	Fragaki et al. (2005)
^{1}H NMR[b]	RHAZ	$CDCl_3$ solutions	^{1}H/400, 500 and 600 MHz	≤10 %	ANOVA, PCA, LDA, LMR	Mannina et al. (2009)
^{1}H NMR[c]	VEO	Neat samples	^{1}H/300 MHz	10 % SUN, SOY; 30 % HAZ	ANOVA, DA	Šmejkalová and Piccolo (2010)
Phenols, FA	RHAZ	Phosphitylation, pyridine/$CDCl_3$ solutions	^{1}H/500 MHz, ^{31}P/202 MHz	1 %	SCDA, CBT	Agiomyrgianaki et al. (2010)
^{13}C NMR[b]	–	$CDCl_3$ solutions	^{13}C/75.5 MHz	–	–	Zamora et al. (1994)
^{13}C NMR[b]	–	Fractionation of polar and non-polar part of oils. $CDCl_3$ solutions	^{13}C/75.5 MHz	–	SDA	Zamora et al. (2002)
FA	–	$CDCl_3$ solutions	^{1}H/300 MHz	–	–	Guillén and Ruiz (2003)

LVOO lampante virgin olive oils, *ROO* refined olive oil, *SUN* sunflower oil, *SOY* soybean oil, *HAZ* hazelnut oil, *RHAZ* refined hazelnut oil, *VEO* vegetable oils; *UFA* unsaturated fatty acids, *DAG* diacylglycerols, *FA* fatty acids, *ANN* artificial neural networks, *ANOVA* analysis of variance, *CBT* classification binary trees, *DA* discriminant analysis, *LDA* linear DA, *SCDA* stepwise canonical DA, *SDA* stepwise DA, *LMR* linear multiple regression, *PCA* principle component analysis

[a]Extra virgin olive oil, unless otherwise specified

[b]Fingerprint

[c]Diffusion spectra

11.9 Geographical and Varietal Characterization of Olive Oil

The characterization of the geographical or varietal origins of EVOOs is becoming increasingly important. This is because false labeling of the origins of olive oil is considered another facet of fraud. With regard to the geographical origin, EVOO is permitted to be marketed under a Protected Designation of Origin (PDO) or Protected Geographical Indication (PGI) label on the basis of the area, cultivar, and methods of production. This labeling protects the reputation of the regional food and eliminates the unfair competition and misleading of consumers by nongenuine products, which may be of inferior quality. Moreover, olive oils made from a certain cultivar (monocultivar olive oils) are being increasingly introduced in markets, and their quality control requires the development of new and effective analytical methodologies to detect fraud. Monocultivar olive oils have certain specific characteristics ascribed to the olive cultivar and are therefore easier to elaborate. Several groups in different countries, especially from countries located in the Mediterranean basin, have investigated the possibility of discriminating olive oils originating from different areas in the same country or from different countries by employing high-field NMR spectroscopy in combination with multivariate statistical analysis. Their efforts have focused on selecting those biomarkers that are capable of discriminating oils. The biomarkers examined ranged from the major olive oil constituents, fatty acids and triacylglycerols, to minor components, sterols, phenols, volatiles, hydrocarbons, etc., or combinations of both. Minor components provide more useful information than major constituents, and they have been used more often to discriminate olive oils according to their geographical or varietal origin. It appears that minor components are prone to greater concentration changes under the influence of various exogenous and endogenous factors (cultivar, ripening conditions, storage, climatic conditions, agricultural practices, and extraction technology). Table 11.5 lists NMR studies concerning olive oil classification according to geographical origin and cultivar. This table contains the biomarkers used, the chemometric treatment, the cultivars analyzed, and their geographical origins, as well as the most important information obtained from each study.

11.10 Future Trends and Perspectives

The NMR techniques described in this chapter and their application to olive oil analysis show the enormous potential of NMR in the quality control and authentication of olive oil. The identification of different olive oil components at the molecular level facilitates current intensive efforts to establish the genuineness and quality of the product in a rapid and reliable way. In addition, application of multivariate statistics to NMR fingerprints increases significantly the efficiency of this technique, especially for the geographical and varietal classification of olive oils. Future trends

Table 11.5 Geographical and varietal classification of extra virgin olive oil by NMR

Biomarker	Nucleus/frequency	Cultivars	Geographical origin	Data treatment[a]	Observations (discrimination)	References
^{13}C NMR[a] (FA)	^{13}C/75.5 MHz	Coratina, Dritta, Grossa di Cassano Moraiolo, Picholine	Abruzzo, Calabria, Lazio, Lombardia, Marche, Molise, Puglia, Tuscany	PCA, PLS, PCR, MLR	70–100 % cultivars and 93–100 % origin	Shaw et al. (1997)
^{13}C NMR[a], (DAG, TAG)	^{13}C/75.5 MHz	Leccino, Moraiolo, Dritta	Abruzzo, Puglia, Tuscany	PLS, PCR	100 % cultivars, ~100 % origin	Vlahov et al. (1999)
^{1}H NMR[a] (FA, phenols)	^{1}H/400, 500 MHz	Coratina, Leccino, Oliarola, Olivastro, Simone	Apulia region	PCA, HCA, DA	FA allowed 100 %. Phenols discriminate northern Apulia	Sacco et al. (2000)
^{1}H NMR[a,b], ^{13}C NMR[a] (TAG), GC(FA)	^{1}H/600 MHz, ^{13}C/150.9 MHz	17 monocultivars	Italy, Argentina	TCA, LDA	100 % origin of cultivars by NMR and GC data	Mannina et al. (2001a)
^{1}H NMR[a,b]	^{1}H/600 MHz	Frantoio, Leccino, Moraiolo, Nerino, Quercetana	Tuscany (Seggiano, Arezzo, Florence, Lucca)	HCA, K-means, DA	Origin discrimination	Mannina et al. (2001b)
^{13}C NMR[a]	^{13}C/75.5 MHz	12 Italian mono- and multicultivar oils	13 Italian areas	PCA	DEPT sequence improved sensitivity; cultivar discrimination	Vlahov et al. (2001)
^{1}H NMR[a], (DAG, TAG) GC (FA)	^{13}C/150.9 MHz	Nocellara del Belice, Tonda Iblea, Cerasuola, Biancollila	Castel Vetrano, Trapani, Delia, Paceco, Caltabellota, Ragusa	MANOVA, PCA, TCA, MDS, LDA	100 % cultivars combining ^{13}C NMR and GC data	Mannina et al. (2003b)
^{13}C NMR[a], GC (FA, TAG)	^{13}C/125.7 MHz	Coratina, Leccino, Peranzana,Oliarola	Apulia area	ANOVA, PCA, HCA, DA	92 % with GC and 88 % with NMR data	Brescia et al. (2003)
^{13}C NMR[a], (TAG, sterols, FA)	^{13}C/125.7 MHz	Coratina, Ogliarola, Peranzana	Terra di Barri, Colline di Brindisi, Foggia	MANOVA, LDA	Origin: 72 % (Dauno) to 90 % (Terra di Bari)	Vlahov et al. (2003)

^{1}H NMR[a]	^{1}H/500 MHz	Uknown	Greece, Italy, Spain, Tunisia, Turkey	LDA, PLS-DA, ANN,	35–92 % depending on origin and harvest year	Rezzi et al. (2005)
^{1}H NMR[a,b]	^{1}H/600 MHz	Casaliva, multicultivar	Garda, Veneto (Valpolicella, Euganei-Berici, Grappa)	ANOVA, PCA	Fine classification origin	Mannina et al. (2005)
^{1}H NMR[a,b]	^{1}H/600 MHz	Casaliva, multicultivar	Garda Lake, Veneto, Lombardia banks	PCA	Satisfactory discrimination by origin	Schievano et al. (2006)
^{1}H- ^{13}C-NMR[a,c]	^{1}H/600 MHz, ^{13}C/62.9 MHz	22 monocultivars	Lazio	ANOVA, PCA, LDA	Origin affected by irrigation and altitude	D'Imperio et al. (2007)
^{1}H NMR (FA,DAG) ^{31}P NMR (phenols)	^{1}H/500 MHz, ^{13}C/100 MHz	Koroneiki	Crete (Heraklion, Sitia, Chania), Peloponnesus (Messinia, Lakonia), Zakynthos	SCDA, CBT	87 % with three and 74 % with six areas, 92 % if harvest year is included	Petrakis et al. (2008)
^{1}H NMR[a], IRMS	^{1}H/500 MHz	Uknown	Italy, Greece, Spain, France, Turkey, Cyprus, Syria	ANOVA, LDA, PLS-DA	Best models for origin and harvest year with PLS-DA; IRMS improves classification results	Alonso-Salces et al. (2010)
^{1}H NMR[a,b]	^{1}H/600 MHz	Uknown	Italy, Liguria, Spain, France, Greece, Turkey	SIMCA, PLS-DA	Selected resonances discriminate Ligurian vs. non-Ligurian oils	Mannina, et al. (2010)

DAG diacylglycerols, *TAG* triacylglycerols, *IRMS* isotope ratio mass spectroscopy, *ANN* artificial neural networks, *ANOVA* analysis of variance, *CART* classification and regression trees, *CBT* classification binary trees, *DA* discriminant analysis, *HCA* hierarchical cluster analysis, *LDA* linear DA, *LMR* linear multiple regression, *MANOVA* multivariate analysis of variance, *PCA* principal component analysis, *PCR* principal component regression, *PLS* partial least squares, *SCDA* stepwise canonical DA, *SDA* stepwise DA, *SIMCA* soft independent modeling and class analogy, *TCA* tree cluster analysis

[a]Fingerprint

[b]Minor compounds

[c]Volatiles, sterols, terpenes, squalene

[d]Bulk oil and unsaponifiable fraction

and perspectives encompass the detailed assessment and standardization of current NMR analytical procedures and accompanying quantitative responses. The NMR analytical approaches discussed in this chapter should be taken properly into consideration by controlling agencies and accredited laboratories in order to support or even substitute some of the old-fashioned analytical protocols now used in olive oil authentication. The MEDEO research project (Development and Assessment of Methods for the Detection of Adulteration of Olive Oils with Hazelnut Oil) funded by the European Union is an important contribution in this direction. Another trend is the development of further innovative analytical NMR procedures that would allow the treatment of more complex problems related to olive oil quality and authentication. For instance, application of NMR metabonomics to more complex systems, such as binary or higher mixtures of olive oils from different monocultivars, would establish subtle variations in the NMR spectral regions (signal intensities or chemical shifts) owing to the presence (or absence) of characteristic metabolites (e.g., phenols) that are unique to each oil component in the mixture. The profiling of the NMR metabonomics data could help to authenticate the coupage (blending) of olive oils, which frequently raises doubts in consumers' minds about the quality of the commercialized olive oil. Another field of exploration would be the investigation of possible existing differences in the deuterium distribution between minor compounds that belong to olive oil and potential adulterants, thus providing a useful NMR index to detect olive oil adulteration. A final trend is the quality examination of olive fruits since the quality of the extracted oils depends ultimately on the quality of olive fruits. A potential, noninvasive method to investigate quality factors or dynamic changes inside the fruit without piercing or slicing is magnetic resonance imaging. This NMR methodology, which has not been exploited in research on olive oil as it should be, could yield information on internal quality defects and disorders due to proharvest treatment and processes leading to quality defects or insect attacks. Also, serial MRI measurements could provide information about developmental processes, such as fruit ripening.

References

Agiomyrgianaki A, Petrakis PV, Dais P (2010) Detection of refined olive oil adulteration with refined hazelnut oil by employing NMR spectroscopy and multivariate statistical analysis. Talanta 80:2165–2171

Alonso-Salces RM, Moreno-Rojas JM, Holland MV, Reniero F, Guillou C et al (2010) Virgin olive oil authentication by multivariate analyses of ^{1}H NMR fingerprints and δ ^{13}C and δ ^{1}H data. J Agric Food Chem 59:5586–5596

Aparicio R, Aparicio-Ruiz R (2000) Authentication of vegetable oils by chromatographic techniques. J Chromatogr A 881:93–104

Berger S, Braun S (2004) 200 and more experiments: a practical course. Wiley VCH, Weinheim

Brescia MA, Alviti G, Liuzzi V, Antonio Sacco A (2003) Chemometric classification of olive cultivars based on compositional data of oils. J Am Oil Chem Soc 80:945–950

Brescia MA, Sacco A (2008a) High-resolution ^{1}H nuclear magnetic resonance in the study of oils. In: Webb GA (ed) Modern magnetic resonance. Part III. Springer, Dordrect, pp 1645–1650

Brescia MA, Sacco A (2008b) High-resolution ^{13}C nuclear magnetic resonance in the study of oils. In: Webb GA (ed) Modern magnetic resonance. Part III. Springer, Dordrecht, pp 1637–1643

Ceci LN, Carelli AA (2007) Characterization of monovarietal argentinian olive oils from new productive zones. J Am Oil Chem Soc 84:1125–1136

Christophoridou S, Dais P (2006) Novel approach to the detection and quantification of phenolic compounds in olive oil based on ^{31}P nuclear magnetic resonance spectroscopy. J Agric Food Chem 54:656–664

Christophoridou S, Dais P (2009) Detection and quantification of phenolic compounds in olive oil by high resolution ^{1}H nuclear magnetic resonance spectroscopy. Anal Chim Acta 633:283–292

Christophoridou S, Dais P, Tseng L-H, Spraul M (2005) Separation and identification of phenolic compounds in olive oil by coupling high-performance liquid chromatography with postcolumn soli-phase extraction to nuclear magnetic resonance spectroscopy (LC-SPE-NMR). J Agric Food Chem 53:4667–4679

Corcoran O, Wilkinson PS, Godejohann M, Braumann U, Hofmann M et al (2002) Advancing sensitivity for flow NMR spectroscopy: LC-SPE-NMR and capillary-scale LC-NMR. Am Lab 5:18–21

Cotte JF, Casabianca H, Lhéritier J, Perrucchietti JC, Sanglar C et al (2007) Study and validity of ^{13}C stable carbon isotopic ratio analysis by mass spectrometry and ^{2}H site-specific natural isotopic fractionation by nuclear magnetic resonance isotopic measurements to characterize and control the authenticity of honey. Anal Chim Acta 582:125–136

D'Imperio M, Mannina L, Capitani D, Bidet O, Rossi E et al (2007) NMR and statistical study of olive oils from Lazio: a geographical, ecological and agronomic characterization. Food Chem 105:1256–1267

Dais P, Spyros A (2007) ^{31}P NMR spectroscopy in the quality control and authentication of virgin olive oil. An account of recent results. Magn Reson Chem 45:367–377

Dais P, Spyros A, Christophoridou S, Hatzakis E, Fragaki G et al (2007) Comparison of analytical methodologies based on ^{1}H and ^{31}P NMR spectroscopy with conventional methods of analysis for the determination of some olive oil constituents. J Agric Food Chem 55:577–584

Exarchou V, Krucker M, van Beek T, Vervoort J, Gerothanassis I et al (2005) LC-NMR coupling technology: recent advancements and applications in natural products analysis. Magn Reson Chem 43:681–687

Fauhl C, Reniero F, Guillou C (2000) ^{1}H.NMR as a tool for the analysis of mixtures of virgin olive oils of different botanical origin. Magn Reson Chem 38:436–443

Fragaki G, Spyros A, Siragakis G, Salivaras E, Dais P (2005) Detection of extra virgin olive oil adulteration with lampante olive oil and refined olive oil using nuclear magnetic resonance spectroscopy and multivariate statistical analysis. J Agric Food Chem 53:2810–2816

Fronimaki P, Spyros A, Christophoridou S, Dais P (2002) Determination of the diglyceride content in Greek virgin olive oils and some commercial olive oils by employing ^{31}P NMR spectroscopy. J Agric Food Chem 50:2207–2213

Gambhir PN (1992) Applications of low-resolution pulsed NMR to the determination of oil and moisture in oilseeds. Trends Food Sci Technol 3:191–196

Gao L, Sedman J, García-González DL, Ehsan S, Sprules T et al (2009) ^{13}C NMR as a primary method for determining saturates, *cis* and *trans*-monounsaturates and polyunsaturates in fats and oils for nutritional labeling purposes. Eur J Lipid Sci Technol 11:612–622

García-González DL, Mannina L, D'Imperio M, Segre AL, Aparicio R (2004) Using ^{1}H and ^{13}C NMR techniques and artificial neural networks to detect the adulteration of olive oil with hazelnut oil. Eur Food Res Technol 219:545–548

Goulas V, Exarchou V, Troganis AN, Psomiadou E, Fotsis T et al (2009) Phytochemicals in olive-leaf extracts and their antiproliferative activity against cancer and endothelial cells. Mol Nutr Food Res 53:600–608

Guillen MD, Ruiz A (2003) Edible oils: discrimination by ^{1}H nuclear magnetic resonance. J Sci Food Agric 83:338–346

Guillen MD, Ruiz A (2006) Study by means of ^{1}H nuclear magnetic resonance of the oxidation process undergone by edible oils of different natures submitted to microwave action. Food Chem 96:665–674

Hatzakis E, Dais P (2008) Determination of water content in olive oil by ^{31}P NMR spectroscopy. J Agric Food Chem 56:1866–1872
Hatzakis E, Koidis A, Boskou D, Dais P (2008) Determination of phospholipids in olive oil by ^{31}P NMR spectroscopy. J Agric Food Chem 56:6232–6240
Hatzakis E, Dagounakis G, Agiomyrgianaki A, Dais P (2010a) A facile NMR method for the quantification of total, free and esterified sterols in virgin olive oil. Food Chem 122:346–352
Hatzakis E, Agiomyrgianaki A, Dais P (2010b) Detection and quantification of free glycerol in virgin olive oil by ^{31}P-NMR spectroscopy. J Am Oil Chem Soc 87:29–34
Hidalgo FJ, Zamora R (2003) Edible oil analysis by high-resolution nuclear magnetic resonance spectroscopy: recent advances and future perspectives. Trends Food Sci Technol 14:499–506
Hidalgo FJ, Gómez G, Navarro JL, Zamora RZ (2002) Oil stability prediction by high-resolution 13C nuclear magnetic resonance spectroscopy. J Agric Food Chem 50:5825–5831
International Olive Council (IOC) (2003) COI/T.15/NC no 3-25. Madrid, Spain
Lercker G, Frega N, Bocci F, Servidio G (1994) Veiled extravirgin olive oils: dispersion response related to oil stability. J Am Oil Chem Soc 71:657–658
Lui A, Casu M, Saba G, Corongiu FP, Dessi MA (1995) NMR investigation of the intramolecular distribution of deuterium nuclei in natural ttiacylglycerols. Magn Reson Chem 33:165–166
Mannina L, Segre A (2002) High resolution nuclear magnetic resonance: from chemical structure to food authenticity. Grasas Aceites 53:22–33
Mannina L, Luchinat C, Emanuele MC, Segre AL (1999a) Acyl positional distribution of glycerol tri-esters in vegetable oils: a ^{13}C NMR study. Chem Phys Lipids 103:47–55
Mannina L, Patumi M, Fiordiponti P, Emanuele MC, Segre AL (1999b) Olive and hazelnut oils: a study by high ^{1}H NMR and gas chromatography. Ital J Food Sci 11:139–149
Mannina L, Fontanazza G, Patumi M, Ansanelli G, Segre AL (2001a) Italian and Argentine olive oils: a NMR and gas chromatography study. Grasas Aceites 6:380–388
Mannina L, Patumi M, Proietti N, Segre AL (2001b) PDO (Protected Designation of Origin): geographical characterization of Tuscan extra virgin olive oils using high-field H-1 NMR spectroscopy. Ital J Food Sci 13:53–63
Mannina L, Luchinat C, Patumi M, Emanuele MC, Rossi E et al (2002) Concentration dependence of 13C NMR spectra of triglycerides: implications for the NMR analysis of olive oils. Magn Reson Chem 38:886–890
Mannina L, Sobolev AP, Segre A (2003a) Olive oil as seen by NMR and chemometrics. Spectr Eur 15:6–14
Mannina L, Dugo G, Salvo F, Cicero L, Ansanelli G et al (2003b) Study of the cultivar-composition relationship in Sicilian olive oils by GC, NMR, and statistical methods. J Agric Food Chem 51:120–127
Mannina L, D'Imperio M, Lava R, Schievano E, Mammi S (2005) Caratterizzazione NMR e analisi statistica di oli di oliva DOP Veneti. Riv Ital Sostanze Grasse 82:59–63
Mannina L, D'Imperio M, Capitani D, Rezzi S, Guillou C et al (2009) ^{1}H NMR-based protocol for the detection of adulterations of refined olive oil with refined hazelnut oil. J Agric Food Chem 57:11550–11556
Mannina L, Marini F, Gobbino M, Sobolev AP, Capitani D (2010) NMR and chemometrics in tracing European olive oils: the case study of Ligurian samples. Talanta 80:2141–2148
Mannina L, Sobolev AP (2011) High resolution NMR characterization of olive oils in terms of quality, authenticity and geographical origin. Magn Reson Chem 49:S3–S11
Martin ML, Martin GJ (1990) Deuterium NMR in the study of site-specific natural isotope fractionation (SNIF-NMR). NMR Basic Princ Prog 23:1–61
Martin ML, Martin GJ (1995) Stable Isotope analysis of food and beverages by nuclear magnetic resonance. Ann Rep NMR Spectrosc 31:81–104
Mavromoustakos T, Zervou M, Theodoropoulou E, Panagiotopoulos D, Bonas G et al (1997) ^{13}C NMR analysis of the triacyglycerol composition of Greek virgin olive oils. Magn Reson Chem 35:S3–S7
Mavromoustakos T, Zervou M, Bonas G, Kolocouris A, Petrakis P (2000) A novel analytical method to detect adulteration of virgin olive oil by other oils. J Am Oil Chem Soc 77:405–411

Medina I, Sacchi R, Giudicianni I, Aubourg S (1998) Oxidation in fish lipids during thermal stress as studied by ^{13}C nuclear magnetic resonance spectroscopy. J Am Oil Chem Soc 75:147–154

Miyake Y, Yokomizo K, Matsuzaki N (1998) Rapid determination of iodine value by ^{1}H Nuclear magnetic resonance spectroscopy. J Am Oil Chem Soc 75:15–19

Montedoro G, Servili M, Baldioli M, Selvaggini R, Miniati E (1992) Simple and hydrolysable compounds in virgin olive oil. 1. Their extraction, separation and semiquantitative evaluation by HPLC. J Agric Food Chem 40:1571–1576

Morelló JR, Romero MP, Motilva MJ (2006) Influence of seasonal conditions on the composition and quality parameters of monovarietal virgin olive oils. J Am Oil Chem Soc 83:683–690

Morris KF, Johnson CS (1992) Diffusion-ordered two-dimensional nuclear magnetic resonance spectroscopy. J Am Oil Chem Soc 114:3139–3141

Official Journal of the Commission of the European Communities (EC) (1991) Regulation No. 2568/91, L248, Sept 5

Official Journal of the Commission of the European Communities (EC) (1995) Regulation No. 656/95, L69, Mar 29

Petrakis PV, Agiomyrgianaki A, Christophoridou S, Spyros A, Dais P (2008) Geographical characterization of Greek virgin olive oils (*Cv*. Koroneiki) using ^{1}H and ^{31}P NMR fingerprinting with canonical discriminant analysis and classification binary trees. J Agric Food Chem 56:3200–3207

Rezzi S, Axelso DE, Héberger K, Reniero F, Mariani C et al (2005) Classification of olive oils using throughput flow 1H NMR fingerprinting with principal component analysis, linear discriminant analysis and probabilistic neural network. Anal Chim Acta 552:13–24

Royer A, Nauler N, Mabon F, Lees M, Martin CJ (1999) Stable isotope characterization of olive oils. II-Deuterium distribution in fatty acids studied by nuclear magnetic resonance. J Am Oil Chem Soc 76:365–373

Sacchi R, Addeo F, Paolillo L, Giudicianni I (1997) ^{1}H and ^{13}C NMR of virgin olive oil. An overview. Magn Reson Chem 35:S133–S145

Sacco A, Brescia MA, Liuzzi V, Reniero F, Guillou C et al (2000) Characterization of Italian olive oils based on analytical and nuclear magnetic resonance determinations. J Am Oil Chem Soc 77:619–625

Schievano E, Arosio I, Lava R, Simionato V, Mammi S et al (2006) Olio di oliva DOP del lago di Garda: uno studio NMR e analisi statistica multivariata. Riv Ital Sostanze Grasse 83:14–17

Sedman J, Gao L, García-González DL, Ehsan S, van de Voort FR (2010) Determining nutritional labeling data for fats and oils by ^{1}H NMR. Eur J Lipid Sci Technol 112:439–451

Shaw AD, di Camillo A, Vlahov G, Jones A, Bianchi G et al (1997) Discrimination of the variety and region of origin of extra virgin olive oils using 13C NMR and multivariate calibration with variable reduction. Anal Chim Acta 348:357–374

Simonova S, Ivanova G, Spassov SL (2003) Alternative NMR method for quantitative determination of acyl positional distribution in triacylglycerols and related compounds. Chem Phys Lipids 126:167–176

Šmejkalová D, Piccolo A (2010) High-power gradient diffusion NMR spectroscopy for rapid assessment of extra-virgin olive oil adulteration. Food Chem 118:153–158

Spyros A, Dais P (2000) Application of ^{31}P NMR spectroscopy in food analysis. Quantitative determination of the mono- and diglyceride composition of olive oils. J Agric Food Chem 48:802–805

Spyros A, Philippidis A, Dais P (2004) Kinetics of diglyceride formation and isomerization in virgin olive oils by employing ^{31}P NMR spectroscopy. Formulation of a quantitative measure to assess olive oil storage history. J Agric Food Chem 52:157–164

Stilbs P (1987) Fourier transform pulsed-gradient spin-echo studies of molecular diffusion. Prog NMR Spectrosc 19:1–45

Van Duynhoven J, Voda A, Witek M, Van As H (2010) Time-domain NMR applied food products. Ann Rep NMR Spectrosc 69:145–197

Vigli G, Philippidis A, Spyros A, Dais P (2003) Classification of edible oils by employing ^{31}P and ^{1}H NMR spectroscopy in combination with multivariate statistical analysis. A proposal for detection of seed oil adulteration in virgin olive oils. J Agric Food Chem 51:5715–5722

Vlahov G (1997) Quantitative ^{13}C NMR method using the DEPT pulse sequence for the detection of olive oil adulteration with soybean oil. Magn Reson Chem 35:S8–S12

Vlahov G (1999) Application of NMR to study of olive oils. Prog NMR Spectrosc 35:341–357

Vlahov G, Shaw AD, Kell DB (1999) Use of ^{13}C nuclear magnetic resonance distortionless enhancement by polarization transfer pulse sequence and multivariate analysis to discriminate olive oil cultivars. J Am Oil Chem Soc 76:1223–1231

Vlahov G, Schiavone C, Simone N (2001) Quantitative ^{13}C NMR method using the DEPT pulse sequence for the determination of the geographical origin (DOP) of olive oils. Magn Reson Chem 39:689–695

Vlahov G, Del Re P, Simone N (2003) Determination of geographical origin of olive oils using ^{13}C nuclear magnetic resonance spectroscopy. I-Classification of olive oils of the Puglia region with denomination of protected origin. J Agric Food Chem 51:5612–5615

Zamora R, Navarro JL, Hidalgo FJ (1994) Identification and classification of olive oils by high-resolution ^{13}C nuclear magnetic resonance. J Am Oil Chem Soc 71:361–464

Zamora R, Gómez G, Hidalgo FJ (2002) Classification of vegetable oils by high-resolution ^{13}C NMR spectroscopy using chromatographically obtained oil fractions. J Am Oil Chem Soc 79:267–272

Zamora R, Gómez G, Hidalgo FJ (2003) Quality control of vegetable oils by ^{13}C NMR spectroscopy. In: Webb GA, Belton PS, Gill AM, Rutledge DN (eds) Magnetic resonance in food science latest developments. The Royal Society of Chemistry, Cambridge, UK, pp 231–238

Zamora R, Alba V, Hidalgo FJ (2001) Use of high-resolution ^{13}C nuclear magnetic resonance spectroscopy for screening of virgin olive oils. J Am Oil Chem Soc 78:89–94

Chapter 12
Olive Oil Characterization and Traceability

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Contents

12.1 Introduction

Some studies have pointed out that Andalusian (Baetican) olive oil was consumed in Germany (Germania) during the last centuries of the Roman Empire (Remesal-Rodríguez 1997). In fact, it has even been possible to determine the regions of Baetica and the producers who sent olive oil to Germania at any given time.

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties*,
DOI 10.1007/978-1-4614-7777-8_12, © Springer Science+Business Media New York 2013

This research highlights not only economic relations inside the Roman Empire, or how the people of Germania became accustomed to eating this product, but also the importance of emphasizing distinctive properties of products coming from a particular region. This broad distribution of goods across Europe since ancient times indicates, in other words, the great importance of characterization and traceability, which ultimately refers to distinctive chemical properties.

Hundreds of chemical compounds have been identified in olive oil, but only about 200 have been used in characterization. With the perspective of such numbers of chemical compounds, researchers have studied the effect of both intrinsic (e.g., cultivar, ripeness) and external (e.g., climate, extraction systems) variables on the evolution of chemical compounds. However, the evolution of only a few compounds has been clearly related to specific agronomic or technological aspects of production. The problem could be localized in the great interaction between different variables no matter what aspect is being characterized. The characterization of a varietal olive oil could be affected by, for example, climate, altitude of olive grove zones, harvesting, ripening, extraction system, methodology of analysis, or the chemical compounds selected. In contrast, various authors have stated that the content of phenols is affected by the altitude of olive grove zones, stage of ripeness at harvesting, extraction system, and variety of cultivar. Thus, it can be concluded that characterization is not an easy task where we put data in one side of a black box (the computer) and get the results from the other side (the result of any statistical analysis). Characterization involves three issues: sampling, analysis, and mathematics. The first and third aspects of characterization will be described first, whereas the results of different kinds of characterization will be discussed later on.

12.2 Mathematical Procedures in Olive Oil Characterization

The procedures of mathematical analysis that are currently applied to empirical investigations have progressed notably in recent years with the widespread dissemination and application of computer science. From the initial application of univariate analyses, researchers have passed on to the intensive and extensive use of multivariate procedures, or beyond artificial intelligence algorithms, in less than one decade. Two aspects are responsible for this qualitative step: first are the new sophisticated analytical instruments that are able to analyze hundreds of chemical compounds in dozens of samples daily; second is the use of personal computers that are less expensive and quicker, day by day, and that can be run with a great diversity of software packages.

(a) The use of multivariate mathematical procedures has allowed conclusions that were unthinkable only three decades ago. However, the conclusions will only be useful if three steps are strictly followed:
(b) Exploratory data analysis (EDA). Since the experience of a researcher cannot be sufficient to single out the significant information, the objective of EDA is to

obtain the maximum useful information from each chemical/physical data set. This step comprises descriptive univariate statistical algorithms (e.g., mean, normality assumption, skewness, kurtosis, variance, coefficient of variation), detection of outliers, cleansing of data matrices, use of basic algorithms such as, for example, box-and-whisker and stem-and-leaf. This study is also the so-called pretreatment of data and is crucial to avoid wrong or obvious conclusions.

(c) Bivariate statistics. The objective of this step is to look for possible relationships between pairs of variables. Pearson's correlation has traditionally been the most widely used procedure, although the analysis of the correlation matrix should be studied before the use of most multivariate statistical procedures.

(d) Multivariate statistics. The objective here is the simultaneous analysis of numerous variables. Mathematicians classify multivariate analyses into two large groups, descriptive and explanatory, while chemometricians call them unsupervised and supervised.

A general question that researchers in food characterization must face is how to organize observed data into meaningful structures. Regardless of the names given to the basic multivariate procedures, the descriptive (unsupervised) group is characterized by the fact that there is no previous hypothesis classifying or defining the objects (samples). The procedures clustered inside this group analyze the given information and explore the data matrix in the search for new knowledge. There are many statistical procedures within this group, such as factorial analysis [e.g., principal component analysis (PCA), maximum likelihood], canonical correlation, multiple partial correlation, clusters, correspondence analysis, procrusters, and multidimensional scaling. The explanatory (supervised) procedures, on the other hand, either have as their objective checking a priori hypothesis, e.g., varietal olive oil characterizing or classifying the samples, or they are simply dependence models that can be subsumed under the general regression theory. Discriminant analysis, multivariate analysis of variance, regressions, log-linear models, and many others are explanatory procedures.

12.2.1 Pretreatment of Data

Statistics and artificial intelligence require a strict process of data selection. Since almost all statistical procedures are based on Bayesian theory, the information should comply with eight conditions:

1. The error of the method (or overall variability) and uncertainty should be known.
2. Chemical or physical analyses should be carried out in triplicate, or at least duplicate.
3. The assumption of normality should be verified with each variable.
4. Outliers (i.e., outlying data) should be detected and corrected if possible.
5. The data set should have information on the whole range of values of the universe of discourse [e.g., a varietal virgin olive oil (VOO)].

6. The validation (test) set should be independent of the training set, if possible.
7. The standardization of data should agree with the objective of the study.
8. The number of variables (e.g., chemical compounds) should be lower than the number of objects (e.g., samples).

Knowing which factors contribute to the overall variability would allow for the control of and, if possible, improvement in methodology. The error is composed of the systematic error or bias, the unspecified random error, and a series of errors produced during the chemical or physical analyses (e.g., laboratory, operator, equipment, calibration, standard recovering). Uncertainty, also expressed as the standard deviation, is the concept for measuring the quality of analytical procedures. Thus, variables must be quantified in duplicate or triplicate.

The vast majority of statistical procedures are based on the assumption of normality of variables. The central limit theorem protects against failures of normality and, furthermore, there are numerous mathematical transformations that are able to reduce skewness or the influence of outlying objects. Although univariate normality does not guarantee multivariate normality, the latter is increased whether or not all the variables have normal distributions; in any event, it avoids the deleterious consequences of skewness and outliers on the robustness of many statistical procedures.

Since statistics works almost exclusively with numbers, abnormal data can cause mathematical procedures to yield obvious or wrong conclusions (e.g., outliers inside a training set of neural networks). Most outliers can be detected and some of them corrected before applying certain mathematical procedures using so-called robust algorithms (Armstrong and Beck 1990), although most statisticians usually remove them when the database is large enough. Those conclusions, however, are mostly due to databases that do not keep all the aspects of the characterization (e.g., partial or skew databases) or that merge data of identical chemical compounds quantified using different techniques (e.g., fatty acids quantified by different chromatographic columns).

Databases should be split into two independent parts: a training set and a test set. The first set is used to obtain mathematical equations, whereas the second set is used to validate them. The selection of objects (samples) for these sets should be carried out with random methods (e.g., random numbers) and the independent test set should have at least 25 % of the total samples. The use of an external validation set does not invalidate the use of some form of internal validation (e.g., cross validation or leverage correction) that is applied with success when the total number of objects is small.

In characterization, chemical or physical data can differ by orders of magnitude (e.g., parts per billion or parts per million); moreover, data are collected from instruments that often give information in different scientific measurements (e.g., degrees Celsius, percentage, or grams per liter). In these cases, scaling (also called standardization) should be applied in order to readjust the individual contributions to the outcome on an equal basis, so as to avoid having some variables with a greater weighting than others in the results.

Overfitting is the most common problem in multivariate statistical procedures when the number of variables is greater than the number of objects (samples); one can fit an elephant with enough variables. Minimum requirements have been

suggested for the multivariate procedures (Tabachnick and Fidell 1983); otherwise, overfitting can occur in a somewhat unpredictable manner regardless of the multivariate procedure chosen.

12.3 Multivariate Statistical Procedures

The primary goal of this section is to provide a summary of the most widely used multivariate procedures in olive oil characterization. Among the vast range currently available are the statistical procedures of well-known computer packages such as MATLAB, SAS, SPSS, and Statistica.

12.3.1 Cluster Analysis

Cluster analysis is a generic name for methods designed to help one understand the information of data matrices, to describe the similarities and dissimilarities among objects (cases or samples), and to single out categories that group similar objects. Cluster analysis is not so much a typical statistical test as a collection of various algorithms that put objects into clusters. Thus, cluster analysis should be used mostly when there are no a priori hypotheses and when the research is still in the explanatory phase. The term cluster analysis encompasses a number of different classification algorithms: K-means clustering, block clustering, and tree clustering. The k-means clustering method moves objects around a user-specified number of clusters with the goal of minimizing within-cluster variability while maximizing between-cluster variability (Jacobsen and Gunderson 1986). Block clustering simultaneously amalgamates objects and variables. Tree clustering, also called joining, is the most widely used one in characterization. It is based on two kinds of subprocedures: distance measures and amalgamation rules. Each statistical library or package has programmed a certain number of algorithms. The pros and cons of tree clustering are described below.

12.3.1.1 Distance Measures in Tree Clustering

The hierarchical clustering method uses the distances (or dissimilarities) between variables when forming the clusters. These distances can be based on a single dimension or multiple dimensions. Cluster analysis can compute various types of distance measures:

- The Euclidean distance is probably the most commonly chosen type of distance. It is simply the geometric distance in the multidimensional space.
- The squared Euclidean distance is similar to the preceding one, but it adds progressively greater weight to objects that are further apart.

- The Chebyshev distance is recommended when the analyst wishes to state that there are objects that are different in any of the dimensions.
- The power distance is applied when one wants to increase or decrease the progressive weight of each dimension. This distance is equal to the Euclidean distance under certain circumstances.
- The Manhattan (city-block) distance is simply the average difference across dimensions. This distance usually yields results that are similar to the simple Euclidean distance, although the effect of outliers is dampened. If this distance is applied, then the outliers should be removed beforehand or corrected.
- The percent disagreement is a distance that is particularly useful if the information is categorical in nature.
- The Mahalanobis distance is a measure between two points in the space defined by two or more correlated variables. If there were two uncorrelated variables, the Mahalanobis distance between the points would then be identical to the Euclidean distance. When variables are correlated, the simple Euclidean distance is not an appropriate measure, whereas the Mahalanobis distance will adequately account for the correlation.

12.3.1.2 Amalgamation Rules in Tree Clustering

Once several objects have been linked together, the next step is to determine the distances between those new clusters. This new procedure is carried out by linkage or amalgamation rules that determine when two clusters are similar enough as to be linked together. Several possibilities exist:

- Single linkage (K-nearest neighbor) is determined by the distance of the two closest objects in the different clusters. Thus, the resulting clusters tend to represent long chains.
- Complete linkage (K-furthest neighbor) is determined by the greatest distance between any two objects in the different clusters. This amalgamation method is inappropriate if the clusters tend to be elongated, although it works reasonably well with naturally distinct objects (or variables).
- Unweighted pair-group average is the average distance between all pairs of objects in two different clusters. It should be used when the objects form natural distinct groups. This amalgamation method is not affected by the shape of clusters (e.g., elongated and chains).
- Weighted pair-group average is identical to the unweighted pair-group average method, except that the size of the respective clusters is used as a weight. This amalgamation method should be used when the cluster sizes are suspected to be largely uneven.
- Unweighted pair-group centroid is the center of gravity for each cluster, and the distance between two clusters is determined to be the difference between centroids.
- Weighted pair-group centroid is identical to the overweighted one, except that it takes into consideration the differences in cluster sizes. This amalgamation method is appropriate when there are appreciable differences in cluster sizes.

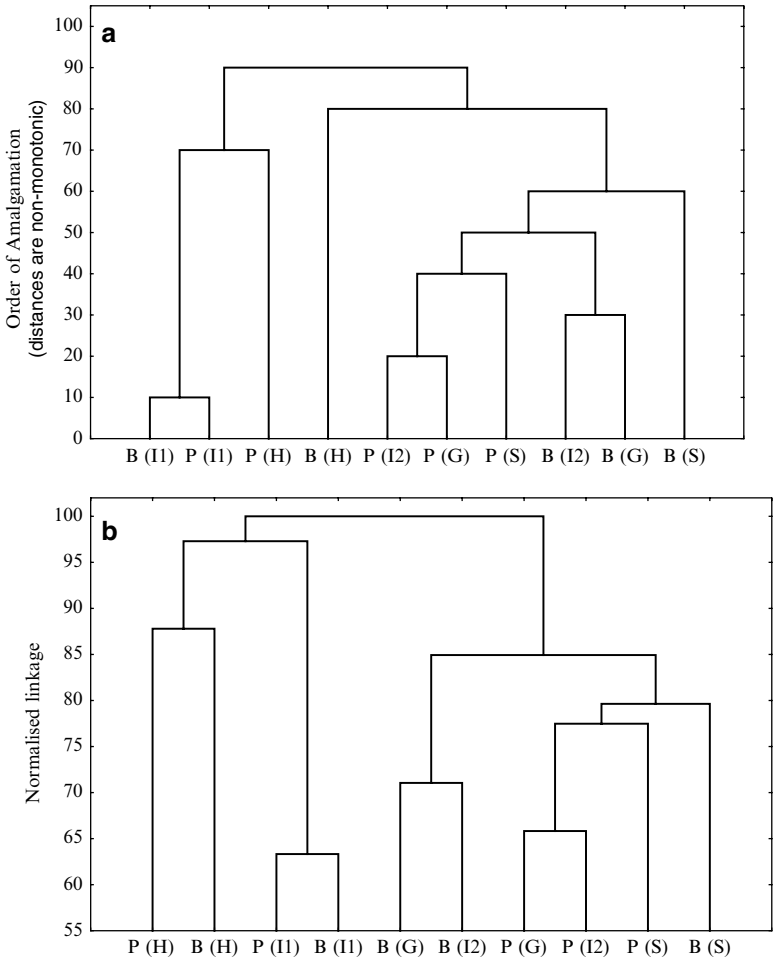

Fig. 12.1 Results of applying cluster analysis to pungent (**a**) and bitter (**b**) sensory descriptors evaluated by Dutch (**H**), Greek (**G**), Italian (**I1**, **I2**), and Spanish (**S**) panels. The Chebyshev distance metric and unweighted pair-group centroid amalgamation rule were used in (**a**), while Euclidean distance and the single-linkage rule were used in (**b**)

- Ward's method is different from all the other methods in that it uses an analysis-of-variance approach to evaluate the distances between clusters. This method is regarded as quite efficient, although it tends to create small clusters.

Cluster analysis has been applied to tasting sensory descriptors (bitter and pungent) that were evaluated in a number of VOOs by panels of different nationalities (Fig. 12.1). Greek (G), Italian (I2), and Spanish (S) panels strictly followed the official methods (EC 1995) for VOO evaluation, while Dutch (H) and Italian (I1) QDA (quantitative-descriptive analysis) panels applied respectively structured (0-9) and unstructured (130 mm) scales. Figure 12.1 shows how the selection of amalgamation rules and the linkage distances determine the result; thus, analysts should

bear in mind this subject before applying cluster analysis. At first glance, both plots (A, B) show that there are two large groups [panels based on official methods (EC 1995) *vs.* nonnormalized QDA panels], but in fact there are important differences that can lead to different conclusions if the multidimensional scaling (MDS) statistical procedure (Sect. 12.3.3) had been applied to the results.

12.3.2 Factor Analysis

A group of techniques known as factor analysis (FA) has been developed for the modeling of complex data. Among the many ways of carrying out FA, PCA is one of the best known, and it is applied in characterization. The objective in applying FA is to obtain a number of unobservable factors, from the original set of observable variables (e.g., sensory perceptions, chemical components), so as to reduce a large raw data matrix to another, smaller one while retaining most of the original information. FA produces several linear combinations of observed variables generally termed *eigenvectors*, but they are more frequently called either factors, when data have not been centered, or components, when data have been autoscaled and applied to only one category. The steps in FA include selecting a group of original variables, building the correlation matrix, determining the number of eigenvectors (components or factors) to be considered, extracting a set of eigenvectors from the correlation matrix, rotating the eigenvectors to increase interpretability, and, eventually, making the conclusions. There are two requirements for the conclusions: they must make sense and they should be supported, when used in characterization, by chemical or physical or biochemical or climatic or agronomic knowledge to demonstrate that results were not obtained by chance.

12.3.2.1 Algorithms for Extracting Eigenvectors That Explain the Variability of Data

Numerous procedures for factor extraction are available; those described below are the most common. All these extraction methods calculate a set of orthogonal factors (or components) that in combination reproduce the matrix of the correlation. The criteria used to generate the factors can differ from method to method, but differences between the solutions may be very small.

- PCA is the most widely used multivariate procedure because it is easy to interpret and permits an explanation for the maximal variability of the initial distribution. Moreover, there is no need to invert a matrix when applying this method.
- Communalities by multiple R-square are a common default method for estimating the communalities for principal FA. In this method, the diagonal of the correlation matrix (communalities) will be computed as the multiple R-square of the respective variable with all other variables prior to factoring.

- The iterated communalities method uses multiple R-square estimates for the communalities, and later the method adjusts the loadings over several iterations. The residual sums of squares are used to evaluate the goodness of fit of the resulting solution.
- The centroid method is the geometrical approach to FA.
- The principal axis method first computes the eigenvalues from current communalities and then the next communalities (sum of squared loadings) are repeatedly recomputed, based on the subsequent extracted eigenvalues and eigenvectors, to determine the minimal changes in communalities.
- The maximum likelihood factors method requires an a priori hypothesis on the number of possible factors, and the method calculates the loadings and communalities that maximize the likelihood of the correlation matrix.

Regardless of the method applied to extract factors, an algorithm should be later used to remove those eigenvectors that are too dependent on the actual objects, which could reduce the validity of the model. Cross validation, the process of assessing the predictive accuracy of a model in a test sample, allows the selection of eigenvectors that give the minimum residual sum of squares for the omitted objects (Piggot and Sharman 1986).

Each of the various methods for extracting factors has a distinct mathematical background, but from an empirical viewpoint, there are no significant differences in the results. Hence, PCA can be recommended because it is simply a mathematical transformation of raw data. Figure 12.2 illustrates the result of applying PCA and the principal axis method to the characterization of varietal VOOs by sensory descriptors.

12.3.2.2 Graphical Representation of Results: Rotation

Eigenvector rotation is applied only as an aid to the interpretation and scientific utility of results, but it does not improve the fit between the observed and reproduced correlation matrices. The four types of rotation are as follows:

- A varimax normalized (or simply varimax) algorithm performs a rotation of the normalized factor loadings, which are the raw factor loadings divided by the square roots of the corresponding communalities. In mathematical terms, it maximizes the variances in the columns of the matrix of the squared normalized factor loadings. It is the most commonly used algorithm.
- The quartimax normalized method maximizes the variances in the rows of the matrix of the squared normalized factor loadings or in the squared raw factor loadings if normalization has not been requested (quartimax raw).
- An equamax rotation is a weighted mixture of the varimax and quartimax rotations. It simultaneously maximizes the variances in the rows and columns of the matrix of the squared raw factor loadings.
- The oblique rotation was developed to rotate factors, without the constraint of orthogonality of factors, although they are often not easily interpreted.

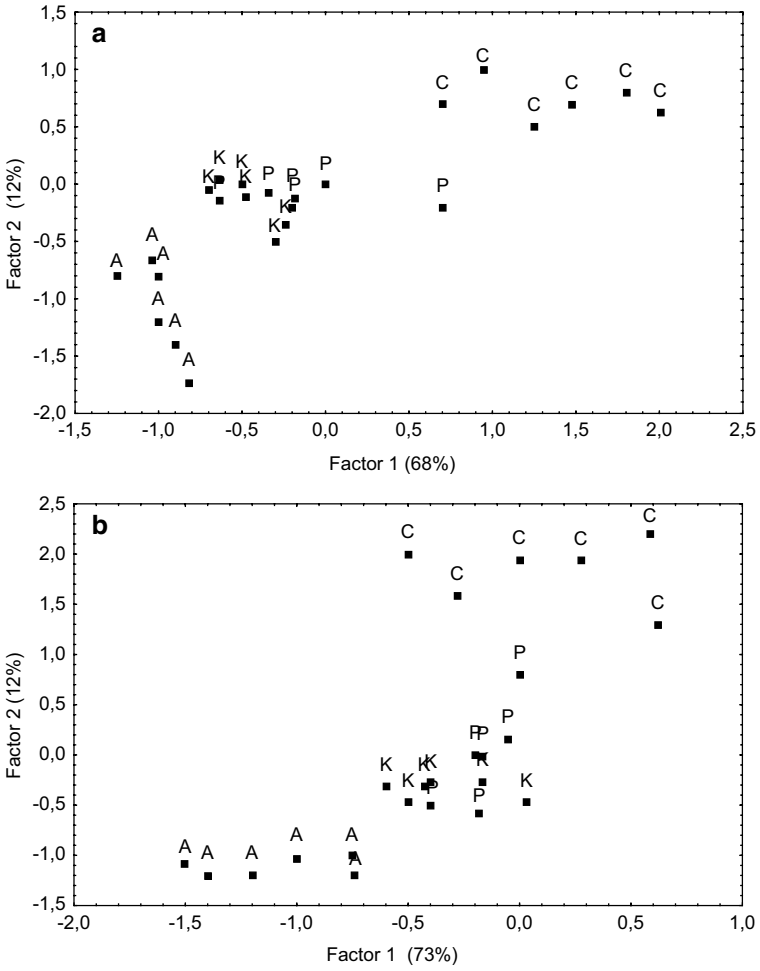

Fig. 12.2 Results of applying factor analysis (*FA*) to sensory descriptors characterizing varietal virgin olive oils Arbequina (*A*), Coratina (*C*), Koroneiki (*K*), and Picual (*P*). (**a**) Maximum likelihood and varimax rotation; (**b**) principal component and varimax rotation

12.3.2.3 Analyzing the Results

With the application of FA, three practical issues should be taken into account: (1) multicollinearity and singularity, (2) outliers among variables and with respect to the solution, and (3) validation of results. Extreme multicollinearity and singularity must be avoided for those algorithms that require matrix inversion. When multicollinearity is present, it might be necessary to eliminate some variables. Variables

that are unrelated to others should be identified as potential outliers. To determine which objects (samples or cases) are multivariate outliers, one should calculate a critical value by looking up the critical χ^2 at the desired alpha level (Tabachnick and Fidell 1983). Confirmatory FA is performed to test hypotheses about the structure of the underlying processes (e.g., which variables in a data set form coherent clusters that are relatively independent of one another). Thus, confirmatory FA requires a validation process that can be carried out with an independent test set of data (external validation) or the same data set (validation set). Algorithms used in the latter validation include cross validation, leverage correction, bootstrap, or Mallows *Cp* (Martens and Næs 1989).

12.3.2.4 Current Problems with FA

Computationally, FA needs to invert the correlation matrix. If this correlation matrix includes variables that are 100 % redundant, the inverse of the matrix cannot be computed; this is the so-called ill-conditioning matrix. In practice, this happens when there are high intercorrelated variables (e.g., variables that are the sum of other variables). Statistical packages can artificially add a small constant to the diagonal of the matrix, thus lowering the whole correlation in the correlation matrix, but researchers usually forget that these resulting estimates will not be exact.

12.3.3 Multidimensional Scaling

Multidimensional scaling (MDS) is an alternative to FA when the goal of the analysis is to explain observed similarities or dissimilarities (distances) between investigated objects (e.g., samples from different geographical origins). MDS can analyze any kind of similarity or dissimilarity matrix, in addition to correlation matrices. It can be applied to the results of cluster analysis; thus, the aforementioned algorithms for the distances and amalgamations can be used to build the similarity (or dissimilarity) matrix.

The MDS procedure allows the researcher to ask questions and to derive from those questions underlying dimensions without the respondents ever knowing the researcher's real interest. This is of great interest for VOO sensory characterization or for studies on the relationship between sensory descriptors and the chemical compounds responsible for them. Thus, MDS is not so much an exact procedure as it is a way to rearrange objects in an efficient manner, so as to arrive at a configuration that best approximates the observed distances. In more technical terms, the program uses a function minimization algorithm that evaluates different configurations with the goal of maximizing the goodness of fit (or minimizing lack of fit). Stress is the commonest measure of goodness of fit. It is used to evaluate how well (or poorly) a particular configuration reproduces the observed distance matrix.

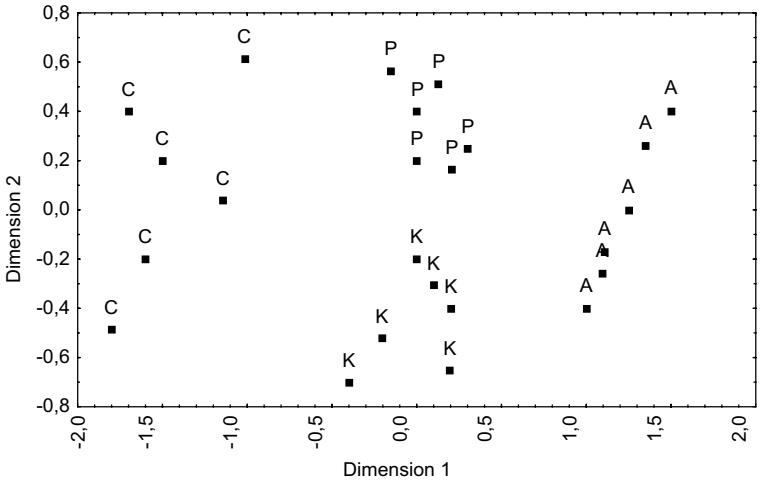

Fig. 12.3 Results of applying multidimensional scaling to hydrocarbon series of varietal virgin olive oils Arbequina (*A*), Coratina (*C*), Koroneiki (*K*), and Picual (*P*). The distance was Manhattan (city-block), and the amalgamation was Ward's method

Thus, the smaller the stress value, the better is the fit of the reproduced distance matrix to the observed distance matrix.

The reproduced distances plotted on the vertical (Y) axis against the original similarities plotted on the horizontal (X) axis configure the Shepard diagram. The result is a step line that represents the so-called D-hat values. The interpretation of dimensions usually represents the final step of this multivariate procedure. As in FA, the final orientation of axes in the plane or space is mostly the result of a subjective decision by the researcher because the distances between objects remain the same regardless of the type of rotation. An analytical way of interpreting dimensions is to use regression techniques to regress some meaningful variables on the coordinates for the different dimensions. Figure 12.3 shows an example of the application of MDS. An interesting application is given by Aparicio et al. (1996a).

12.3.3.1 Factor Analysis Versus Multidimensional Scaling

Although similarities exist between MDS and FA, the two procedures are fundamentally different in their essence. FA requires that the underlying data be distributed as multivariate normal and that the relationships be linear, whereas MDS imposes no such restrictions. Because FA tends to extract more factors than MDS, the latter often yields more-interpretable solutions. MDS can be applied to any kind of distances or similarities, but FA requires the computation of a correlation matrix. Concerning sensory analysis and characterization, MDS can be directly applied to

the analysis of stimuli, whereas FA requires the consideration of stimuli on certain sensory descriptors (Schiffman et al. 1981).

12.3.4 Discriminant Analysis

Discriminant analysis is used not only to determine which variables discriminate between two or more naturally occurring groups, but also to determine which variables are the best predictors discriminating between groups. The discriminant model may be interpreted as a special type of FA that extracts orthogonal factors. This procedure is disparaged by certain chemometricians who say that it capitalizes on chance because it "picks and chooses" the variables to be included in the model. If the procedure is applied with rigor (Fisher for the selection of initial variables, adequate values of F-to-Enter and F-to-Remove, Jackknife algorithm, and a validation set), however, the procedure can render results as good as the unsupervised procedures.

The objective of the procedure is to build a model in which all of the variables can better predict to which group an object (sample or case) belongs (e.g., a sample of VOO *cv.* Picual with respect to the Picual variety group). Forward stepwise analysis can be used to build a model of discrimination step by step. At each step, all unselected variables are evaluated to determine which one contributes most to the discrimination between groups, and that variable will then be included in the model. The backward stepwise analysis first includes all the variables in the model and then, at each step, eliminates the variable that contributes least to the prediction of group membership. The model retains only the important variables, that is, those variables that contribute the most to the discrimination between groups. The control of the variables included or excluded from the model is carried out by the a priori F-to-enter and F-to-remove values. The F value is a measure of the extent to which a variable makes a unique contribution to the prediction of group membership. These values must be fixed with strictness; the higher the values, the lesser the variables included but the better the validation results. F-to-enter values can be taken from an F-distribution (m × n) table at 99 %, where m is the number of groups and n the number of samples of the smallest group.

12.3.4.1 Current Tests Before Assuming the Final Conclusions

It is assumed that the variance/covariance matrices of variables are homogeneous across groups. It is suggested, however, to review the within-groups variances and correlation matrices before accepting final conclusions and rerun the analyses excluding one or two groups that are of less interest when in doubt. The a priori probabilities, which can greatly affect the accuracy of the classification, are additional factors that need to be considered when analyzing the objects to be classified. If the analyst sees that there are more objects (samples) in one group than in any

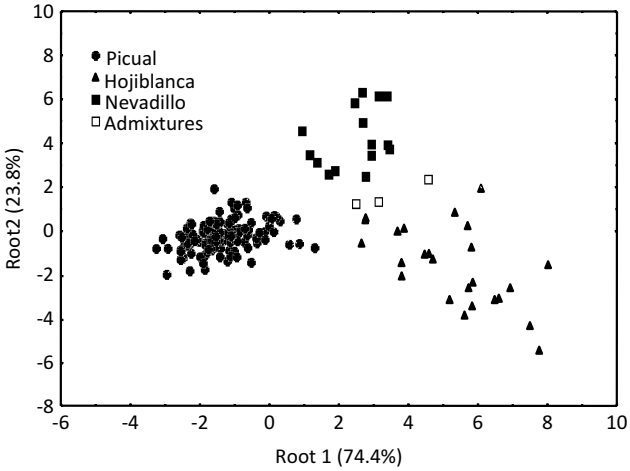

Fig. 12.4 Results of applying stepwise linear discriminant analysis (*SLDA*) to some Andalusian virgin olive oils. Classification was carried out by seven variables: (1) muurolene, tridecene, and heptadecene hydrocarbons; (2) triterpene dialcohol phytol; (3) aliphatic alcohol ethyllophenol; (4) β-sitosterol and stigmasterol

other, the discriminant analysis should be readjusted with a priori probabilities proportional to the sizes of the groups, or another user-defined algorithm.

Once the procedure has computed the classification scores, the analyst should know the posterior probabilities by the Mahalanobis distances. The probability that an object (i.e., a sample) can be classified within a group is proportional to the Mahalanobis distance from the hypothetical sample location to the group centroid.

Another assumption of discriminant function analysis is that the variables used to discriminate between groups are not completely redundant. If any one of the variables is completely redundant with respect to the other variables, then the matrix is ill-conditioned and cannot be inverted.

A few extreme outliers have a large impact on the means and increase the variability as well. An inspection of the means and standard deviations or variances could assist in detecting outliers that must be removed or the methods that must be used to correct their influence.

The number of discriminant functions, which makes reliable groups differ, is typically one or two, so that the remaining orthogonal discriminant functions scarcely provide additional information about group membership and are, in general, better ignored.

Finally, the major "real" threat to the validity of significance tests occurs when the means for variables across groups are correlated with the variances (or standard deviations). Intuitively, if there is high variability in a group with particularly high means on some variables, those high means are not reliable. The overall significance tests, however, are based on pooled variances, that is, the average variance across all the groups. Thus, the significance tests on the relatively larger means

(with the large variances) would be based on the relatively smaller pooled variances, resulting, erroneously, in statistical significance.

Figure 12.4 shows the results of applying stepwise linear discriminant analysis (SLDA) to some Andalusian VOOs characterized by 55 variables (chemical compounds), although the procedure used only 7 of them. Thus, SLDA can be applied to select the best variables characterizing a priori clusters, and then PCA works with only those selected variables (Baeten et al. 1996). This two-step procedure would avoid building a model from pure noise or where noise has a great influence (Aparicio 1997).

12.3.4.2 Other Statistical Multivariate Procedures in Food Characterization

Multivariate calibration was developed to find the relationship between one or more dependent variables and a group of independent variables. The objective is to design a model for the relationship. In practice, a linear model is usually used for explaining the relationship, but other possibilities also exist. Stepwise multiple linear regression, partial least-squares regression, principal component regression, and piecewise linear regression are the most commonly used procedures for linear solutions. Nonlinear regression procedures, like logistic models, growth models, and probit and logit models, among others, are used as well.

Ridge regression (RR) analysis is used when the independent variables are highly interrelated and stable estimates for the regression coefficients cannot be obtained via ordinary least-squares methods (Pfaffenberger and Dielman 1990).

Correspondence analysis is a descriptive/exploratory technique designed to analyze simple two-way and multiway tables containing some measures of correspondence between the rows and columns. The results provide information similar in nature to those produced by FA techniques.

Another interesting statistical procedure is canonical correlation, which allows one to determine the correlation between two sets of objects and to know the redundancy between them; this procedure is particularly useful in sensory analyses. Procluster analysis (Arnold and Williams 1986) is also able to analyze the behavior of each panelist in sensory analysis. Statistical sensory wheel (SSW) (García-González et al. 2009a), based on directional statistics (Mardia 1972) and PCA, allows for clustering inside a circle of not only the sensory descriptors but also of the chemical compounds responsible for them. This algorithm is able to determine the relationship between sensory descriptors and chemical compounds, certain synergies and antagonisms between chemical compounds, and the interaction between sensory descriptors and basic stimuli.

12.4 Artificial Intelligence Methods in Food Characterization

When the analyst faces a characterization problem and makes the decision to analyze it using artificial intelligence methods, the most common methods chosen are expert systems (ESs), artificial neural networks (ANNs), and fuzzy logic. Usually, one wonders which method would give the best conclusions. Each one of these methods (models) has its advantages and disadvantages. Although they are dissected in subsections below, simple definitions can shed light on their possible application in food characterization. Neural networks are models that can learn from past experiences through adaptive programs, which means that they do not reqeuire knowledge about the nature of the relationship of the process parameters, but they have some of the disadvantages of discrimination systems. The theory of fuzzy sets provides a convenient means to deal with imprecision, vagueness, and ill-defined and doubtful data (Aparicio et al. 1996a; Aparicio 2000). ESs are intelligent, efficient, and reliable programs that are able to solve a particular problem based on knowledge bases of heuristic rules. They also have the ability to self-learn and to correct inaccurate stored information.

12.4.1 Expert Systems

Statistical packages are generally employed because they provide users with the mechanics of data analysis; however, they do not help very much with analytical strategies. A user must think about different aspects simultaneously while conducting an analysis, which is not easy because data analysis must be done by structuring the analysis processes into various successive steps. Despite these problems, probability theory has been extensively applied in olive oil characterization, whereas too few applications of ESs have been made (Aparicio 1988; Aparicio and Alonso 1994; Aparicio et al. 1994a; Adler et al. 1993; Betteridge et al. 1988; González-Andujar 2009), perhaps because of the lack of mathematical background among analysts.

The ability to make decisions on the basis of knowledge makes ESs different from statistical programs. Their essential characteristics are the self-learning, look-ahead, and back-propagation to the point that they are sine qua non conditions for true ESs. The most important tasks in building an ES are to extract knowledge rules and to design an algorithm for controlling them. The resulting ES is composed of a knowledge base and an inference engine. The former contains the domain knowledge in the form of facts and rules, while the latter is able to cluster only the knowledge related to a specific universe of discourse (e.g., a varietal VOO, the geographical origin of a VOO) and to apply the rules in the appropriate order to infer correct conclusions.

ESs work with rules that have been built independently. Each rule has propositions that are related to a parameter, or a ratio between parameters or an equation classifying categories (e.g., two varietal VOOs) of the universe of discourse (e.g., a varietal olive oil) to be characterized. The SEXIA TM has a knowledge base of more than 400 rules that compile more than 1,400 samples of European VOOs characterized by 65 chemical compounds (Aparicio and Alonso 1994). The knowledge base stores rules related to geographical origin, cultivar, ripeness, extraction systems, and so on.

12.4.2 Artificial Neural Networks

Artificial neural networks (ANNs) have become one of the most applied artificial intelligence algorithms. They are adaptive algorithms capable of learning from past experiences. The algorithm, based on a binary threshold unit (McCulloch and Pitts 1943), allows universal computation under certain conditions. ANNs, like the human brain, process information through a training process using a set of known samples to reduce the error of a predicting model. The training process makes it possible to determine the efficiency of a model and can be repeated (retrained) in the case of a significant change in the nature of the data.

ANNs have been applied to analyze complex nonlinear data with satisfactory outputs. Thus, ANNs are particularly useful for processing data that are difficult to interpret because of the absence of chemical assignments, such as those registered by sensors or spectrometers. Two of the main advantages of ANNs over other algorithms are the high adaptability and the high noise tolerance (Gardner et al. 1990), which make this mathematical procedure adequate for processing chemical data affected by repeatability or sensitivity problems. Furthermore, ANNs are able to compensate the signal shift in quantification due to errors in calibration or to drift problems of some instruments (Lazzerini and Maggiore 1998). ANNs also have some disadvantages, such as the difficulty of selecting an appropriate training system and the ANN architecture (Singh et al. 1996). The values of the parameters at the beginning of training are randomly selected, and consequently the training process is subjected to noncontrolled variations that make it nonreproducible (Lazzerini and Maggiore 1998). As a consequence, the training time to obtain optimum results is unpredictable to some extent since it depends on the initial values. The procedures for exploring data prior to training are necessary to know the nature of the information, namely, variability, linearity, and noise, among other features. PCA can be used and its principal components can even be submitted as input variables in ANNs.

The structure of neural networks is a set of nodes at different levels (or layers) where the node of a layer is linked with all the nodes of the next layer. This structure is the so-called multilayer perceptron, or MLP (Huang 2009), and it is a procedure commonly used for classification tasks. Figure 12.5 shows the general structure of a MLP. The perceptron is a set of input terminals – nodes, units, or neurons (i and j) – that are distributed in three layers: input layer, hidden layer, and output layer.

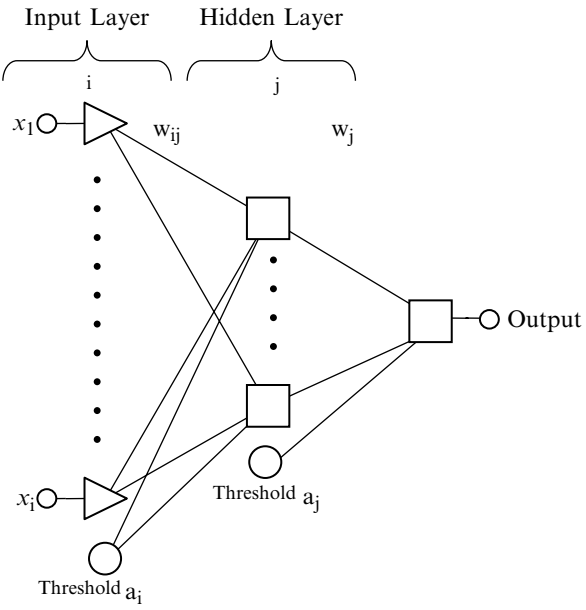

Fig. 12.5 General scheme of a multilayer perceptron (*MLP*)

The input layer feeds input patterns of one or more intermediate layers of units, commonly called hidden units. The hidden layers are followed by an output layer where the result of the computation is read off. Each of these units or neurons computes a weighted sum of its inputs from other neurons of the previous layer. When the MLP is designed to classify samples into two groups or classes, the output layer is formed by a single unit. In that case, the final output is typically assigned a binary value (1 or 0) according to whether the sum is above or below a certain threshold. This classical definition has been generalized in the equation

$$n_i := f(\Sigma w_{ij} n_j - a_i), \textit{ for } i, j = 1 \textit{ to } n,$$

where the number n_i represents the state of the unit and is continuous-valued; $f(x)$ is a general nonlinear function called activation function; w_{ij} represents the strength of the connection between unit i and unit j (if it is zero, then there is no connection, but if it is positive or negative, then it indicates an excitatory or inhibitory connection); a_i is the specific threshold value for unit i; and the sign := emphasizes that the equation is not a function of time since it is updated asynchronously.

Figure 12.6 shows the details of a node or neuron and the synaptic connections with other nodes. As was explained earlier, the synaptic connection between neurons is defined by an equation in which each node has a weight value associated with it (w_{ij}). Thus, the input value of each node is obtained by summing up all the output values of the nodes of the previous layer, multiplied by the corresponding weights (w_{ij}) and subtracting the threshold values (a_i and a_j). Then the resulting

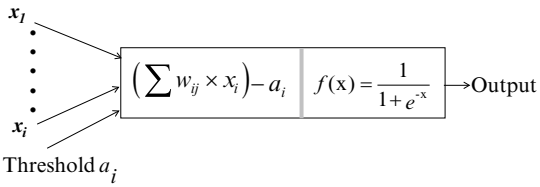

Fig. 12.6 Scheme of synaptic connections in a node or unit within a multilayer perceptron (*MLP*)

value is submitted to an activation function [f(x)] to yield the output of this node. This output will be the input value for the next layer. The activation function shown in Fig. 12.6 is the sigmoid function that is appropriate for classification tasks.

The design and training of an ANN involve choosing the right network architecture (organization of layers and nodes) and the right weight and threshold values (w_{ij}, a_i, and a_j). The most common solution is the iterative adjustments of the w_{ij} strengths, which may be done by supervised or unsupervised learning. The former is based on the comparison of the network output with known correct answers. The differences between the ANN output and the right answers are represented by the error factor, which is used for generating the weight adjustment in the output and hidden layers. In general terms the design and the training includes three basic steps:

1. Selection of the best input variables before starting the training. This selection can be carried out by genetic algorithms. These algorithms imply the evolution or small changes of an initial population (initial randomly selected data set). These small changes are called mutations. The algorithm is run until the best possible classification rates are reached.
2. Selection of the best network architecture. As a general rule, the structure of the network is defined by taking into account the notion that the number of units of the hidden layer should be similar to half of the sum of input and output units. The network architecture is very important because each application requires its own design. To get good results, one should build into the network as much knowledge as possible and use criteria for optimal network architecture such as the number of units, number of connections, learning time, cost, and so on. A genetic algorithm can be used to search the possible architectures (Whitley and Hanson 1989). Figure 12.7 shows some networks of unsupervised and supervised procedures.
3. Network training. Once the structure of the network is decided, the weight and threshold values that define the connection between layers must be established through a training process that minimizes the prediction error. The error of a particular MLP is obtained by comparing the outputs of the N training samples with the expected results (for example, 0 and 1 for two classes). The error function can be the root mean square error (RMSE) function in which all the errors are squared and summed up, resulting in a single value that represents the error of the neural network. The RMSE function is expressed as

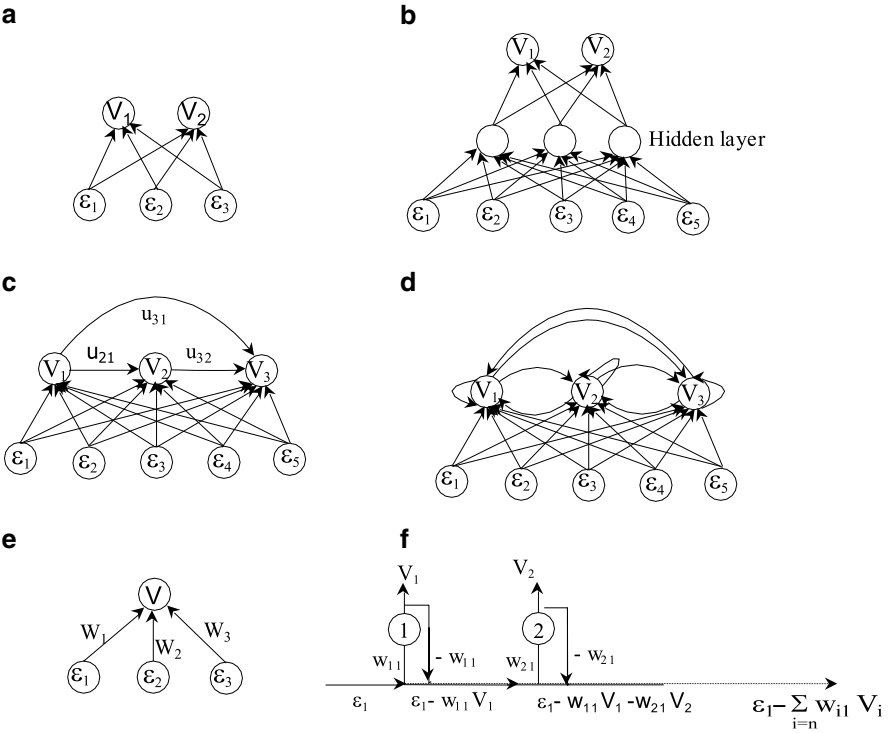

Fig. 12.7 Networks of different kinds of artificial neural networks (*ANN*): (**a**) one-layer perceptron; (**b**) two-layer feedforward perceptron; (**c**) network for PCA with anti-Hebbian learning; (**d**) v_1 is the first PCA, v_2 the second PCA, and so on; a simple competitive learning network; (**e**) simple Hebbian learning; (**f**) Sanger's unsupervised learning

$$RMS = \sqrt{\frac{1}{N}\sum_{n=1}^{N}\left[\hat{f}_o^n(x) - f_o^n(x)\right]^2}$$

where N is the number of training samples,

$\hat{f}_o^n(x)$ is the expected value of the activation function of the output node for sample n, and

$f_o^n(x)$ is the observed value of the activation function of the output node for sample n.

Although there are many kinds of training algorithms to minimize RMSE values, two of the most widely used in MLP are backpropagation (BP) and conjugated gradient descent (CGD) (Bishop 1995). These two supervised algorithms use

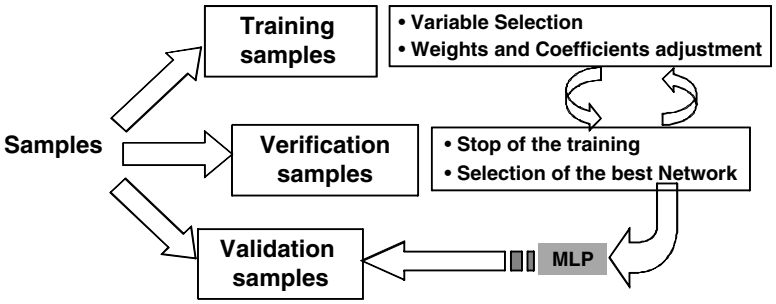

Fig. 12.8 Sample sets required for designing and testing a multilayer perceptron (*MLP*)

the concept of error surface. An error surface is a surface of p + 1 dimensions that represents the error of a model for p parameters. These p parameters are the weights and threshold values (*w* and *a*). These algorithms starts with an initial set of *w* and *a* values randomly selected. The algorithms search for a global minimum error by computing the slope at each point on the error surface and moving toward *w* and *a* values, which provide lower RMSE. The algorithms run until a local minimum error is obtained where any change in *w* and *a* results in no improvement in RMSE. The training algorithms also stop if the differences between the RMSE of the training set of samples and a verification set of samples are too high to avoid an overfitting of the weight and threshold values.

Once the network is trained, the performance of the designed network can be checked by an external validation set of samples. Therefore, in general terms, the design of MLPs requires three sets of samples (Fig. 12.8): the training set to select the best variables (weights and thresholds), the verification set to avoid overfitting or overlearning, and the validation set to test the performance when working with unknown samples.

The network can also be trained with unsupervised procedures in which there is no pattern with which results can be matched and the network is expected to create categories from the correlation between the input data. Thus, there is no feedback saying what the outputs should be or whether they are correct. This means that unsupervised learning can only do something useful when there is redundancy in the input data (Barlow 1989). Unsupervised learning can be based on Hebbian learning (Hebb 1949) and extension rules (Oja 1989; Sangers 1989), where multiple output units are often active together, or on competitive learning in which only one output unit per group is on at a time. The former learning offers different possibilities such as procedures close to statistical algorithms of principal components and cluster analysis. The objective of competitive learning is to categorize the input data by different models such as Willshaw and von der Masburg or Kohonen's algorithm. Unsupervised learning is rapid as it does not use BP, which can be

extremely slow, and it is advisable to apply it before training a network with a supervised procedure with BP. This unsupervised learning works in the case of problems where similar input vectors produce similar outputs. Counterpropagation networks use unsupervised procedures for the connection of the inputs with the hidden layer and supervised procedures for the connection of the hidden layer with the outputs.

In regard to traceability, Schierle and Otto (1992) and Lohninger and Stancl (1992) also carried out comparative studies of ANNs and multivariate statistical procedures. Although ANNs have been extensively applied in many kinds of application to characterize foods, this procedure works better than classical statistical techniques in complex classification tasks, where the data sets are large and have a significant noise component. For that reason, ANNs are particularly useful in sensory quality characterization and geographical traceability of olive oils.

12.5 State of the Art in Virgin Olive Oil Traceability

The many factors that affect the traceability of VOOs can be clustered into four broad groups: (1) environmental (soil, climate); (2) agronomic (irrigation, fertilization); (3) cultivation and harvesting (cultivar, ripeness); and (4) technological factors (postharvest storage and extraction). The diversity and interrelationship of these factors make it very difficult to carry out a complete traceability of VOOs by either their chemical composition or sensory descriptors.

The first step for correct traceability is to define the strategy for olive oil sampling. General objectives look for intercluster differences taking into consideration the maximum intracluster variability. A geographical origin characterization, for example, requires samples from all the sources of variation (cultivar, altitude, extraction systems, harvesting time, or ripeness, among others). Specific objectives, by contrast, look for samples with the minimum intracluster variability. The ripeness characterization, for example, should avoid VOO samples obtained by different extraction systems but collect samples from different altitudes and climates. At any rate, how information is managed is crucial to avoid obvious or wrong conclusions. In general, the mathematical problems are limited to the improper use of procedures or the nonexistence of a validation process.

Another big problem concerns the distribution of the values of the chemical parameters over the years. A high degree of data dispersion for some chemical parameters over time might decrease the coefficients of correct classification obtained by statistical procedures applied to a crop from a single year. Studies using information from only one crop can yield a high value of misclassifications when they are verified with data from other crops.

Finally, the quantification of chemical compounds is crucial for arriving at plausible results, although there are classical problems affecting the analyses. Thus, the use of unidentified compounds can lead to the selection of artifacts as potential sources of discrimination. The use of redundant information, such as

Table 12.1 Content of some chemical compounds of cv. Picual at different altitudes

	Altitude		
Chemical compound	<400 m	400–700 m	>700 m
Palmitoleic acid (%)	0.82	0.68	0.62
Linoleic acid (%)	5.20	5.43	5.73
β-sitosterol (mg/kg)	1,329.22	1,250.68	1,103.38
Sigmasterol (mg/kg)	16.87	14.06	12.26
Campesterol	48.82	46.42	41.23
Sum of sterols (mg/kg)	1,459.77	1,380.26	1,217.96
Cycloarthenol (mg/kg)	104.81	161.55	194.39
24-methylene cycloarthanol (mg/kg)	610.43	979.14	1,203.55
Phytol (mg/kg)	32.68	27.97	39.43
Copaene (mg/kg)	0.26	0.34	0.59
Tridecene (mg/kg)	5.68	5.05	3.65
Heptadecene (mg/kg)	0.029	0.027	0.23
Heneicosane (mg/kg)	0.43	0.41	0.36

chemical compounds and their sum, or fatty acids and certain triacylglycerols (TAGs), can be a useless activity, particularly because it may also produce noise in the course of discrimination. A real problem is also the use of data sets obtained by different chemical methodologies (e.g., capillary, semicapillary, and packed chromatographic columns), which leads to problems when the information is cross-tabulated.

12.5.1 Characterization of Certain Agronomic and Pedoclimatic Aspects

It is generally accepted that climate has a great influence on ripeness and, hence, on the chemical composition of vegetable oils. For example, the concentration of β-sistosterol increases and those of campesterol and stigmasterol decrease when the climate becomes colder. These conclusions, which had been extensively demonstrated with oilseeds, have also been studied in olives.

Many studies have reported significant statistical differences between oils from olive groves cultivated near the Mediterranean Sea and those harvested in the mountains. Thus, VOOs of fruits collected from low altitudes have higher amounts of sterols, tocopherols, and phenols and lower contents of chlorophylls and unsaturated fatty acids (García-González et al. 2009a; Moussa et al. 1996) than oils from the mountains. Beyond this point, it has been verified that linoleic acid, 24-methylene-cycloartanol, β-sitosterol, and copaene are distinguishing features among VOOs from elevations below 400 m and above 700 m (Table 12.1), even using a test set with samples from different crop years (Aparicio et al. 1994a; Paz

Aguilera et al. 2005). Because 24-methylene-cycloartanol increases and β-sitosterol decreases during ripening (Frega and Lercker 1986), the test results also indicate that olives at high altitudes reach maturity more slowly.

The fact that the ratio of unsaturated/saturated fatty acids rises with the altitude of olive grove zones has great importance from the standpoint of either shelf life or sensory quality. Paz Aguilera et al. (2005) *corroborated* the findings of Aparicio et al. (1994a) that olive oils from low elevations have higher oxidative stability than those from high altitudes because the percentage of unsaturated fatty acids increases as the temperature decreases within the same growing area. This oxidative stability is also related to the content of α-tocopherol, which has a pronounced antioxidant activity (Blekas et al. 1995), and phenolic compounds, which have traditionally been considered the most important antioxidants (Papadopoulos and Boskou 1991). Cimato (1991) reported that the concentration of tocopherols is not affected by altitude, unlike the total content of polyphenols, which is double in coastal zones (elevation <100 m) for all the stages of ripeness studied in the varieties Frantoio, Leccino, Moraiolo, and Coratina.

The amount of unsaturated acids also has great importance from a sensory quality viewpoint because the most important volatiles are produced from linoleic and linolenic acids through the lipoxygenase pathway. Moussa et al. (1996) found that the content of linoleic acid was higher at high altitudes (approximately 800 m), but, in contrast, that of linolenic acid was higher at lower elevations (approximately 100 m). This difference based on altitude was also observed in later studies (Aparicio et al. 1994a; Issaouia et al. 2010). VOOs obtained from monovarietal olive groves at high altitudes are, in general, sweeter and have an herbaceous fragrance compared to the corresponding oils from lower elevations. A plausible explanation could lie in the linoleic/linolenic ratio since linoleic acid gives rise to hexanal and hexyl-acetate volatile compounds, which are responsible for green desirable perceptions, while (E)-2-hexenal (green, astringent) is produced from linolenic acid.

In addition to altitude, soils and climatic variables of olive grove zone affect VOO chemical composition. The influence of temperature and rainfall on the biosynthesis of oil was studied by Angerosa et al. (1996a), who took into account the maximum and minimum values of these variables. Major TAGs (OOO, POL, SOO, OLL) and phytol contents were tentatively correlated, by multivariate procedures, with the autumn temperatures and relative humidity of summer months and the rainfall of the whole year. Concerning soils, Cresti et al. (1994) reported the influence of salinity in olive oil composition. The contents of aliphatic and triterpenic alcohols and the linoleic/linolenic ratio increase considerably with salinity, showing an accelerated ripening of fruits induced by salinization.

With regard to soil, Montedoro et al. (1993) reported significant differences in the total content of polyphenols according to the type of soil, e.g., 155.7 ppm for clayey soils and 314.2 ppm for calcareous soils. The improvements in soil fertilization impact on sensory quality and chemical characteristics of olive oil (Dag et al. 2009; Fernández-Escobar et al. 2006).

Studies on the effect of soil, salinity, and irrigation regimes on chemical composition have increased in importance because of the new agricultural practices of

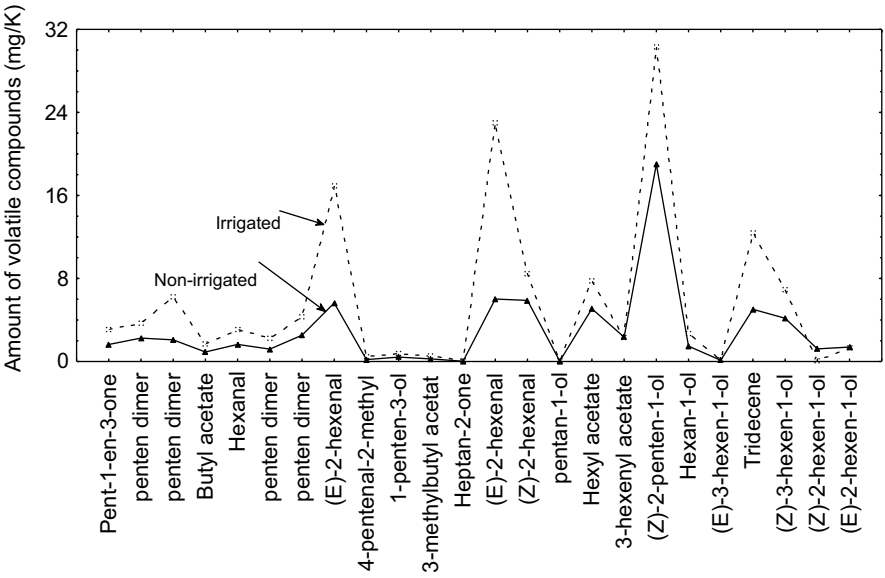

Fig. 12.9 Volatile profiles of virgin olive oils, cv. Koroneiki, obtained from irrigated and nonirrigated olive trees

high-density orchards (Stefanoudaki et al. 2009). Until recently, the olive tree was a crop of dry regions, and traditional agricultural practices did not require irrigation. The increase in olive oil prices and recent droughts in the Mediterranean basin have altered traditional agricultural practices, and irrigated olive groves are exponentially increasing in all producer countries. The balance between the irrigation pros and cons is described in Chap. 2. The differences between irrigated and nonirrigated olive trees can also been found by analyzing their VOO chemical and sensory characteristics. In general, irrigation affects chemical components related to bitterness and even the fatty acid profile, and the total content of polyphenols, partially responsible for bitter taste, is lower in VOOs from irrigated groves. The decrease in bitterness with irrigation is of great importance for varieties of cultivar characterized by high values of astringent, throat-catching, or bitter descriptors, for example, *cv.* Coratina. Furthermore, the sensory profiles of VOOs from irrigated and nonirrigated olive trees are quite different (Salas et al. 1997; Gómez-Rico et al. 2006). Figure 12.9 shows the most remarkable volatile compounds responsible for VOO aroma. If the sensory descriptors characterizing these volatile compounds (García-González et al. 2009a) are taken into consideration, then that lends more scientific support to the notion that VOOs from irrigated olive trees are less bitter and astringent than oils from nonirrigated olive grove zones.

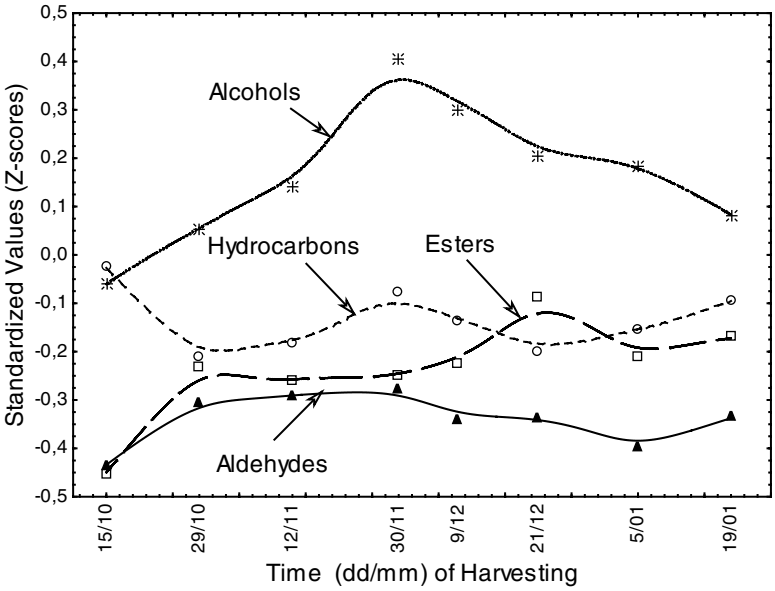

Fig. 12.10 Evolution of different series of volatile compounds during ripeness of *cv*. Arbequina

12.5.2 Characterization of Stages of Ripeness

The ripeness of olives is important for their harvesting because the accumulation of fatty acids rises with maturity and, hence, the extraction yield. For ages, olives were bought mostly for their oil content, and hence farmers were interested in harvesting when olives were mature enough. But studies on the evolution of chemical compounds during ripeness have allowed us to determine not only the best time for harvesting, but also to delineate the importance of olive maturity to sensory quality. VOO obtained from overripe olives has higher yield, but the chlorophyll content is relatively low (Criado et al. 2007) and it is poorer in phenols (Kalua et al. 2005) and has smaller amounts of some aromatic compounds (García-González et al. 2010b). In addition, late harvesting may also increase the likelihood of damage to the fruit, and this irremediably produces inferior olive oil quality, organoleptic defects and higher peroxidation, if not higher free acidity.

Several physical and chemical methods have been proposed for the evaluation of the optimum time of harvesting or, at least, for the characterization of the olive ripeness process. These simple and reliable physical methods can be easily applied in the field to determine the optimum harvest time, although they can have serious flaws due to their great variability with the ripeness of olive varieties. The chemical

methods are not rapid and straightforward, but they are precise enough and give plausible explanations about the evolution of olive oil chemical composition. For example, Fig. 12.10 shows the evolution of the series of volatiles compounds with time. A good correlation (adjusted-$R^2 = 0.98$) was found between the content of hexanal and the percentage of dry yield of olives (Aparicio and Morales 1998). This aldehyde, characterized by the sensory perception green-sweet, was also well correlated with the overall grading of the former European Community Regulation (EC 1995). This means that there is a good agreement between the best sensory quality, hexanal, and the highest industrial yield in the case of healthy olives and a sound extraction process.

However, studies on the best date for harvesting are only a part of the research devoted to a better knowledge of the evolution of chemical composition during olive ripening. Frega and Lercker (1986) studied the lipid extracts from epicarp and kernel mesocarp of the olive drupe obtained in the last 6 months of ripening. The epicarp was found to be coated mostly with the triterpenic acids oleanolic, ursolic, and maslinic (Jancini and Fedeli 1972), while the most mature olives revealed the presence of 18- and 16-carbon-atom fatty acids in addition to 28-, 26-, and 24-carbon atoms. The kernel mesocarp contains certain fractions of unsaponifiable matter (alcohols, 4-methyl-sterols, and sterols) that are modified during the ripening process of the drupe. There is an increase in triterpenic alcohol 24-methylen-cycloartanol, which corresponds to a decrease in butyrospermol and cycloartenol (Mariani et al. 1991), a decrease in 4-methyl-sterol citrostadienol that produces an increase in 3-methyl-sterol gramisterol and an increase in sterol Δ^5-avenasterol due to a decrease in β-sitosterol. Later, Esti et al. (1996a) verified the Δ^5-avenasterol to β-sitosterol ratio analyzing three different cultivars (Coratina, Leccino, Frantoio). This relation could be explained by the fact that the percentage of stones in the total weight of olive diminishes with ripeness, and olive oil from stones has a high concentration of campesterol and β-sitosterol but a low of Δ^5-avenasterol content (Christopoulou et al. 1996). These authors completed their studies by showing that erythrodiol, uvaol, and aliphatic alcohols are accumulated in the flesh and skin, with the concentration of erythrodiol being particularly high in the skin. More recent studies have centered on the combination effect of cultivar, irrigation, and maturity and their effect on quality and chemical characteristics (Anastasopoulos et al. 2011; Salvador et al. 2001; Stefanoudaki et al. 2000).

Although the evolution of sterols with ripeness has a certain importance (Δ^5-avenasterol is an unofficial test of quality), the evolution of other variables with ripeness, e.g., PUFA or polyphenols, is of greater relevance. Thus, Moussa et al. (1996) found that oils from high altitudes had higher chlorophyll contents compared with those from low altitudes, which is explained by the fact that olives grown in high altitudes reach maturity more slowly. Their conclusions highlight the fact that the loss of chlorophylls parallels the formation of other pigments responsible for the purple color of olives (Chap. 7). Analyzing the phenolic fraction, the authors found that protocatechuic and gallic acids could be good markers for the stages of ripeness because they are easily degraded during the ripening process. Thus, phenolic compounds have been proposed to design predictive models to determine the maturity

stage, tyrosol and oleuropein derivatives being the most informative phenols (Kalua et al. 2005). Esti et al. (1996a) also found that the content of oleic acid increased at the expense of palmitic acid during ripening of the cultivars Leccino and Frantoio, while the content of linoleic acid rose with ripening in the cultivar Coratina and decreased in the cultivars Leccino and Frantoio. Working with *cv.* Frantoio, Tacchino and Borgogni (1983) found that the contents of aliphatic C22, C24, C26 alcohol and C28 diminished with the ripeness of this cultivar. The maturity dependence of aliphatic alcohols has been corroborated by more recent studies where the importance of cultivar and geographical origin has also been highlighted (Lazzez et al. 2008; Ranalli et al. 2002).

The evolution of chemical composition during ripening can also be studied from a sensory quality viewpoint. The decrease in total phenols and tocopherols during ripening [15–35 % for tocopherols and 52–66 % for phenols in the last 4 months of fruit maturation Moussa et al. (1996)] is directly responsible for the decrease in bitterness. Thus, the effects of ripening on sensory quality are obvious and have been studied with either sensory descriptors or volatiles and phenol compounds. Morales et al. (1996) studied the influence of ripening on the concentration of green aroma compounds. Nine volatile compounds, produced through the lipoxygenase cascade, were quantified in four varieties of cultivar with the objective of looking for general markers characterizing basic stages of olive ripeness. Arbequina and Picual (Spain), Coratina (Italy), and Koroneiki (Greece) were the varieties selected. They represent a substantial proportion of the bottled VOO trade, and they are completely different from either a sensory evaluation or by chemical composition, and the evolution of their ripeness is also rather different. The authors found that there are markers for unripe olives – (Z)-hex-3-en-1-ol and (E)-hex-2-en-1-ol – and for normal ripe – hexyl acetate – regardless of the variety, though the total content of volatile compounds decreases during ripening (Aparicio and Morales 1998). In the light of the evolution of unsaturated fatty acids during ripening (Moussa et al. 1996; Esti et al. 1996), the linoleic/linolenic ratio determines the bitterness and green perception of oils through the contribution of volatile compounds to VOO flavor. For example, (E)-hex-2-enal contributes to green odor but also to intense bitter taste (Aparicio 2000; García-González et al. 2009a).

The concentration of phenols, responsible for taste, also varies with ripening, and this variation strongly depends on the genotype (García-González et al. 2010b). In addition to the organoleptic change (mainly bitterness) over maturation, the change in the concentration of phenols also leads to a change in stability against oxidation (Nieto et al. 2010). A significant decrease in concentration is observed for secoiridoid derivatives, in particular for secoiridoid derivatives of hydroxytyrosol (Gómez-Rico et al. 2006).

Ripeness can also be observed using emergent techniques such as ^{13}C-NMR (Chap. 11). Vlahov (1996b) studied the assumption that the content of diacylglycerols (DAGs) can be related to the maturation stage of olives. On the basis of total diglyceride content and the DAG 1,2/DAG 1,3 ratio, Vlahov obtained a complete compositional glyceride picture for olive cultivars that provides useful information

Table 12.2 Mean content of sterols and alcohols in virgin olive oils, cv. Koroneiki and Coratina, obtained by centrifugation systems of two (A) and three (B) phases

Chemical compound (%)	Coratina		Koroneiki		Chemical compound (mg/kg)	Coratina		Koroneiki	
	A	B	A	B		A	B	A	B
Cholesterol	0.19	0.28	0.42	0.52	Docosanol	12	6	31	27
Stigmasterol	0.69	0.77	0.88	0.93	Tetracosanol	11	11	76	65
Δ^7-Campesterol	0.74	0.34	0.28	0.24	Hexacosanol	23	22	112	91
Δ^5-Avenasterol	7.28	6.98	18.73	20.92	Octacosanol	11	11	39	34
Stigmastadienol	0.54	0.62	0.48	0.70	Total aliphatic alcohols	63	58	267	243
Δ^7-Stigmastenol	0.17	0.21	0.52	0.45	Cycloarthenol	414	427	201	219
Δ^7-Avenasterol	0.25	0.27	0.37	0.39	24-methylene-cycloarthanol	229	252	311	324
β-Sitosterol	84.19	83.90	69.80	69.50	Citrostadienol	70	75	217	228

regarding the ripeness stages of the olives processed. The DAG content enabled a possible classification of *cv.* Nebio and Coratina as late cultivars, unlike Dritta, Caroleo, and Cassano, which were early maturing fruits.

12.5.3 Characterization of Extraction Systems

Characterization of the extraction process has been a recurrent topic of research each time a new extraction system has been implemented/developed (Chap. 3). The research has usually been focused on either the chemical composition or the sensory quality of olive oils obtained by the extraction systems used at that moment. In the 1980s, the interest pointed to differences between pressure and three-phase centrifugation systems (Martel and Alba 1981; Lanzón et al. 1986; Nergiz and Ünal 1990) by the quantification of triterpenic and aliphatic alcohols. Today, however, the research is focused on the production of good-quality olives by centrifugation systems characterized by lower content of certain volatile compounds (n-octane, 2-methyl-1-propanol, 2-methyl-1-butanol, and acetic acid) that are responsible for defects like fustiness and winey taste (Morales et al. 1995) and the problems with the volume of wastewaters and alperujo (Capasso et al. 1992). The three-phase centrifugation systems evolved into two-phase centrifugal decanters that avoid the use of added water in the process (Chap. 3). From the first adoptions of this system in industrial olive processing in the early 1990s, numerous researchers have studied their differences also in terms of VOO sensory descriptors. VOOs obtained by two-phase decanters have high contents of (1) phenols (Jiménez-Márquez et al. 1995; Gimeno et al. 2002); (2) *ortho*-diphenols, hydroxytyrosol, tocopherols (Angerosa and Di Giovacchino 1996); and (3) (E)-2-hexenal and total volatile substances (Di Giovacchino et al. 2001). By contrast, these oils are characterized by low values of pigments, aliphatic and triterpenic alcohols, steroid hydrocarbons, and waxes (Ranalli and Angerosa 1996). Tables 12.2 and 12.3 show some chemical compounds

Table 12.3 Mean values of sensory descriptors and content of some volatile compounds and phenols of virgin olive oils, cv. Koroneiki and Coratina, obtained by centrifugation decanters of two (A) and three (B) phases

Sensory descriptor[a]	Varieties of cultivar				Chemical compound	Varieties of cultivar			
	Koroneiki		Coratina			Koroneiki		Coratina	
	A	B	A	B		A	B	A	B
Cut green lawn	1.60	0.69	1.37	1.47	Methyl acetate[b]	42	35	5	3
Twig	0.63	0.66	0.72	0.87	Ethyl acetate[b]	52	23	17	12
Green olives	1.53	1.02	1.49	1.46	(Z)-3-hexenal[b]	753	84	444	325
Wild flowers	0.31	0.15	0.64	0.36	(E)-2-hexenal[b]	19,040	16,006	27,435	25,280
Green banana	0.46	0.33	0.58	0.30	Pentan-1-ol[b]	58	29	47	37
Green tomato	1.27	0.68	0.62	0.45	Hexyl acetate[b]	723	700	29	28
Almond	0.30	0.21	1.19	0.81	3-hexyl acetate[b]	263	91	14	14
Bitter almond	0.48	0.09	0.73	0.56	(Z)-2-penten-1-ol[b]	2,052	917	61	54
Walnut husk	0.30	0.26	1.03	0.18	Hexan-1-ol[b]	4,052	41	1,060	912
Artichoke	nd	0.38	0.28	0.55	(E)-3-hexen-1-ol[b]	77	52	29	28
Apple	0.39	0.41	0.78	0.22	(Z)-3-hexen-1-ol[b]	2,360	423	150	138
Green hay	0.58	0.35	nd	0.92	(Z)-2-hexen-1-ol[b]	75	tr	47	10
Butter/cream	1.37	1.75	1.29	1.27	(E)-2-hexen-1-ol[b]	917	849	2,567	1,767
Bitter	0.49	0.30	1.65	1.06	σ-diphenols[c]	12.1	7.0	21.1	14.7
Pungent	1.33	0.48	2.07	1.18	Total phenols[c]	82.3	50.58	220.3	192.6

A two-phase decanter, *B* three-phase decanter, *nd* none detected, *tr* traces

[a]Structured scale 1–5

[b]μg/kg

[c]mg/L as gallic acid

and sensory descriptors of VOOs *cv.* Koroneiki and Coratina obtained by centrifugation systems of two and three phases.

From the viewpoint of sensory quality, malaxation (Chap. 3) is the most important step of a centrifugation system whatever the number of phases. Malaxation temperature is the main factor, and it has been the most studied parameter, together with time (Aparicio et al. 1994b; Alba 1996; Stefanoudaki et al. 2011). The values of temperature range between 25 °C and 35 °C; the former corresponds to the cold extraction conditions while the latter is in the neighborhood of the maximum value suggested by experts. The malaxation time varies according to ripeness, variety, and sanitary conditions of olives. In general terms, a range of time between 15 and 90 min covers almost all published studies. From a sensory viewpoint, low temperatures ($T \leq 25$ °C) and medium times (35–45 min) are the best extraction conditions to promote the formation of green volatile compounds responsible for desirable sensory perceptions (Aparicio et al. 1997). Some studies (Morales and Aparicio 1999; Morales et al. 1999) have shown that at a high temperature (35 °C) the production of alcohols is promoted compared to esters that are increased at a low temperature (25 °C). The amount of aldehydes depends on the malaxation time. Thus, high temperatures ($T \geq 35$ °C) during the mixing process promote the formation of volatile compounds responsible for undesirable sensory perceptions (e.g., bitter tasting) while, in

contrast, low temperatures ($T \leq 25$ °C) favor the production of desirable green sensory perceptions (e.g., cut grass and pleasant odor) (Aparicio 2000).

Other volatile compounds that significantly vary with malaxation temperature are hexanal, 1-penten-3-ol, (E)-2-hexenal, octane, and (Z)-2-penten-1-ol (Kalua et al. 2006). The advantages of cold extraction also affect the concentration of erythrodiol and aliphatic alcohols. Christopoulou et al. (1996) extracted (with hexane) samples of olives *cv.* Koroneiki by applying two different temperatures of extraction (cold and hot) in the laboratory. The composition of oils showed that the total concentrations of aliphatic alcohols and erythrodiol were more than twice as high at hot temperatures. Phenols (total content and composition), which affect the bitterness and stability of the oils, also varied significantly with malaxation temperature; they are present at lower concentrations when high temperatures are used. Thus, Kalua et al. (2006) determined that phenolic compounds that are strongly affected by malaxation conditions are 3,4-dihydroxyphenyl ethyl alcohol-decarboxymethyl elenolic acid dialdehyde (3,4-DHPEA-DEDA), tyrosol, vanillic acid, and (+)-acetoxypinoresinol. Nevertheless, the variation in all these compounds depends on the cultivar, and it is not possible to reach general conclusions about the malaxation effect on the chemical characteristics of oils (Stefanoudaki et al. 2011).

12.5.4 Characterization of Monovarietal Virgin Olive Oils

Perhaps because of the enormous number of different cultivars (Bartolini et al. 1998) or perhaps because farmers have a great fondness for their own traditional cultivars, the characteristics of varietal VOOs have been profusely described in the literature on the basis of either chemical or sensory aspects (Aparicio and Luna 2002; García-González and Aparicio 2010). Until recently, the olive cultivar was clearly linked to geographical origin or climatic aspects, and the autochthonous cultivar was, in fact, in the majority in each olive grove zone only 50 years ago. Through modern agricultural practices (Chap. 2), however, plastic cultivars have been disseminated to the point where they are now major cultivars in olive grove zones where they were unknown only 20 years ago. As examples, the autochthonous Northern Spain *cv.* Arbequina is extensively cultivated in countries as far away as Chile, Argentina, Australia, and New Zealand. These changes have meant that studies on the characteristics of the same cultivar under different pedoclimatic conditions have exponentially risen in recent decades (Aparicio et al. 1991; Vlahov 1996a; Salas et al. 1997; Ceci and Carelli 2007; Baccouri et al. 2008; García-González et al. 2010a).

From a mathematical point of view, the correct characterization of varietal VOOs requires sampling where the different parameters affecting the final product (e.g., extraction systems, geographical origin, ripeness) are represented in, more or less, their exact proportion as in the overall production. From chemical and technical points of view, almost all the chemical compounds identified in VOO have been used in this characterization. Some authors have used certain series of chemical compounds (Zunin et al. 2005; Giuffrida et al. 2011), whereas other authors have

Table 12.4 Sensory descriptors of various European olive oil varieties obtained by centrifugation systems from normal ripe olives

Descriptor[a]	Varieties of cultivar							
	K	C	P	A	T	Cb	M	F
Fruity	42	31	39	33	37	30	50	28
Green	41	31	34	26	32	26	32	14
Bitter	39	48	42	16	35	37	70	30
Pungent	45	62	45	22	45	55	62	62
Sweet	35	30	31	40	30	34	23	39
Cut grass flavor	39	29	38	23	26	32	20	55
Tomato flavor	16	8	22	26	12	8	21	24
Throat-catching	39	56	39	18	41	48	38	20
Green appearance	36	31	30	22	39	19	28	–
Fresh after mouthfeel	25	25	29	21	23	20	51	–
Apple smell	21	21	30	21	16	25	28	26
Flowers aroma	22	18	24	19	15	24	27	25

K Koroneiki, *C* Coratina, *P* Picual, *A* Arbequina, *T* Tsunnati, *Cb* Cima di Bitonto, *M* Moraiolo, *F* Frantoio

[a]Unstructured scale of 100 cm

Table 12.5 Concentrations of some volatile compounds of various European olive oil varieties

Volatile compound	Varieties of cultivar							
(μg/kg)	K	C	P	A	T	Cb	M	F
Hexanal	275	478	189	300	110	42	48	30
(Z)-Hex-3-enal	658	390	316	175	422	214	218	119
(E)-Hex-2-enal	5,416	27,464	1,882	10,282	9,737	21,158	5,058	11,728
Hexyl acetate	524	20	212	78	80	39	66	171
(Z)-Hex-3-enyl acetate	289	25	66	14	40	55	35	61
Hexan-1-ol	349	866	242	446	294	496	226	526
(E)-Hex-3-en-1-ol	17	19	17	13	7	15	7	22
(Z)-Hex-3-en-1-ol	756	696	601	424	311	314	612	683
(E)-Hex-2-en-1-ol	221	1,388	157	266	367	870	204	498

K Koroneiki, *C* Coratina, *P* Picual, *A* Arbequina, *T* Tsunnati, *Cb* Cima di Bitonto, *M* Moraiolo, *F* Frantoio

preferred to work with several series (e.g., hydrocarbons, sterols, fatty acids) (Ollivier et al. 2006; García-González et al. 2009a) together with sensory descriptors (Tena et al. 2007).

The relationship between cultivar and sensory quality has engaged researchers since Gutiérrez (1964) published an organoleptic method for the evaluation of VOO aromatic characteristics and Flath et al. (1973) described a method for the quantification of volatile compounds in VOO. In further works carried out with both methodologies, Gutiérrez et al. (1974) introduced two parallel but alternative ways to study VOO sensory quality through either sensory descriptors or the chemical compounds responsible for flavor.

The sensory and chemical characteristics of most European monovarietal VOOs – Arbequina and Picual (Spain), Koroneiki and Tsunnati (Greece), and Moraiolo, Frantoio, Coratina, and Cima de Bitonto (Italy) – were studied covering different

stages of ripeness, extraction systems, and shelf life (Tables 12.4 and 12.5). A statistical sensory wheel (Fig. 8.7) was designed in order to reduce the large number of VOO descriptors to the most noteworthy (Aparicio and Morales 1995). The results were then applied to the individual characterization of cultivars (Aparicio et al. 1996a) by means of fuzzy logic profiles (Calvente and Aparicio 1995).

The same varieties were also characterized by volatile compounds quantified by dynamic headspace and thermal desorption cold trap injector GLC (Aparicio and Luna 2002). This information was used to build a consensus plot where volatile compounds explain basic perceptions of VOO flavor inside the statistical sensory wheel (Aparicio et al. 1996b; García-González et al. 2009a). The studies of the volatile composition of monovarietal oils has clearly benefited from the widespread use of solid-phase microextraction (SPME), a rapid and easy technique for the concentration of volatile compounds prior to chromatographic injections. Thus, many studies have been carried out on Spanish (García-González et al. 2010b), Greek (Kandylis et al. 2011), Italian (Kotti et al. 2011), Turkish (Kaftan and Elmaci 2011), and Tunisian (Tena et al. 2007; Issaouia et al. 2010; Dabbou et al. 2010) varieties cultivated in different geographical locations.

These cultivars have also been analyzed by nonvolatile compounds. Gandul-Rojas and Mínguez-Mosquera (1996) found that mono- and diesterified xanthophylls and pheophorbide were the pigments exclusive to the Arbequina cultivar when they analyzed the quantitative differences in the contents of chlorophylls and carotenoids of nine single Spanish VOOs. Aparicio-Ruiz et al. (2009) established differences and similarities in the pigment composition of Spanish varieties (see Chap. 7 for more information). Giuffrida et al. (2011) characterized the pigments of Italian cultivars, finding auroxanthin only in olive oils from the Umbria and Molise regions. Esti et al. (1996a) found that the total content of alcohols could be used to distinguish among varieties since, for example, it varies from 178 mg/kg for Frantoio to 62 mg/kg for Coratina. Uceda and Hermoso (1996) measured the total content of phenols and tocopherols of 27 varieties. They found that the major source of variation of these compounds is the variety, 79 % in the case of tocopherols but only 46 % in the case of phenols, whose interaction with the stage of ripeness is around 35 %. Nevertheless, the quantitative composition of phenols strongly depends on irrigation (Stefanoudaki et al. 2009) and pedoclimatic conditions (García-González et al. 2010a). The comparative analyses of the varieties Arbequina, Coratina, Koroneiki, and Picual – among the major cultivars – have allowed knowing their distinctive chemical properties (Aparicio et al. 1997):

- Arbequina has high concentrations of major sterols, phytol, and notably high amounts of linoleic acid and low amounts of oleic acid.
- Coratina has high concentrations of phenols, methyl sterols, and triterpenic alcohols but low amounts of major sterols and aliphatic alcohols.
- Picual has a low content of triterpenic alcohols except for 24-methylene cycloarthanol and a low content of linoleic acid.
- Koroneiki has high concentrations of erythrodiol, almost all aliphatic alcohols, and linolenic and arachidic fatty acids.

Table 12.6 Mean concentration of some fatty acids, phytol, erythrodiol, sums of sterols, alcohols and methyl sterols of various European olive oil varieties

Varieties	P	S	O	L	Ln	PH	ER	ST	MST	AA	TA
Arbequina	12.54	2.12	75.33	8.52	0.59	564.6	22.6	1,432.6	154.7	204.9	1247.8
Coratina	9.73	3.15	76.90	7.46	0.61	81.4	26.2	1,192.3	217.1	65.0	712.2
Cornicabra	8.36	2.59	78.66	7.04	0.64	237.2	45.5	1,519.3	152.4	181.0	715.4
Farga	9.41	2.44	71.72	8.61	0.54	97.0	43.1	1,551.6	177.1	360.8	841.0
Frantoio	9.53	2.91	78.20	7.36	0.64	192.3	26.2	1,325.3	245.3	177.2	872.3
Hojiblanca	8.97	3.29	75.21	9.51	0.71	44.9	29.6	1,946.2	260.5	175.8	1,146.5
Imperial	9.67	2.27	80.09	4.63	0.90	90.7	29.8	1,481.0	180.6	188.3	945.7
Koroneiki	13.27	2.04	71.90	8.83	0.71	147.2	44.9	1,965.2	-	249.3	593.8
Leccino	14.24	1.83	77.70	7.72	0.81	177.6	18.9	1,271.5	117.4	121.8	1,071.5
Lechín	10.51	1.42	70.83	13.89	1.14	108.1	26.6	1,806.9	327.4	135.9	734.7
Moraiolo	10.51	2.18	77.70	7.48	0.59	101.2	26.1	1,184.7	148.3	134.3	836.4
Morrut	7.57	2.04	73.03	13.73	0.11	113.4	47.2	1,463.7	120.3	135.8	639.8
Nevadillo	11.02	3.02	75.32	7.32	0.69	50.1	30.7	1,596.1	186.1	205.1	1,069.7
Picual	9.91	3.21	77.83	5.02	0.65	24.5	18.5	1,310.2	160.1	227.1	1,162.7
Redondilla	12.46	1.68	68.28	14.06	1.08	118.6	37.2	2,032.3	387.4	121.4	1,066.6
Sevillenca	11.13	1.65	66.49	17.06	1.04	215.0	32.1	2,002.3	300.3	89.3	998.2
Verdial[a]	9.01	2.23	66.72	8.38	0.83	228.7	69.1	1,606.3	337.4	162.4	878.3

Note: The contents of chemical compounds are given in milligrams per kilogram, except for fatty acids, which are given in percentages

P palmitic acid, *S* stearic acid, *O* oleic acid, *L* linoleic acid, *Ln* linolenic acid, *PH* phytol, *ER* erythrodiol, *ST* total content of 4-desmethylsterols, *MST* total content of 4-monomethylsterols, *AA* total content of aliphatic alcohols, *TA* total content of 4,4'-dimethylsterols

[a] Verdial de Huevar

Other cultivars have also been studied. VOOs *cv.* Verdial are characterized by high values of erythrodiol and phytol but also by other compounds such as, for example, 24-methylene-24-dihydro-lanosterol, citrostadienol, butyrospermol. VOOs *cv.* Hojiblanca are easily detected by high contents of hydrocarbon α-copaene (Aparicio and Alonso 1994; Guinda et al. 1996), while *cv.* Cornicabra is characterized by methyl sterols, cycloeucalenol, and 24-ethyllophenol. Table 12.6 shows the values for some chemical compounds of different European cultivars.

The total content of phenols (mg/kg) is a good marker for distinguishing between varieties at similar stages of ripeness. Thus, the concentration ranges at a medium maturity stage are 110–273 mg/kg for Frantoio, 67–228 mg/kg for Leccino, 78–199 mg/kg for Moraiolo, and 163–555 mg/kg for Coratina, although the upper limits for Frantoio and Leccino are much lower when samples from low altitudes are not taken into account (Cimato 1991).

The varieties Mignola, Orbetana, and Leccino have also been characterized according to their phenol content and oxidative stability (Servili et al. 2007). Thus, VOOs from

- *cv.* Mignola contained 706–900 mg/kg of total phenols, had a peroxide number between 5.8 and 7.4, and their oxidative stability ranged from 4.84 to 6.0 h;
- *cv.* Orbetana contained 419–745 mg/kg total phenols, had a peroxide number between 7.0 and 9.2, and an oxidative stability ranging from 3.28 to 4.9 h; and
- *cv.* Leccino contained 370–433 mg/K total phenols, had a peroxide number between 5.8 and 10.5, and an oxidative stability ranging from 2.4 to 3.82 h.

Esti et al. (1996a) studied fatty acids, sterols, and aliphatic alcohols of varietal VOOs (Gentile, Frantoio, Leccino, Coratina, and Cellina) produced in Molise. Ranalli et al. (2002) also checked the use of aliphatic alcohols as genuineness markers of cultivars, together with sterols and triterpene alcohols. Seven categories were characterized in this study (Leccino, Dritta, Caroleo, Coratina, Castiglionese, Carboncella, and Nebbio). Pigments also allow characterization of varieties. Thus, the content of violaxanthin, lutein, and total pigments are cultivar-dependent parameters (Aparicio-Ruiz et al. 2009). Koprivnjak and Conte (1996) used fatty acids and hydrocarbons (unsaturated and aliphatic) to compare the Croatian varieties Bianchera, Carbonazza, and Busa with the Italian Leccino. These authors found that low values of aliphatic hydrocarbon C_{24} characterize autochthonous Croatian varieties of cultivar, while high values of C_{25} and C_{35} do so for Italian Leccino.

This kind of characterization has been carried out using spectroscopic techniques such as nuclear magnetic resonance (Chap. 11). Thus, Vlahov (1996a) studied the stereospecific distribution of major fatty acids between the 1,3- and 2-positions of glycerol in oil samples *cv.* Leccino from different geographical origins (Tuscany, Abruzzo, Campania, Marche, and Lazio) by ^{13}C-NMR spectroscopy. Later, she applied ^{13}C NMR to quantify monoacylglycerols, DAGs, and TAGs of VOOs from different varieties of cultivar, Nebbio, Coratina, Leccino, Dritta, Caroleo, and Grossa di Cassano (Vlahov 1996b). Vlahov concluded that DAG contents were significantly lower in the Nebbio and Coratina varieties.

Furthermore, FT-Raman and infrared spectroscopy (Chap. 10) has also been used, although the most innovative application is the attempt to identify VOOs by DNA technology, although so far it has met with very limited success. Thus, Ben-Ayed et al. (2012) extracted DNA and analyzed microsatellite markers in a series of monovarietal olive oils from Tunisia, Spain, Italy, and Greece. Alleles with very slight differences in molecular weight or partially degraded, because they are usually extracted from olive oil, can be differentiated by capillary electrophoresis and might be used to differentiate olive oils by cultivar, although much more work must be carried out before determining the usefulness of the methodology.

12.5.5 Traceability of Virgin Olive Oils from Their Geographical Origins

Since ancient times, the characterization of geographical origin has been a perennial research issue. Thus, the characterization of the geographical origin of VOO has a

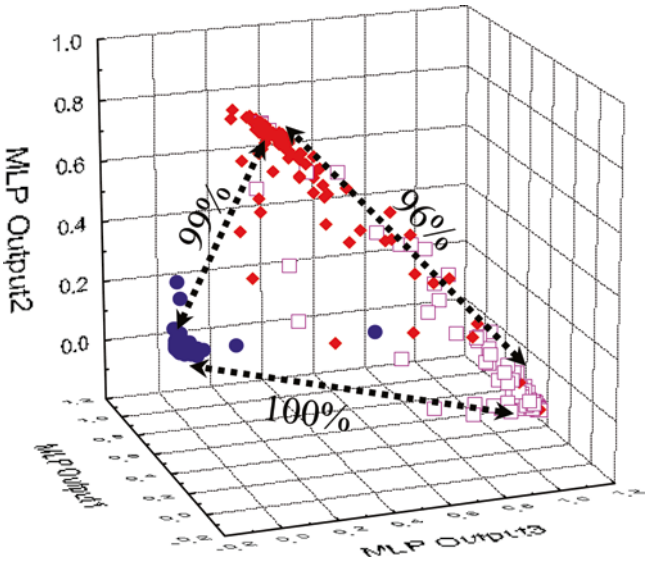

Fig. 12.11 Results of applying the multilayer perceptron (*MLP*) to Italian (*red color*), Portuguese (*blue color*) and Spanish (*pink color*) samples of the data set. The classification percentages between classes are shown on the plot

significant economic interest (Gerber 1995). The sensory and chemical characterization of the geographical origin of VOOs is needed not only to protect designations of origin, but also to delineate the best olive oil for some consumers. In addition, it also helps analysts to develop powerful tools against fraud.

This issue requires the establishment of strong relationships between chemical and sensory characteristics with the agronomic, climatic, and botanical aspects in such a way that it would be almost impossible for these relationships to occur in other regions. This means that the traceability of geographical origins should not be carried out with a few series of chemical compounds or a simply data manipulation. The study should be carried out with a large number of chemical compounds and data analyzed by statistical techniques or artificial intelligence algorithms. The series of chemical compounds and the type of mathematical procedures will depend on the objectives. Major components (fatty acids) can give only basic information or characterize clusters that are very different (Tsimidou and Karakostas 1993; Alonso and Aparicio 1993). If a proper characterization is claimed, many more minor compounds should be added to the study (García-González et al. 2009b).

Thus, the studies can be divided according to the chemical compounds used (minor or major chemical compounds) and the mathematical procedures applied (statistical or artificial intelligence). The methodologies for good characterization include many possible combinations, from the analysis of major compounds with

multivariate statistical procedures to the application of artificial intelligence algorithms to a great number of minor compounds.

A large panoply of multivariate data analyses and artificial intelligence algorithms has been used in recent years (Longobardi et al. 2012; Chiavaro et al. 2011; García-González et al. 2009b; Calvente and Aparicio 1995; Angerosa et al. 1996b). These mathematical procedures should not be applied as a philosopher's stone but always as specific tools for each problem and objective. However, the greatest problem concerns the distribution of the values for individual chemical and sensory parameters over the years. A high degree of data dispersion for some parameters over time may decrease the coefficients for correct classification, but it increases the certainty factor in the validation step. In fact, studies using information from only one crop can yield a high value of misclassification when they are verified with data from other crops.

The complexity of chemical information required to identify the geographical origin of olive oils also demands powerful mathematical tools, such as MLP. Thus, the geographical identification of VOOs must be addressed by a complete chemical profiling of olive oil samples (e.g., 64 compounds analyzed by GC and HPLC including fatty acids, sterols, hydrocarbons, and alcohols) and the design of artificial neural network (ANN) models for each of the levels of a proposed classification scheme, from country to protected designations of origin (García-González et al. 2009b, 2012). Figure 12.11 shows the results of classifying olive oil samples from Italy, Portugal, and Spain by means of fatty acid composition and a MLP designed for identifying samples from each country.

12.5.5.1 Mediterranean Basin

Eddid and Nickless (1987) found clear statistical differences between the fatty acid composition of olive oils produced in the Mediterranean countries Libya, Tunisia, Turkey, and Italy. Fatty acids, together with TAGs, were also used to distinguish VOOs from Italy, Greece, Morocco, Tunisia, and Spain (Gigliotti et al. 1993). In both studies, the content of linoleic and palmitic acids were greater in the African oils, thereby delineating the effect of latitude on the accumulation of linoleic acid at the expense of a lower content of oleic acid. This series has also been used to carry out a characterization of Italian, Portuguese, and Spanish VOOs by mean of statistics and an ES (Alonso and Aparicio 1993). With respect to the level of significance, oleic and linoleic acids are highly correlated with Italian oils, palmitic acid with Spanish oils, and linolenic acid with Portuguese oils.

12.5.5.2 Italian Virgin Olive Oils

From the viewpoint of characterization, Italy means not only the second world producer and first consumer of VOO (Chap. 2) but also the challenge of

characterizing olive grove zones with a large number of cultivars. Italy, in contrast to Spain, has its olive grove zones spread throughout the country, but perhaps without the intensive and extensive cultivation developed in Spain.

Fatty acids and TAGs have been widely used to identify geographical origins of Italian VOOs. Gigliotti et al. (1993) delineated a certain possibility of distinguishing oils by latitude – Northern (Garda, Liguria, Tuscany) versus southern (Puglia, Calabria, Sicily) – using fatty acid and TAG compositions, while Montedoro et al. (1995) were able to discriminate between four regions (Liguria, Umbria, Marche, Apulia). Alonso and Aparicio (1993) made further advances using the SEXIA ES. Eight Italian regions (Basilicata, Calabria, Liguria, Puglia, Sardinia, Sicily, Tuscany, Umbria) were initially clustered into three large groups (Northern, Southern, Sardinia) with correct classifications of between 77.4 % and 99 %. The ES has decision rules that are able to distinguish not only between producer countries but also inside them. These works established the basis for a characterization of Italian olive oils in relation to oils produced in other countries (Chiavaro et al. 2011; Kotti et al. 2011) and between particular producing regions like Sicily, Basilicata, and Liguria, among others (D'Imperio et al. 2007; Zunin et al. 2005; Drava et al. 1994).

Tuscan Olive Grove Zones

The Tuscan VOO has had a good reputation on the world market since ancient times, and hence, Tuscany has been, without doubt, the most studied of Italian regions on the basis of either chemical or sensory characterization. From the first studies, where only the fatty acid profile was used in the characterization, to the latest contributions, where ESs and sets of over 75 chemical compounds were used, many papers have been published. The papers cover the whole set of variables (e.g., varieties, geographical origin, extraction systems, climate) studied from different series of chemical compounds, sensory descriptors, and mathematical procedures.

Aparicio et al. (1994c) described the chemical compounds that could characterize the VOOs produced inside olive grove zones, which can, more or less, coincide with the provinces of Tuscany. This study analyzes the results in the light of geographical (e.g., mountains, valleys) and pedoclimatic information. For example, the valley zones of the province of Lucca, influenced by the proximity of the mountain range to the north, produce VOOs characterized by 24-methylene cycloarthanol and the lowest values of erythrodiol; in contrast, the chemical composition of VOOs produced in Arezzo is fairly homogeneous and its VOOs can be distinguished from the rest of Tuscany by stigmasterol concentrations.

12.5.5.3 Greek Virgin Olive Oils

Although Greece is the third producer country and its VOOs have gained a fully deserved reputation for high quality, fewer papers today are published characterizing Greek regions or their varieties in comparison with papers studying Italian

ones. Fatty acids, in combination with PCA, have been used to separate oils from the north and south, thus confirming once again the relationship between latitude of olive grove zones and olive oil fatty acid profile (Tsimidou and Karakostas 1993). TAG composition has confirmed the results obtained by fatty acid analysis, and the researchers were able to distinguish olive oils from Crete and the Peloponnese from those from Sterea Hellas, Corfu, and Evros. On the other hand, oils produced from different Greek Ionian islands were characterized according to free acidity, peroxide content, spectrophotometric parameters, chlorophyll content, phenols, sterols, and fatty acid and TAG compositions (Longobardi et al. 2012). Oils produced in southern Greece, such as Cretan oils, have received attention by researchers who characterized them in terms of phenols, seterols, and fatty acid content, among other chemical variables (Vekiari et al. 2010). Other variables used to study Greek oils include spectrophotometric parameters and volatile compounds (Longobardi et al. 2012; Poliarekou et al. 2011; Spyros and Dais 2009). However, no study has focused on compiling Greek VOO production with all chemical and agronomic information as described for Spanish oils (see the section "Andalusian Virgin Olive Oils").

12.5.5.4 Portuguese Virgin Olive Oils

Portugal is the fourth European producer, but Portuguese olive grove zones are few and far between. Forina et al. (1983) were the first who examined the fatty acid and sterol compositions of Portuguese olive oils, and they were able to distinguish two great zones (Douro Valley and southern Portugal). Alonso and Aparicio (1993) also distinguished VOOs from northern regions from oils produced in the south by multivariate procedures applied to fatty acids. These authors were able to differentiate the oils from three great zones – North (Minho, Tras-os-Montes, Beira Alta), mid-South (Algarve, Alentejo, Ribatejo), and West Coast (Douro, Beira, Litoral) – by applying possibility theory (Aparicio 1988). Later, Aparicio et al. (1994c) were able to obtain better results by applying an ES. They explained the results by, for instance, the geographical origin of samples. The values of palmitic and palmitoleic acids increase in olive oils as one goes from north to south; the lowest values belong to Minho and the highest to Algarve. The geographical longitude is characterized by stigmasterol, whose values decrease as one goes from the coast to the inland regions. VOOs from the central coastal region of Portugal are very different from those of the other areas. Oils from Tras-os-Montes constitute a fairly homogeneous unit extending to some parts of the north of Beira Alta, while the South produces oils chemically similar to Beira Baja. Ribatejo, which represents the midland, is affected by its neighbors, while oils from the South of Portugal are a group apart, except for VOOs from the Beja zone, which are rather different. Oils from the Northeast have also been characterized by tocopherols, sterols, tocotrienols, and fatty acids (Matos et al. 2007; Cunha et al. 2006). A characterization of olive oil from different regions in Portugal has been addressed with DNA markers (Martins-Lopes et al. 2008). The new super-intensive orchards, mostly based on cv. Arbequina, modify the

sensory characteristics and the composition linked to a particular region. Consequently, the conclusions of previous studies could be modified if they were run using the new agricultural practices.

12.5.5.5 Spanish Virgin Olive Oils

Spain is by far the major world producer and exporter of olive oil (Chap. 2). In Spain the most innovative extraction systems have been tested or designed, which has resulted in the concentration of olive oil production in about 500 cooperative societies. More than 1,000 samples were used by Aparicio and collaborators (Aparicio 1988; Aparicio and Alonso 1994; Aparicio et al. 1994c) to characterize the various Spanish VOOs and compare them with VOOs from other producing countries. Each sample was characterized by 65 chemical compounds, including fatty acids, methyl sterols, sterols, prenols, aliphatic and terpenic alcohols, and aliphatic and sesquiterpenic hydrocarbons (Aparicio et al. 1994c). In addition, the most representative samples were characterized by 85+ volatile compounds (Morales et al. 1995), sensory descriptors (Aparicio et al. 1992; 1996a, 1996b), and TAG composition (García-Pulido and Aparicio 1993). With such a large amount of information, Aparicio et al. (1991c) described the enormous possibilities of ESs in olive oil characterization through a practical example. The objective was to distinguish VOO of *cv.* Hojiblanca, produced in Málaga (a province of Andalusia), from all other VOOs, either from the same variety produced in its neighboring orchards or, for example, as far away as Italy (Liguria and Tuscany). The validation test was carried out with a test set of 26 samples that belonged to a different crop year; 15.8 % of the samples from outside the zone were classified inside, and 85.7 % of samples produced inside the zone were correctly classified.

A general study of the geographical origin of Spanish VOOs shows that coastal oils are mainly distinguished by the content of triterpenic alcohols, while sterols and fatty acids distinguish the oils from the inland provinces. When the characterization is inside a province or a particular orchard, then hydrocarbons are the most valuable compounds. The characterization of Spanish Protected Designation of Origin (PDO) was carried out using a whole set of chemical compounds (Aparicio et al. 1994c). As mentioned below, the application of ANNs to the same chemical data makes it possible to obtain a classification with optimum reliability (García-González et al. 2009b).

Andalusian Virgin Olive Oils

Andalusia produces more than a quarter of the total world production of olive oil, the Andalusian province of Jaén being the first world producer and exporter. Dozens of different varieties are cultivated in this region, although Picual represents more than 60 %, followed by Hojiblanca, Lechin, Nevadillo, Verdial, Arbequina, and others. Aparicio and Alonso (1994) used 340 samples to characterize the Andalusian

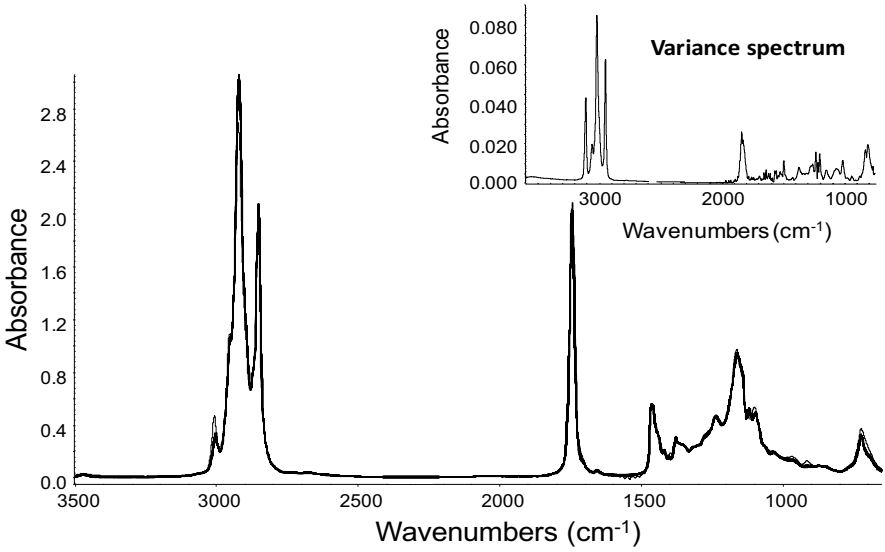

Fig. 12.12 Overlay of 16 FTIR spectra corresponding to different categories (virgin olive oil, refined olive oil, and olive-pomace oil) and different cultivars (Hojiblanca, Royal, Picual, Coratina, Arbequina, Frantoio, Nevado, Verdial, Morruda, Cornicabra, Manzanilla, Coratina, Koroneiki, Chemlali, Chami, Tsounati). The variance spectrum computed for all these spectra is shown as *insert*

provinces and olive grove zones. Some of the provinces were easily characterized because their VOOs are monovarietal, such as as Jaén (Picual), Malaga (Hojiblanca), and Huelva (Verdial and Manzanilla). The provinces of Jaén (which accounts for 60 % of Andalusian production) and Cordoba (a mosaic of different varieties and microclimates) were deeply, widely, and profusely studied using the SEXIA ES. Today there is enough chemical, sensory, and mathematical background information to defend their protected designations of origin against false copies or to predict the international markets where these oils would be well accepted by either regular or potential consumers. The province of Jaén, being 99 % monovarietal, has also been used to detect the influence of pedoclimate variables (e.g., climate, altitude, soils) on the chemical and sensory parameters of VOOs using a large number of samples instead of partial and subjective observations. In a recent work, the oils produced in three traditional regions, corresponding to PDO Estepa, PDO Montoro-Adamuz, and Campiña de Jaén, were characterized in terms of the chemical composition of major and minor compounds (García-González et al. 2012). This study was conducted to point out the distinctive properties of these oils in relation to oils produced in surrounding areas.

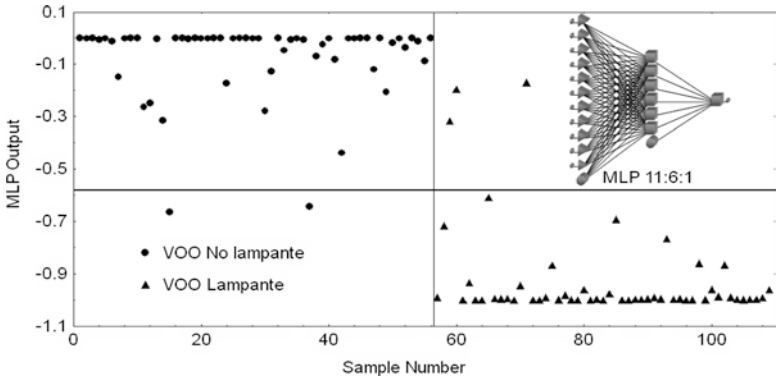

Fig. 12.13 Results of applying multilayer perceptron (MLP) to lampante and nonlampante virgin olive oils, and schematic of MLP designed for this purpose

12.5.6 Characterization of Olive Oil Categories

If there were no clear boundaries between characterization and authentication concepts, the paradigm would be the study of olive oil categories. Chromatographic techniques have been successfully used to determine the characteristics of each olive oil category (Chap. 16), although spectroscopic techniques have also been used with identical or even greater success. Since the infrared absorption spectra of several unsaturated fatty acids and methyl esters were collected in the early 1950s, the characterization of olive oil categories has been carried out with infrared (Lai et al. 1994) and by FT-Raman (Aparicio and Baeten 1998) spectroscopies. Lai et al. (1994) applied principal component analysis (PCA) and linear discriminant analysis (LDA) to investigate the potential of FT-MIR in distinguishing extra virgin from refined olive oils. Almost all the samples (12 out of 13) were correctly classified using a calibration set, and no error was obtained in an independent validation set. Figure 12.12 shows the FTIR bands of EVOO, refined olive oil, and olive-pomace oil from olives of different cultivars. Differences were found among these olive oil categories, as shown in the variance spectrum showing that FT spectroscopy is a powerful spectroscopic technique for olive oil characterization.

The characterization of quality categories has also been addressed with sensor systems and ANNs. Thus, MLP has been designed and trained to classify samples of VOOs between lampante and nonlampante oils by means of an array of metal oxide sensors, with the aim of making it a screening method of early detection of sensory defects (García-González and Aparicio 2003). Figure 12.13 shows the MLP outputs for each of the training samples that covered a wide range the median of defects.

12.6 Challenges and Future Trends

Instrumental advances have led to greater success in the development of traceability. However, geographical traceability is still in need of intense research to incorporate new "omic" procedures for better characterizations. The information obtained from these and other procedures should be compiled to build a large database that would make it possible to determine the geographical origin of the most representative VOOs around the world with fine certainty factors and avoid the in-vogue tree structure designs that lack mathematical support and might lead to more problems than solutions. Future work on geographical traceability will focus on building an olive oil map, where the most productive cultivars and all the approved PDOs will be characterized by chromatographic, spectroscopic, and isotopic techniques. The resulting databases, in conjunction with new procedures of classification and visualization techniques, would make it possible to evaluate the best combination of these techniques in VOO traceability.

References

Adler B, Schütze P, Will J (1993) Expert system for interpretation of x-ray diffraction spectra. Anal Chim Acta 271:287–291

Alba J (1996) Elaboración de Aceite de Olive Virgen. In: Barranco D, Fernández-Escobar D, Rallo L (eds) El cultivo del Olivo. Mundi-Prensa, Madrid, pp 511–537

Alonso V, Aparicio R (1993) Characterization of European virgin olive oils using fatty acids. Grasas Aceites 44:18–24

Anastasopoulos E, Kalogeropoulos N, Kaliora AC, Kountouri A, Andrikopoulos NK (2011) The influence of ripening and crop year on quality indices, polyphenols, terpenic acids, squalene, fatty acid profile, and sterols in virgin olive oil (Koroneiki cv.) produced by organic versus non-organic cultivation method. Int J Food Sci Technol 46:170–178

Angerosa F, Di Giovacchino L (1996) Natural antioxidants of virgin olive oil obtained by two and tri-phase centrifugal decanters. Grasas Aceites 47:247–254

Angerosa A, Giacinto LD, Basti C, Serraiocco A (1996a) Influenza della variabile "ambiente" sulla composizione degli oli vergini di oliva. Riv Ital Sostanze Grasse 73:461–467

Angerosa F, Di Giacinto L, Vito R, Cumitini S (1996b) Sensory evaluation of virgin olive oils by artificial neural network processing of dynamic head-space gas chromatography data. J Sci Food Agric 72:323–328

Aparicio R (1988) Characterization of foods by inexact rules: the SEXIA expert system. J Chemometr A 3:175–192

Aparicio R (1997) Internal report. Results of project FAIR-CT94-1967, 3 out of 4 years project. Instituto de la Grasa, Spain

Aparicio R (2000) Characterization: mathematical procedures for chemical analysis. In: Harwood J, Aparicio R (eds) Handbook on olive oil: analysis and properties, 1st edn. Aspen, Gaitherburg, pp 285–354

Aparicio R, Alonso V (1994) Characterization of virgin olive oils by SEXIA expert system. Prog Lipid Res 33:29–38

Aparicio R, Baeten V (1998) Fats and oils authentication by FT-Raman. OCL 5(3):14–16

Aparicio R, Luna G (2002) Characterisation of monovarietal virgin olive oils. Eur J Lipid Sci Technol 104:614–627

Aparicio R, Morales MT (1995) Sensory wheels: a statistical technique for comparing QDA panels. Application to virgin olive oil. J Sci Food Agric 67:247–257
Aparicio R, Morales MT (1998) Characterization of olive ripeness by green aroma compounds of virgin olive oil. J Agric Food Chem 46:1116–1122
Aparicio R, Ferreiro L, Leardi R, Forin M (1991) An approach for building decision rules by chemometric analysis. An application on olive oil. Chemo Intell Lab Syst 10:349–358
Aparicio R, Gutiérrez F, Rodríguez-Morales J (1992) Relationship between flavour descriptors and overall grading of analytical panels for virgin olive oil. J Sci Food Agric 58:555–562
Aparicio R, Ferreiro L, Alonso V (1994a) Effect of climate on the chemical composition of virgin olive oil. Anal Chim Acta 292:235–241
Aparicio R, Calvente JJ, Alonso MV, Morales MT (1994b) Good control practices underlined by an on-line fuzzy control database. Grasas Aceites 45:75–81
Aparicio R, Alonso V, Morales MT (1994c) Detailed and exhaustive study of the authentication of European virgin olive oils by SEXIA expert system. Grasas Aceites 45:241–252
Aparicio R, Calvente JJ, Morales MT (1996a) Sensory authentication of European extra-virgin olive oil varieties by mathematical procedures. J Sci Food Agric 72:435–447
Aparicio R, Morales MT, Alonso V (1996b) Relationship between volatile compounds and sensory attributes by statistical sensory wheel. J Am Oil Chem Soc 73:1253–1264
Aparicio R, Morales MT, Alonso V (1997) Authentication of European virgin olive oils by their chemical compounds sensory attributes and consumers attitudes. J Agric Food Chem 45:1076–1083
Aparicio-Ruiz R, Gandul-Rojas B, Roca M (2009) Pigment profile in non-Spanish olive varieties (*Olea europaea* L. Var. Coratina, Frantoio, and Koroneiki). J Agric Food Chem 57:10831–10836
Arnold GM, Williams AA (1986) The use of generalised procruters techniques in sensory analysis. In: Pigott JR (ed) Statistical procedures in food research. Elsevier, London, pp 233–254
Armstrong RD, Beck PO (1990) An algorithm to assist in the Identification of Multiple. Multivariate outliers when using a Least Absolute Value Criterion. In: Lawrence KD, Arthur JL (eds) Robust Regression: Analysis and Applications. Marcel Dekker, New York, pp 89–104
Baccouri O, Bendini A, Cerretani L, Guerfel M, Baccouri B et al (2008) Comparative study on volatile compounds from Tunisian and Sicilian monovarietal virgin olive oils. Food Chem 111:322–328
Baeten V, Meurens M, Morales MT, Aparicio R (1996) Detection of virgin olive oil adulteration by Fourier Transform Raman Spectroscopy. J Agric Food Chem 44:2225–2230
Barlow HB (1989) Unsupervised learning. Neural Comput 1:295–311
Bartolini G, Prevost G, Messeri C, Carignani G (1998) Olive germplasm. In: Manini UG (ed) Cultivars and world-wide collections. FAO, Roma
Ben-Ayed R, Grati-Kamoun N, Sans-Grout C, Moreau F, Rebai A (2012) Characterization and authenticity of virgin olive oil (*Olea europaea* L.) cultivars by microsatellite markers. Eur J Food Res Technol 234:263–271
Betteridge D et al (1988) Development of an expert system for the selection of sample points for moisture analysis. Anal Chem 60:1534–1539
Bishop C (1995) Neural networks for pattern recognition. Oxford University Press, Oxford
Blekas G, Tsimidou M, Boskou D (1995) Contribution of α-tocoferol to olive oil stability. Food Chem 52:289–294
Calvente JJ, Aparicio R (1995) A fuzzy filter for removing interferences among membership grade functions. an application to pre-treatment of data in olive oil authentication. Anal Chim Acta 312:281–294
Capasso R, Cristinzio G, Evidente A, Scognamiglio F (1992) Isolation, spectroscopy and selective phytotoxic effects of polyphenols from vegetable waste waters. Phytochemistry 31: 4125–4128
Ceci LN, Carelli AA (2007) Characterization of monovarietal Argentinian olive oils from new productive zones. J Am Oil Chem Soc 84:1125–1136
Chiavaro E, Cerretani L, Di Matteo A, Barnaba C, Bendini A et al (2011) Application of a multidisciplinary approach for the evaluation of traceability of extra virgin olive oil. Eur J Lipid Sci Technol 113:1509–1519

Christopoulou E, Lazaraki M, Alexiou F, Synouri S, Fragisco E (1996) Influence of certain factors on the composition of olive-pomace oils. Part II. Sterols, triterpenic diacohols and aliphatic alcohols. Riv Ital Sostanze Grasse 73:201–211

Cimato A (1991) La caratterizazzione dell'olio Extravergine "Tipico Toscano. IIº Anno di Ricerca. Firenze, Italy: Croevott (Bandechi, Pontedera), pp 1–158

Cresti M, Ciampolini F, Tattini M, Cimato A (1994) Effect of salinity on productivity and oil quality of olive (*Olea Europea L.*) plants. Adv Hortic Sci 8:211–214

Criado MN, Motilva MJ, Goñi M, Romero MP (2007) Comparative study of the effect of the maturation process of the olive fruit on the chlorophyll and carotenoid fractions of drupes and virgin oils from Arbequina and Farga cultivars. Food Chem 100:748–755

Cunha SC, Amaral JS, Fernandes JO, Oliveira MBPP (2006) Quantification of tocopherols and tocotrienols in Portuguese olive oils using HPLC with three different detection systems. J Agric Food Chem 54:3351–3356

D'Imperio M, Dugo G, Alfa M, Mannina L, Segre AL (2007) Statistical analysis on Sicilian olive oils. Food Chem 102:956–965

Dabbou S, Issaoui M, Servili M, Tatichini A, Sifi S, Motedoro GF, Hammami M (2010) Effect of pedoclimatic conditions on the chemical composition of the Sigoise olive cultivar. Chem Biodivers 7(4):898–908

Dag A, Ben-David E, Kerem Z, Ben-Gal A, Erel R et al (2009) Olive oil composition as a function of nitrogen, phosphorus and potassium plant nutrition. J Sci Food Agric 89:1871–1878

Di Giovacchino L, Costantini N, Serraiocco A, Surricchio G, Basti C (2001) Natural antioxidants and volatile compounds of virgin olive oils obtained by two or three-phases centrifugal decanters. Eur J Lipid Sci Technol 103:279–285

Drava G, Forma M, Lanteri S, Lupoli M (1994) Development of the chemical model of a typical food product: olive oil from an Italian region (Basilicata). J Sci Food Agric 65:21–30

Eddid O, Nickless G (1987) Elucidation of olive oil classification by chemometrics. Analyst 112:391–395

Esti M, Cinquanta L, Carrone A, Trivisonno MC, La Notte E et al (1996) Caratteristiche qualitative e compositive di oli vergini di oliva prodotti in Molise. Riv Ital Sostanze Grasse 73:101–106

European Communities (EC) (1995) Official Journal of the Commission of the European Communities, Regulation nº 656/95, L260, 29 Mar 1995

Fernández-Escobar R, Beltrán G, Sánchez-Zamora MA, García-Novelo J, Aguilera MP et al (2006) Olive oil quality decreases with nitrogen over-fertilization. HortScience 41:215–219

Flath R, Forrey RR, Guadagni DG (1973) Aroma components of olive oil. J Agric Food Chem 21:948–952

Forina M, Armanino C, Lanteri S, Calcagno C, Tiscornia E (1983) Valutazione delle caratteristiche chimiche dell'olio di oliva in funzione dell'annata di produzione mediante metodi di classificazione multivariati. Riv Ital Sostanze Grasse 60:607–613

Frega N, Lercker G (1986) Componenti lipidici minori della drupa di olivo in diversi stadi di maturazione. Riv Ital Sostanze Grasse 63:393–398

Gandul-Rojas B, Mínguez-Mosquera MI (1996) Chlorophyll and carotenoid composition in virgin olive oils from various Spanish olive varieties. J Sci Food Agric 72:31–39

García-González DL, Aparicio R (2003) Virgin olive oil quality classification combining neural network and MOS sensors. J Agric Food Chem 51:3515–3519

García-González DL, Aparicio R (2010) Research in olive oil: challenges for the near future. J Agric Food Chem 58:12569–12577

García-González DL, Aparicio-Ruiz R, Aparicio R (2009a) Olive oil. In: Moreau RA, Kamal-Eldin A (eds) Gourmet and health-promoting oils. AOCS Press, Champaign, pp 33–72

García-González DL, Luna G, Morales MT, Aparicio R (2009b) Stepwise geographical traceability of virgin olive oils by chemical profiles using artificial neural network models. Eur J Lipid Sci Technol 111:1003–1013

García-González DL, Romero N, Aparicio R (2010a) Comparative study of virgin olive oil quality from single varieties cultivated in Chile and Spain. J Agric Food Chem 58:12899–12905

García-González DL, Tena N, Aparicio R (2010b) Quality characterization of the new virgin olive oil var. Sikitita by phenols and volatile compounds. J Agric Food Chem 58:8357–8364
García-González DL, Tena N, Aparicio R (2012) Describing the chemical singularity of the Spanish protected designations of origin for virgin olive oils in relation to oils from neighbouring areas. Grasas Aceites 63:26–34
García-Pulido J, Aparicio R (1993) Triacylglycerol determination based on fatty acid composition using chemometrics. Anal Chim Acta 271:293–298
Gardner JW, Hines EL, Wilkinson M (1990) Application of artificial neural networks to an electronic olfactory system. Meat Sci Technol 1:446–451
Gerber D (1995) The Christian Rosi mill at Mouries. Olive oil: the little orchard. Process 1102:20–21 (in French)
Gigliotti C, Daghetta A, Sidoli A (1993) Indagine conoscitiva sul contenuto trigliceridico di oli extra vergini di oliva di varia provenienza. Riv Ital Sostanze Grasse 70:483–489
Gimeno E, Castellote AI, Lamuela-Raventós RM, De la Torre MC, López-Sabater MC (2002) The effects of harvest and extraction methods on the antioxidant content (phenolics, α-tocopherol, and β-carotene) in virgin olive oil. Food Chem 78:207–211
Giuffrida D, Salvo F, Salvo A, Cossignani L, Dugo G (2011) Pigments profile in monovarietal virgin olive oils from various Italian olive varieties. Food Chem 124:1119–1123
Gómez-Rico A, Salvador MD, La Greca M, Fregapane G (2006) Phenolic and volatile compounds of extra virgin olive oil (*Olea europaea* L. Cv. Cornicabra) with regard to fruit ripening and irrigation management. J Agric Food Chem 54:7130–7136
González-Andujar JL (2009) Expert system for pest, diseases and weeds identification in olive crops. Expert Syst Appl 36:3278–3283
Guinda A, Lanzon A, Albi T (1996) Differences in hydrocarbons of virgin olive oils obtained from several olive varieties. J Agric Food Chem 44:1723-1726
Gutiérrez R (1964) Application des Essais Organoleptiques a quelques Problems de l'huile d'olive. Inf Olèicoles Int 28:93–99
Gutiérrez R, Cabrera MJ, Gutiérrez RF, Olías JM (1974) Correlación entre métodos subjetivos y objetivos en la determinación de la calidad. Problemas del aceite de oliva. J Afinidad 31:335–350
Hebb DO (1949) The organization of behaviour. Wiley, New York
Huang Y (2009) Advances in artificial neural networks – methodological development and application. Algorithms 2:973–1007
Issaouia M, Flaminib G, Brahmia F, Dabboua S, Ben Hassinea K et al (2010) Effect of the growing area conditions on differentiation between Chemlali and Chétoui olive oils. Food Chem 119:220–225
Jacobsen T, Gunderson RW (1986) Applied cluster analysis. In: Piggot JR (ed) Statistical procedures in food research. Elsevier Applied Science, London, pp 361–408
Jancini G, Fedeli N (1972) Acidi triterpenici dell'olio di oliva. Rc Ist Lomb Sci Lett 106:446–451
Jiménez-Márquez A, Hermoso Fernández M, Uceda OM (1995) Elaboración del aceite de oliva virgen mediante sistema continuo en dos fases. Influencia de diferentes variables del proceso en algunos parámetros relacionados con la calidad del aceite. Grasas Aceites 46:299–303
Kaftan A, Elmaci Y (2011) Aroma characterization of virgin olive oil from two Turkish olive varieties by SPME/GC/MS. Int J Food Prop 14:1160–1169
Kalua CM, Allen MS, Bedgood DR Jr, Bishop AG, Prenzler PD (2005) Discrimination of olive oils and fruits into cultivars and maturity stages based on phenolic and volatile compounds. J Agric Food Chem 53:8054–8062
Kalua CM, Bedgood DR Jr, Bishop AG, Prenzler PD (2006) Changes in volatile and phenolic compounds with malaxation time and temperature during virgin olive oil production. J Agric Food Chem 54:7641–7651
Kandylis P, Vekiari AS, Kanellaki M, Grati Kamoun N, Msallem M, Kourkoutas Y (2011) Comparative study of extra virgin olive oil flavor profile of Koroneiki variety (*Olea europaea*

var. *Microcarpa alba*) cultivated in Greece and Tunisia during one period of harvesting. LWT- Food Sci Technol 44:1333–1341
Koprivnjak O, Conte LS (1996) Caratteristiche della frazione idrocarburica e composizione degli acidi grassi degli oli d'oliva vergini provenienti dalla zona di Pola (Croazia). Riv Ital Sostanze Grasse 73:317–320
Kotti F, Cerretani L, Gargouri M, Chiavaro E, Bendini A (2011) Evaluation of the volatile fraction of commercial virgin olive oils from Tunisia and Italy: relation with olfactory attributes. J Food Biochem 35:681–698
Lai YW, Kemsley EK, Wilson RH (1994) Potential of Fourier transform infrared spectroscopy for the authentication of vegetable oils. J Agric Food Chem 42:1154–1159
Lanzón A, Albi T, Gracián J (1986) Influencia del sistema de extracción en algunos componentes del aceite de oliva. Primeros ensayos. Grasas Aceites 37:254–258
Lazzerini B, Maggiore A (1998) Linguistic fuzzy classification of olfactive signals. IEEE international conference on fuzzy systems proceedings. In: IEEE world congress on computational intelligence (CAT No. 98CH36228), IEEE Service Center, Piscataway NJ, 2:1518–1523
Lazzez A, Perri E, Caravita MA, Khlif M, Cossentini M (2008) Influence of olive maturity stage and geographical origin on some minor components in virgin olive oil of the chemlali variety. J Agric Food Chem 56:982–988
Lohninger H, Stancl F (1992) Comparing the performance of neural networks to well-established methods of multivariate data analysis: the classification of mass spectral data. Fresen J Anal Chem 344:186–189
Longobardi F, Ventrella A, Casiello G, Sacco D, Tasioula-Margari M et al (2012) Characterisation of the geographical origin of Western Greek virgin olive oils based on instrumental and multivariate statistical analysis. Food Chem 133:169–175
Mardia KV (1972) Statistics of directional data. Academic, New York
Mariani C, Fedeli E, Grob K, Artho A (1991) Indagine sulle variazioni dei componenti minori liberi de esterificati di oli ottenuti da olive in funzione della maturazione e dello stoccaggio. Riv Ital Sostanze Grasse 68:179–186
Martel J, Alba J (1981) Influencia del metodo de obtencion de aceites de oliva por centrifugacion de pastas sobre su contenido en alcoholes grasos superiores. Grasas Aceites 32:233–237
Martens H, Næs T (1989) Multivariate calibration. Wiley, Chichester, pp 237–266
Martins-Lopes P, Gomes S, Santos E, Guedes-Pinto H (2008) DNA Markers for Portuguese Olive Oil Fingerprinting. J Agric Food Chem 56:11786–11791
Matos LC, Cunha SC, Amaral JS, Pereira JA, Andrade PB et al (2007) Chemometric characterization of three varietal olive oils (*cv*. Cobrançosa, Madural and Verdeal Transmontana) extracted from olives with different maturation indices. Food Chem 102:406–414
McCulloch WS, Pitts W (1943) A logical calculus of ideas immanent in nervous activity. Bull Math Biophys 5:115–133
Montedoro G et al (1993) I potenziali modelli che definiscono la tipicita degli oli extra vergini di oliva. Ind Aliment 32:618–631
Montedoro G et al (1995) Characterization of some Italian virgin olive oils in relation to origin area. Riv Ital Sostanze Grasse 72:403–413
Morales MT, Aparicio R (1999) Effect of the extraction conditions on virgin olive oil sensory quality. J Am Oil Chem Soc 76:295–300
Morales MT, Alonso MV, Rios JJ, Aparicio R (1995) Virgin olive oil aroma: relationship between volatile compounds and sensory attributes by chemometrics. J Agric Food Chem 43:2925–2931
Morales MT, Aparicio R, Calvente JJ (1996) Influence of olive ripeness on the concentration of green aroma compounds in virgin olive oil. Flavour Fragr J 11:171–178
Morales MT, Angerosa F, Aparicio R (1999) Effect of the extraction conditions of virgin olive oil on the lipoxygenase cascade: chemical and sensory implications. Grasas Aceites 50:114–121
Moussa YM, Gerosopoulos D, Metzidakis I, Kiritsakis A (1996) Effect of altitude on fruit and oil quality characteristics of "Mastoides" olives. J Sci Food Agric 71:345–350
Nergiz C, Ünal K (1990) The effect of extraction systems on triterpene alcohols and squalene content of virgin olive oil. Grasas Aceites 41:117–121

Nieto LM, Hodaifa G, Peña JLL (2010) Changes in phenolic compounds and Rancimat stability of olive oils from varieties of olives at different stages of ripeness. J Sci Food Agric 90:2393–2398

Oja E (1989) A simplified neuron model as a principal component analyzer. J Math Biol 15: 267–273

Ollivier D, Artaud J, Pinatel C, Durbec JP, Guéreère M (2006) Differentiation of French virgin olive oil RDOs by sensory characteristics, fatty acid and triacylglycerol compositions and chemometrics. Food Chem 97:382–393

Papadopoulos G, Boskou D (1991) Antioxidant effect of natural phenols on olive oil. J Am Oil Chem Soc 68:669–671

Paz Aguilera M, Beltran G, Ortega D, Fernández A, Jiménez A et al (2005) Characterisation of virgin olive oil of Italian olive cultivars: Frantoio and Leccino grown in Andalusia. Food Chem 89:387–391

Pfaffenberger RC, Dielman TE (1990) A comparison of regression estimators when both multicollinearity and outliers are present. In: Lawrence KD, Arthur JL (eds) Robust regression: analysis and applications. Marcel Dekker, New York, pp 243–270

Piggot JR, Sharman K (1986) Method to aid interpretation of multidimensional data. In: Pigott JR (ed) Statistical procedures in food research. Elsevier Applied Science, London, pp 181–192

Pouliarekou E, Badeka A, Tasioula-Margari M, Kontakos S, Longobardi F et al (2011) Characterization and classification of Western Greek olive oils according to cultivar and geographical origin based on volatile compounds. J Chromtogr A 1218:7534–7542

Ranalli A, Angerosa F (1996) Integral centrifuges for olive oil extraction. The qualitative characteristics of products. Am Oil Chem Soc 73:417–422

Ranalli A, Pollastri L, Contento S, Loreto GD, Lannucci E et al (2002) Sterol and alcohol components of seed, pulp and whole olive fruit oils. Their use to characterise olive fruit variety by multivariates. J Agric Food Chem 82:854–859

Remesal-Rodríguez J (1997) Heeresversongung und die wirtschaflichen beziehungen der Baetica und Germanien. Konrad Theiss Verlag, Stuttgart, pp 1–20

Salas J et al (1997) Influencia del riego sobre la composicion y caracteristicas organolepticas del aceite de oliva. Grasas Aceites 48:74–82

Salvador MD, Aranda F, Fregapane G (2001) Influence of fruit ripening on 'Cornicabra' virgin olive oil quality: a study of four successive crop seasons. Food Chem 73:45–53

Sanger TD (1989) Optimal unsupervised learning in a single-layer linear feedforward neural network. Neural Netw 2:359–473

Schierle C, Otto M (1992) Comparison of a neural network with multiple linear regression for quantittative analysis in ICP-atomic emission spectroscopy. Fresen J Anal Chem 344:190–194

Schiffman S, Reynolds ML, Young FW (1981) Introduction to multidimensional scaling. Theory, methods and applications. Academic, Orlando, pp 3–85

Servili M, Esposto S, Lodolini E, Selvaggini R, Taticchi A et al (2007) Irrigation effects on quality, phenolic composition, and selected volatiles of virgin olive oils cv. leccino. J Agric Food Chem 55:6609–6618

Singh S, Hines EL, Gardner JW (1996) Fuzzy Neural Computing of Coffee and Tainted Water Data from an Electronic Nose. Sensors and Actuators 30:190–195

Spyros A, Dais P (2009) 31P NMR spectroscopy in food analysis. Prog Nucl Magn Reson Spectrosc 54:195–207

Stefanoudaki E, Kotsifaki F, Koutsaftakis A (2000) Sensory and chemical profiles of three European olive varieties (*Olea europea* L); an approach for the characterisation and authentication of the extracted oils. J Sci Food Agric 80:381–389

Stefanoudaki E, Williams M, Chartzoulakis K, Harwood J (2009) Olive oil qualitative parameters after orchard irrigation with saline water. J Agric Food Chem 57:1421–1425

Stefanoudaki E, Koutsaftakis A, Harwood JL (2011) Influence of malaxation conditions on characteristic qualities of olive oil. Food Chem 127:1481–1486

Chapter 13
Olive Oil Oxidation

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Contents

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties*,
DOI 10.1007/978-1-4614-7777-8_13, © Springer Science+Business Media New York 2013

13.1 Introduction

Lipids are the structural components of all living cells and play a number of critical roles. They have an important role in the metabolism of cells by providing a source of energy and reserve storage materials and a pool of essential fatty acids. Any healthy diet should contain an adequate amount of lipids that can be obtained from a variety of foods (WHO 2003).

Food lipids are usually complex mixtures that can be affected by external variables rendering changes to their chemical composition that can be associated with a loss of sensory and nutritional quality. The main processes leading to a deterioration of lipids are hydrolytic rancidity or lipolysis and oxidative rancidity or oxidation. Rancid fat derives from a wide variety of chemical substances. Human taste buds are highly sensitive to some compounds such as lactones and free fatty acids, so only minute amounts of these compounds need to be produced to spoil the taste of a food (Ho and Shahidi 2005). Although lipolysis, which is caused by the release of free fatty acids (FFAs) from glycerides, is extremely important in determining how a product tastes, it is unlikely to be of any nutritional significance because fats are in any case enzymatically hydrolyzed in the small bowel before they are absorbed. In some cases hydrolytic rancidity is regarded as desirable. However, oxidation leads to the formation of both unpalatable and toxic compounds and is thus nutritionally undesirable (Sanders 1983; Matthaeus 2010).

Lipid oxidation has been recognized as the major problem affecting edible oils, as it is the cause of important deteriorative changes in their chemical, sensory, and nutritional properties (Velasco and Dobarganes 2002).

Autoxidation and photooxygenation proceed due to the presence of oxygen in air and are virtually inevitable. Understanding lipid oxidation has been impeded by the fact that lipids are usually complex mixtures containing minor components that may catalyze or inhibit oxidation and because primary oxidation products are labile and easily convert into secondary products (Knothe et al. 2007).

As lipids oxidize, they may form hydroperoxides, which are susceptible to further oxidation or decomposition into secondary reaction products such as aldehydes, ketones, acids, and alcohols. In many cases, these compounds adversely affect flavor, aroma, taste, nutritional value, and overall quality (Vercellotti et al. 1992; Matthaeus 2010). Many catalytic systems can oxidize lipids. Among these are light, temperature, enzymes, metals, metalloproteins, and microorganisms. Most of these reactions involve some type of free radical or oxygen species. The oxidation may be

produced either in the dark (autoxidation) or in the presence of light (photooxidation), which differ in their oxidation pathway due to the action of external variables (Velasco and Dobarganes 2002).

Virgin olive oil (VOO), one of the few oils being consumed without any chemical treatment, has high resistance to oxidative deterioration due to both a fatty acid composition characterized by a high monounsaturated-to-polyunsaturated fatty acid ratio and a pool of minor compounds with high antioxidant activity composed mainly of polyphenols and tocopherols. Most of these minor compounds are removed or drastically reduced during the refining process and, consequently, are present in much lower amounts in edible refined oils than in virgin oils. However, even if VOO generally has a high resistance to oxidation, some minor compounds that are also eliminated during refining, i.e., FFAs and photosensitizers, are prooxidants and consequently will contribute to a high variability in the stability of different VOOs (Velasco and Dobarganes 2002).

Olive oil lipolysis usually begins while the oil is in the fruit, while oxidation is mainly produced during the oil extraction process and storage (Kiritsakis 1990; Velasco and Dobarganes 2002). Postharvest and storage effects promote gradual lipid oxidation with decreases in shelf life stability due to continuing organic chemical processes set up by the initial peroxidations (Heath and Reineccius 1986). The effects of postharvest storage and processing of the raw commodities on lipid oxidation are of great interest in the case of olive oil. Olive oil is oxidized when it comes into contact with oxygen, although certain substances retarding oxidation (antioxidants) are present in plant tissue.

The final composition of VOOs is the result of a high number of variables affecting all stages from oil formation in the olive tree up to the status of the oil at consumption. Some of these variables have important effects on the concentration of compounds modifying the stability toward oxidation, and they can be divided as follows (Velasco and Dobarganes 2002):

1. Variables acting before oil extraction: numerous factors such as, for example, olive variety, environmental, climatic, soil and cultivation conditions, tree age, olive ripeness, and olive health are involved in the composition differences in VOO during its formation in the fruit.
2. Variables acting during oil extraction: including the influence of the various stages of the process such as milling, olive paste preparation, oil extraction system, and filtering on the quality and stability of VOOs.
3. Variables acting after oil extraction during storage: once the olive oil has been extracted, oxidative deterioration can be influenced by external variables related to oil storage and retail packing, among which oxygen availability, temperature, light, and possible metal contamination during storage stand out.

An important effect on the status of the oil at consumption can also be attributed to the culinary processes that usually contribute to the thermal degradation of the oils, with frying being one of the most common processes. In addition to many other uses, olive oil is considered to be excellent for applications involving high temperatures (Casal et al. 2010) since it fulfills all the fatty acid criteria of the stable healthful frying oils (Kochhar 2000) with the added advantage of its relatively low melting point.

Several parameters related to olive oil deterioration are nowadays included in official regulations with regard to olive oil and olive-pomace oils (IOC 1996a; EC 1997, 2007, 2008, 2011). They include determination of peroxide value (PV), ultraviolet absorbance, sensory analysis, and free acidity as quality indicators of these kinds of oils. Tables 16.6, 16.7 and 16.8 show the established values related to oxidation. They are currently used to establish the different oil categories in EC regulations (EC 2011).

13.2 Oxidative Deterioration of Olive Oil

VOO is considered to be resistant to oxidative degradation due to the low content of polyunsaturated fatty acids (PUFAs). It is characterized by a high monounsaturated-to-polyunsaturated fatty acid ratio and by the presence of natural antioxidant minor components such as α-tocopherol and phenolic compounds: hydroxytyrosol, tyrosol, caffeic acid, and others (Aparicio et al. 1999; Boskou et al. 2005). The presence of linoleic and small amounts of linolenic acids makes olive oil susceptible to oxidation, similar to other vegetable oils. Most of the undesirable changes in olive oil can be attributed to the reactions caused by oxidative degradation. The rate of these reactions is affected by many factors, and some of them will be discussed later in detail. The presence of oxygen in air is the main factor causing oil quality deterioration. Oxidative degradation in olive oil is the most important cause of negative changes and happens by both enzymatic and chemical reaction pathways.

13.2.1 Enzymatic Oxidative Deterioration

Degradation of lipids in plant tissues occurs by a process often called the PUFA cascade (Feussner and Wasternack 2002; Wang and Hammond 2010). Typically, different enzymes are involved in the oxidative decomposition of unsaturated fatty acids (Fig. 13.1). The sequence begins with the hydrolysis of various acylglycerides by lipases, lipolytic acyl hydrolases, and phospholipases, and free PUFAs are released. Then specific lipoxygenases convert unsaturated fatty acids into two main hydroperoxides, namely 9 and 13 isomers, which have poor stability. In the last step of the cascade, lyases, isomerases, and dehydrogenases degrade hydroperoxides into a variety of volatile and nonvolatile products. The flavor components formed, such as aldehydes, ketones, and alcohols, are usually directly responsible for off-flavors in oils (Fig. 13.1). VOO is unique because the same enzymatic processes are also responsible for the formation of its genuine and appreciated flavor. In olives the formation of 13-hydroperoxides from linoleic and linolenic acids is promoted, leading to the formation of C_6 aldehydes and alcohols, which are the main contributors to VOO sensory perception (Morales et al. 1996; Aparicio and Morales 1998). Free unsaturated fatty acids, particularly linoleic and linolenic in plants, are the preferred substrates

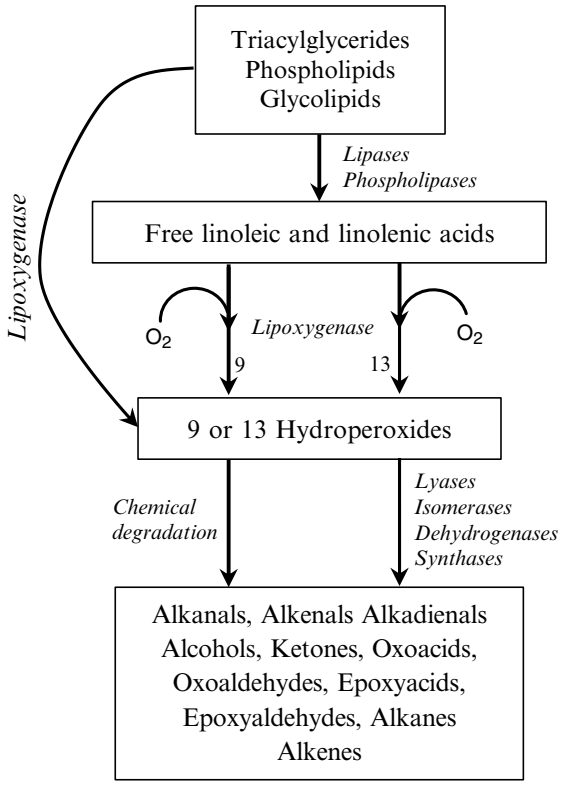

Fig. 13.1 Enzymatic oxidation of unsaturated fatty acids. *Numbers* with lipoxygenase represent enzymes producing specific isomers of hydroperoxide

oxidized by lipoxygenases. A certain type of lipoxygenases is able to catalyze the oxidation of unsaturated fatty acids when they are part of triglycerides (Christopher et al. 1972). For an efficient and high rate of oxidation, enzymes involved in oxidative degradation of PUFA require the constant presence of oxygen (Siedow 1991). Lack of oxygen does not terminate oxidative degradation since some lipoxygenases and other enzymes involved can oxidize fatty acids in the absence of oxygen, in this case forming free radicals instead of hydroperoxides (Feussner et al. 2001). The presence of trace amounts of hydroperoxides accelerates the oxidation of unsaturated fatty acids by lipoxygenase, particularly under anaerobic conditions (Siedow 1991).

Enzymes catalyzing PUFA degradation are very active during olive maturation. During olive harvest, storage, and processing, damage to the fruit occurs often, and the contents of the broken cells are exposed to oxygen-stimulating enzyme activity. During the production of VOO these pathways are activated at a relatively high rate, forming desirable volatile compounds, when olives are crushed and oil pressed. As can be seen

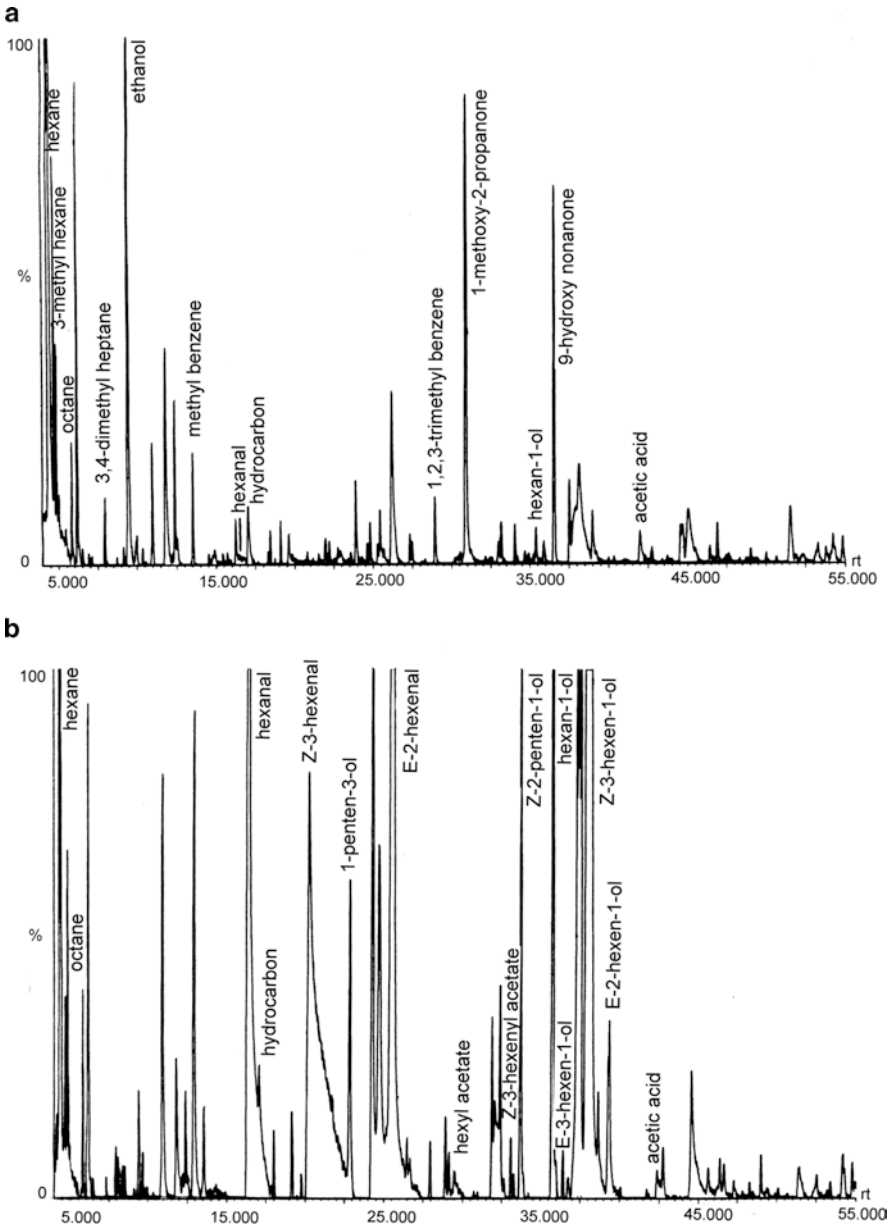

Fig. 13.2 Chromatograms showing volatile compounds of intact (**a**) and cut olive fruits (**b**). (Courtesy of SEXIA Group, Instituto de la Grasa, Sevilla, Spain)

in Fig. 13.2, the profile of volatile compounds of intact and cut olives is quite different, with the volatile compounds being produced by the action of lipoxygenase and other enzymes immediately after the olives are cut, thus exposing cell contents to oxygen. It is noteworthy that in intact fruits (Fig. 13.2a) significant amounts of hydrocarbons are

present. However, typical VOO volatile compounds, such as hexanal and hexan-1-ol, were observed at very low levels, indicating that they are formed by oxidative degradation of PUFAs when olive cells are damaged. The volatile profile of cut olives (Fig. 13.2b) showed a considerable change due to the production of volatile compounds (aldehydes and alcohols) offering characteristic green flavors, coming from oxidative degradation products formed from both linoleic and linolenic acids.

The breakdown enzymes can be native to the olive fruit/plant, whereas microbial enzymes cannot be excluded as an additional deterioration factor. The formation of small amounts of the hydroperoxides can have an accelerating effect on oxidation in the finished oil. Free radicals formed from the decomposition of hydroperoxides can escalate further oxidation, causing earlier-than-expected off-flavor formation and lowering oil storage stability.

Nonvolatile decomposition oxidation products, particularly hydroxylated and oxylated fatty acids, are responsible for the bitter taste of oils. In soybean, isomers of trihydroxyoctadecanoic acid were identified as being responsible for bitterness formation (Baur et al. 1977). These acids probably originated from the free radical decomposition of hydroperoxides of linoleic and linolenic acids degraded by nonenzymatic and enzymatic oxidation (Biermann et al. 1980).

13.2.2 Lipid Autoxidation

Over the past few decades many comprehensive reviews have been written on the mechanism of lipid oxidation (Frankel 1989, 2005). Many catalysts or initiators accelerate the oxidation of lipids. These include light, temperature, enzymes, metals, metalloproteins, pigments, and microorganisms. In these reactions free radicals or reactive oxygen species such as singlet oxygen are formed. The main substrates for these reactions are unsaturated fatty acids and oxygen. The free radical mechanism of lipid oxidation is usually divided into three stages: initiation, propagation, and termination (Ingold 1969). In the initiation stage free radicals are formed directly from fatty acids with the help of initiators such as temperature, light, other free radicals, and heavy metals (Fig. 13.3). As mentioned earlier, fatty acid oxidation can proceed by hydrogen abstraction, and the carbon-centered radical formed is then stabilized by spontaneous rearrangement to form conjugated dienes.

During the propagation stage, lipid radicals react with oxygen to form peroxyl radicals. Formed at this stage, peroxyl radicals react with another molecule of lipid, forming a lipid radical and a hydroperoxide (Fig. 13.3). This stage of lipid radical and hydroperoxide formation is autocatalytic and progresses at a fast rate. The hydroperoxides formed are not stable and decompose easily to produce a wide range of nonvolatile and volatile products. Among these products, radicals are also present that can initiate or stimulate the oxidation process (Fig. 13.4). The volatile and nonvolatile products formed can decompose and be further oxidized to produce the whole range of secondary products (Schieberle and Grosch 1981). With time the amount of PUFAs decreases, while the proportion of hydroperoxides increases (Fig. 13.4). However, the onset of off-flavor compounds is delayed

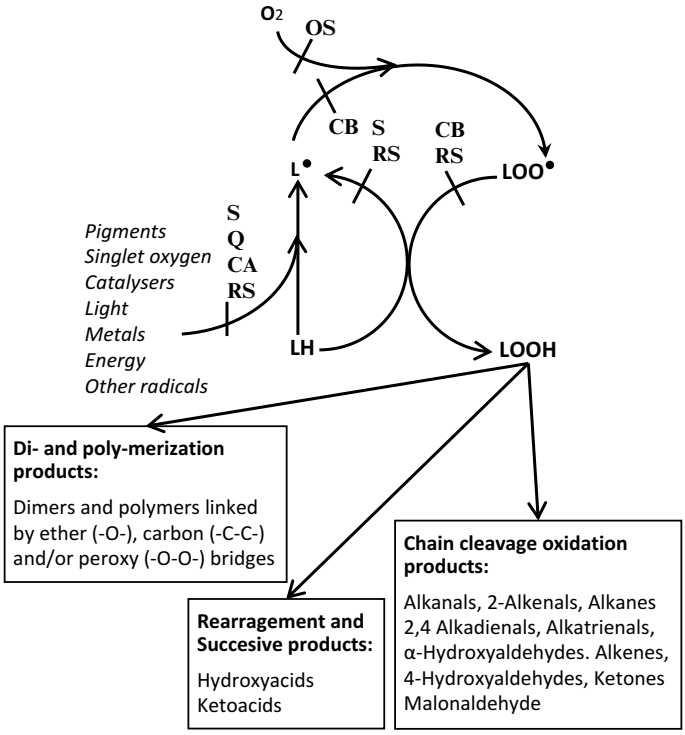

Fig. 13.3 Mechanism of unsaturated fatty acid oxidation including effect of antioxidants and chelating agents. *Note*: Lipid compounds: *LH*, lipid; *L*, lipid radical; *LOO*, peroxy radical; *LOOH*, hydroperoxide. Antioxidants: *OS* oxygen scavenger, *CB* chain reaction breaker, *S* synergist, *Q* quencher, *CA* chelating agent, *RS* radical scavenger/blocker (*Source*: Shahidi and Wanasundra (1992), with permission of Taylor & Francis Group)

because these are secondary products of oxidation and are formed when primary oxides decompose. The formation of nonvolatile oxidation products starts earlier since primary oxidation compounds belong to this group (Fig. 13.4). All these products directly affect the quality of oils, and many of them are the major cause of off-flavor formation and changes in taste, as described earlier for enzymatic oxidation (Fig. 13.3).

During the termination stage, radicals react with each other and form nonradical products. Any reaction that prevents the propagation of peroxidation or removes free radicals from the system plays a key role in the termination mechanism (Fig. 13.3). Chain-breaking antioxidants, such as phenolic components, react with lipid radicals and form nonreactive radicals, stopping the propagation chain (Simic et al. 1992). Examples of phenolic antioxidants include tocopherols, butylated hydroquinone, and propyl gallate.

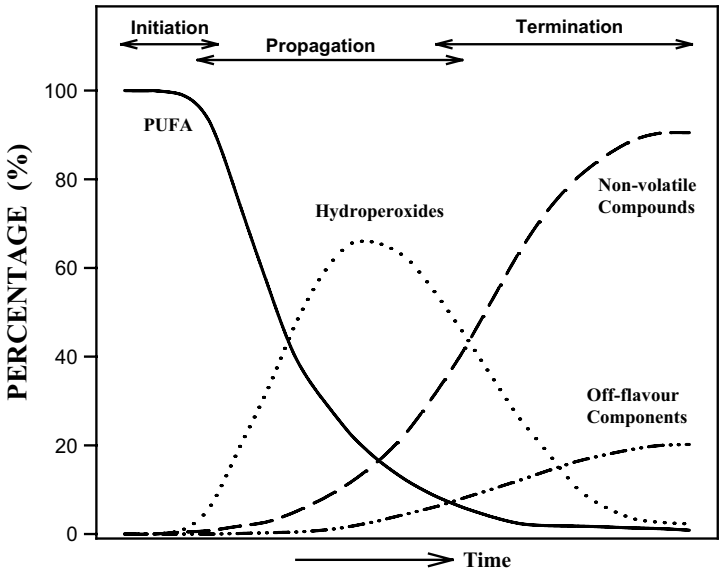

Fig. 13.4 Oxidation of polyunsaturated fatty acids (*PUFA*) and formation of primary (hydroperoxides) and secondary (nonvolatile and off-flavor component) products (*Source*: Gardner (1987), with permission of Elsevier)

The relative rate of oxidation of oleate:linoleate:linolenate was reported to be on the order of 1:12:25 based on peroxide formation (Frankel 1983). The rate of hydroperoxide decomposition is related to the number of double bonds in the oxidized fatty acid. Linolenate hydroperoxides decompose faster than linoleate and oleate and can also propagate the oxidation of other fatty acids (Frankel 1962).

Figure 13.5 shows the change in the fatty acid composition of VOO during oxidation. The fatty acid percentage was determined by periodically removing aliquots during the oxidative process from 0 to 33 h of oxidation (Morales et al. 1997). The quantification was based on the major saturated acid (C 16:0) initially present to show the real content of unaltered fatty acids (Dobarganes and Perez-Camino 1988). As expected, mainly unsaturated fatty acids were altered during the process; thus oleic, linoleic, and linolenic acids were those most affected. Linolenic acid showed a faster decomposition than linoleic acid, followed by monounsaturated fatty acids, which showed a similar behavior. The most important decrease was observed after 21 h of oxidation, when a higher amount of volatiles was detected. Linolenic acid practically disappeared after 33 h of oxidation, linoleic acid content was drastically reduced, and oleic acid was also affected by the oxidative deterioration. These results were in accordance with the volatile compounds found in the oxidized samples, as many aldehydes were detected after 21 h of oxidation. Their concentrations increased further during the following hours, meaning that the main volatile decomposition products found in oxidized olive oils were produced from monohydroperoxides of the unsaturated oleic, linoleic, and linolenic fatty acids as the total content of unaltered fatty acids was reduced from 98.2 % (0 h) to 80.8 % (21 h) and, later, to 49.2 % (33 h).

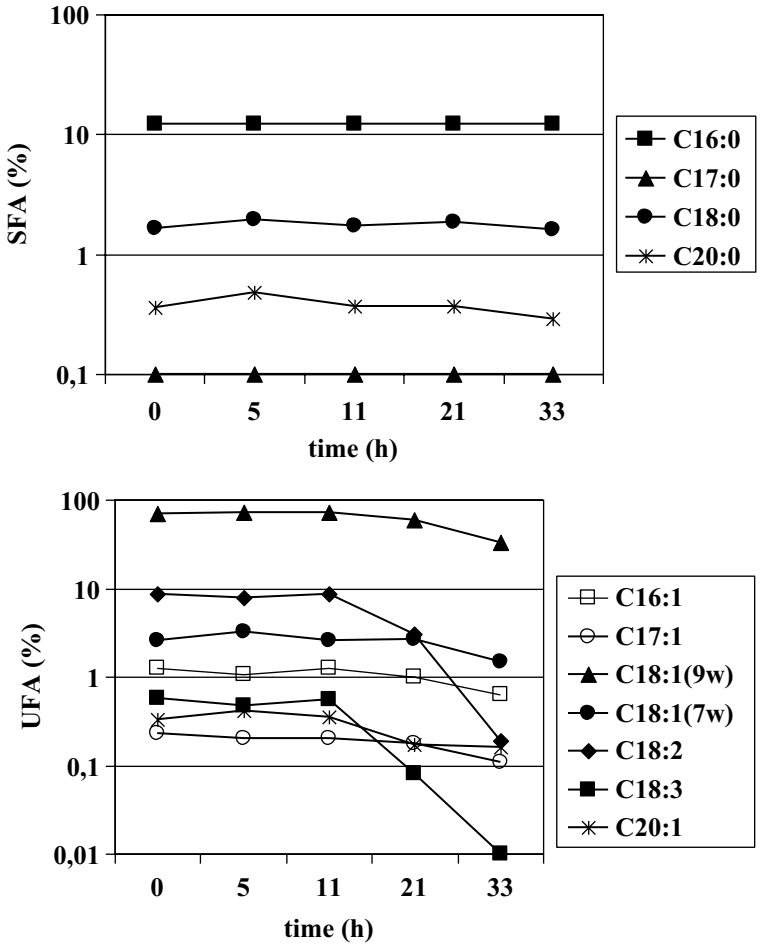

Fig. 13.5 Change of saturated and unsaturated fatty acids (%) during virgin olive oil oxidative deterioration process. (Courtesy of SEXIA Group, Instituto de la Grasa, Sevilla, Spain)

13.2.3 Photosensitized Oxidation

Photosensitized oxidation has been recognized as being most detrimental to the oxidative stability of vegetable oils. Most oils contain natural photosensitizers that are activated when oils are exposed to light during processing and commercial distribution (Carlsson et al. 1976). Exposure to light can cause the formation of hydroperoxides when both oxygen and the photosensitizer are present. Usually, the amount of oxygen dissolved in oil as a result of processing is sufficient to oxidize oil in a commercial container to a PV value of 10 meq/kg (Przybylski and Eskin 1988). The process of

formation of hydroperoxides can take place by photooxidation, and transfer of energy occurs as described in the following mechanism (Foote 1968):

$$\text{Sensitizer}_{\text{Ground}} + h\nu \rightarrow \text{Sensitizer}_{\text{Excited}}, \tag{13.1}$$

$$\text{Sensitizer}_{\text{Excited}} + RH \rightarrow \text{Sensitizer}_{H} + R^{*}, \tag{13.2}$$

$$\text{Sensitizer}_{\text{Excited}} + {}^{3}O_{2} \rightarrow \text{Sensitizer}_{\text{Ground}} + {}^{1}O_{2}, \tag{13.3}$$

$${}^{1}O_{2} + RH \rightarrow ROOH + {}^{3}O_{2}. \tag{13.4}$$

Energy ($h\nu$) is transferred from light onto the sensitizer (Sensitizer $_{\text{Excited}}$), which in turn can react directly with a lipid (RH), forming radicals (R^{*}) that in turn initiate autoxidation (Eq. 13.2). More damaging is the reaction of an excited sensitizer with ground state oxygen to form singlet oxygen (Eq. 13.3). The transferral of energy onto an acceptor, an unsaturated fatty acid, causes the formation of a hydroperoxide. Singlet oxygen has been found to react with linoleic acid about 1,500 times faster than normal oxygen (Rawls and Van Santen 1970). Therefore, this reactive component is defined as the most important initiator in the free radical autoxidation of vegetable oils. The effectiveness of light initiation is inversely proportional to the wavelength, where shorter wavelengths are the most effective. This process is propagated by the free radical mechanism discussed earlier, and similar hydroperoxides are formed (Frankel 1983).

Singlet oxygen is more often produced by the activity of natural photosensitizers such as chlorophyll, its degradation products, and other pigments usually present in oils (Bradley and Min 1992). Hydroperoxides formed from singlet oxygen oxidation decompose easily to form a variety of alkoxy and peroxy radicals, which in turn accelerate free radical autoxidation.

Oxidation of unsaturated fatty acids by singlet oxygen can be inhibited by compounds that react faster with this initiator or by quenchers that deactivate singlet oxygen to its ground state form (Foote 1976). Among the most efficient natural quenchers known are α-tocopherol and β-carotene. Other known quenchers include amino acids, proteins, sulfides, phenols, and metal chelators (Bellus 1978).

13.3 Primary Oxidation Products

The main primary oxidation products formed from unsaturated fatty acids are various isomers of hydroperoxides whose formation is dependent upon the number of double bonds and the mechanism of oxidation (Table 13.1). Linolenic acid produces the largest number of hydroperoxide isomers during both oxidation mechanism followed by linoleic and oleic acids. Photooxidation was found to be the most efficient oxidation, particularly for linolenic acid where the most isomers were formed. Among primary products fatty acids with conjugated double bond configurations are also formed. However, these compounds are very unstable (Frankel 1985).

Among vegetable oils, olive oil contains a variety of minor components that can also be affected by oxygen present in oil and may undergo oxidative degradation,

Table 13.1 Isomers of hydroperoxides formed from oxidation of fatty acids (%)

Hydroperoxide isomer	Oleic			Linoleic			α-Linolenic	
	FR	PH	E	FR	PH	E	FR	PH
8-OOH	26–28							
9-OOH	22–25	48–51	X^A	48–53	32	X^A	28–35	20–23
10-OOH	22–24	49–52			16–17			13
11-OOH	26–28							
12-OOH					17		8–13	12–14
13-OOH			X^A	48–53	34–35	X^A	10–13	14–15
15-OOH								12–13
16-OOH							41–52	25–26

Data from Frankel (1983)

X^A during enzymatic oxidation the proportion of isomers is fatty-acid-composition dependent; however, 9 and 13 isomers of linoleic and linolenic acids will dominate (Feussner et al. 2001), Oxidation: *E* enzymatic, *FR* free radical oxidation, *PH* photooxidation

which leads to the formation of a variety of oxidation products. Chlorophyll, an olive oil quality indicator, is affected by oxidation in the phytyl chain forming hydroperoxides and small-molecule hydroxy degradation products. These products change the color of the oil and form radicals that, in turn, may initiate oxidation of other oil components (Rontani et al. 2007).

Phytosterols usually form the majority of minor components in vegetable oils including olive oil. These components are prone to oxidative degradation, forming a variety of oxides belonging to the following component groups: epoxides, keto, hydroxy derivatives. Each of these compounds is formed through a free radical mechanism similar to that described earlier for fatty acids. The oxides are unstable and degrade forming a variety of free radicals and off-flavor components, similar in structure to ones formed during degradation of fatty acids (Rudzinska et al. 2009).

13.4 Secondary Oxidation Products

Primary oxidation compounds are unstable and easily decompose into a variety of compounds with lower molecular weights than the parent fatty acid (Fig. 13.3). Among degradation products, off-flavor components are formed that are the most detrimental to quality when rancidity is developed in oils, fats, oilseeds, and oil containing fruits and food products (Przybylski and Eskin 1995; Morales et al. 1997, 2005). Another group of hydroperoxide degradation products are nonvolatile molecules of hydroxy acids, keto acids, and hydroxyaldehydes. These compounds are usually thought to be the most detrimental to human health (Kasai and Kawai 2008; Guichardant and Lagarde 2009). All of the oxidative degradation products are very reactive chemically compounds and are precursors of oligomer formation and a variety of interaction products.

13.5 Minor Components and Vegetable Oil Oxidation

Several minor components have been identified in oils and fats from plants, including tocopherols, tocotrienols, sterols, phospholipids, FFAs, phenols, chlorophylls and derivatives, carotenoids, mono- and diacylglycerides, and trace metals (Perrin 1992). The amount of these components present in oil is determined by the plant species and by the oil processing conditions. Their proportion is usually higher in crude oils when compared with oils that are refined and deodorized because most of these components are removed or volatilized during the processing steps, resulting in only trace amounts in the refined, bleached, and deodorized oils (Jawad et al. 1984; Jung et al. 1989; Velasco and Dobarganes 2002).

These minor components have been reported to affect the oxidative stability of vegetable fats and oils by various mechanisms, but the effect of many of these minor components on oil oxidation, either by autoxidation or photooxidation, is still controversial or remains unclear.

13.5.1 Free Fatty Acids

The addition of different fatty acids to purified substrates has demonstrated a prooxidant effect of FFAs on edible oils. FFAs added to soybean oil have been shown to reduce its stability (Miyashita and Takagi 1986; Mistry and Min 1987). The prooxidant effect of FFAs was explained as being due to the presence of a carboxyl group in their structure, and the probable mechanism was attributed to the catalytic action of the carboxyl group on the decomposition of a small amount of hydroperoxides formed in the initial stages of autoxidation. Olive oils with high acidity have been found to be less resistant to oxidation than those having lower acidity values (Kiritsakis and Tsipeli 1992). The influence of oleic acid addition to VOOs on oxidative stability has been evaluated and a prooxidant effect, dependent on the FFA concentration in filtered VOOs, was found, but the opposite behavior was found when the oil was not filtered. The authors suggested that suspended-dispersed materials in cloudy virgin oils exert such a beneficial effect that they proposed avoidance of filtration to extend the olive oil shelf life (Frega et al. 1999).

13.5.2 Phospholipids

Olive oil contains the following phospholipids: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) in a range of 40 to 135 ppm (Alter and Gutfinger 1982).

Several studies have shown an antioxidant effect of PE, PC, PI, and phosphatidic acid (PA) when added to vegetable oils at levels of up to 3 % (Pokorny 1990).

The antioxidant activity of phospholipids was attributed to their capacity to chelate metals and, thus, inactivate their prooxidant effect. PE appeared to be the most effective. The greater potential as an antioxidant of this phospholipid was attributed to its amino group, which has the capacity to chelate metals.

Phospholipids can also act as synergists with phenolic compounds and tocopherols to improve their effectiveness (Hudson and Ghovami 1984). There is evidence that phospholipids have stronger synergistic activity when used at temperatures over 50 °C (Husain et al. 1986).

However, phospholipids isolated from crude oils by column chromatography with silicic acid have shown prooxidant activity when added to vegetable oils. The loss of antioxidant activity was attributed to changes in polarity and geometrical structure during the isolation procedure.

At a high temperature their beneficial action has also been demonstrated, although they may cause foaming and darkening and thereby decrease the oil's shelf life in practice. However, their presence at a low concentration (<0.01 %) does not have an adverse effect on the oil color and foaming, but it can improve the frying stability of oils (Velasco and Dobarganes 2002).

13.5.3 Phenolic Compounds

Phenolic compounds are present in olive mesocarp, but they are water soluble, although small quantities have been found in VOO. The amount of phenolic compounds in VOO can be influenced by various factors including the olive cultivar and degree of ripeness, as well as by production and extraction technologies (Gallina-Toschi et al. 2005). Polyphenols and tocopherols are the two main groups of phenolic compounds acting as primary antioxidants to inhibit oxidation in VOOs. The antioxidant activity of natural phenolic compounds has been related to their free radical scavenging properties, particularly their ability to donate a hydrogen atom to the lipid radical formed during the propagation phase of lipid oxidation; they mainly act as chain breakers by donating a radical hydrogen to alkylperoxyl radicals and subsequently forming a stable radical (Shahidi and Wanasundra 1992; Velasco and Dobarganes 2002).

13.5.3.1 Polyphenols

Polyphenols constitute the active hydrophilic antioxidant group of compounds in olive oil. Most hydrophilic phenols in olive oil are exclusive to the *Olea europaea* species (El Riachy et al. 2011). The prevalent classes of hydrophilic phenols found in VOO are phenolic alcohols, phenolic acids, flavonoids, lignans, and secoiridoids. The latter include glycosidic derivatives of oleuropein, demethyloleuropein, and ligstroside, which are the most abundant phenolic antioxidants of VOO (Servili et al. 2004).

Natural polyphenols, either simple compounds such as phenolic acids or their esters, or more complex; for example, lactones, chalcones, and flavonoids all have antioxidant activity and protect vegetable oils from oxidation (Shahidi and Wanasundra 1992; Boskou et al. 2005).

Early studies evaluating the antioxidant activity of oat extracts suggested that antioxidant activity was largely attributable to phenolics present in the grain, mainly caffeic acid (Daniels and Martin 1961). The extended shelf life of VOO compared to other vegetable oils that similarly contain chlorophyll was attributed to the presence of phenolics. The active phenols in the oil were mainly *o*-diphenols, such as hydroxytyrosol, protocatechuic, caffeic, *p*-coumaric, sinapic, cinnamic, and syringic acids (Papadopoulos and Boskou 1991).

At least two or three neighboring phenolic hydroxy groups and a carbonyl group in the form of an aromatic ester, lactone, chalcone, flavanone, or flavone are considered the essential molecular features required to achieve a high level of antioxidant activity (Dziedzic and Hudson 1984). Therefore, the antioxidative efficiency of monophenols increases substantially when one or two methoxy substitutions at the *ortho*-position in the phenol structure are present. The bonds in the *p*-position in cinnamic acids ensure even better efficiency than the carboxyl group in benzoic acid (Cuvelier et al. 1992).

The presence of hydrophilic phenolic compounds in VOO and their high antioxidant activity can be explained by the fact that polar antioxidants are more effective in nonpolar lipids whereas nonpolar antioxidants are more active in polar lipid emulsions (Bendini et al. 2007). In a bulk oil system the hydrophilic antioxidants, such as polar phenols, are oriented in the air–oil interface (a low quantity of air is always trapped in the oil) and becomes more protective against oxidation than the lipophilic antioxidants, like tocopherols, which remain in solution in the oil (Frankel 1996).

13.5.3.2 Tocopherols

Tocopherols constitute the lipophilic antioxidant group of olive oil and are noted for their effective inhibition of lipid oxidation in all vegetable oils (Sayago et al. 2007). The alpha isomer of tocopherols is the major endogenous antioxidant present in olive oil and is found in a range of 12–150 ppm. Olive oil produced from olive kernel showed higher amounts of tocopherols (Ruiz-Lopez et al. 1995). Tocopherols are considered to be the major natural antioxidants present in most vegetable oils. However, in VOOs, they compete with polyphenols at the early stages of oxidation, and their contribution to VOO stability is considered to be of minor importance with respect to that of polyphenols (Velasco and Dobarganes 2002).

Their antioxidant activities are dependent mainly on their concentration and the presence of synergistic compounds (Pokorny 1991). Tocopherols have been reported to act essentially as proton donors and free radical scavengers based on the tocopherol-tocopheryl semiquinone redox system (Sayago et al. 2007). Their maximum activity was observed when present in oil at levels between 400 and 600 ppm

(Clark et al. 1990). Higher levels of tocopherols added to the oil did not improve stability significantly. Moreover, in some oils they can act as prooxidants (Huang et al. 1994). This prooxidant activity of tocopherols was always found when these components were added to oil. Several studies have shown that the antioxidant activity of natural tocopherols is higher than that of the synthetic tocopherols (Wu and Weng 2001) since distribution and amalgamation of these components into oil play an important role in their antioxidant or prooxidant activity.

Tocopherols also act as singlet oxygen quenchers via a charge-transfer quenching mechanism. Tocopherols increased oxidative stability of vegetable oils during storage with light and when chlorophyll was present. The antioxidant activity decreased from α-tocopherol to β- and γ-tocopherols (Yamaguchi and Matsushita 1977; Jung and Min 1991).

The effect of several external agents – temperature, irradiation, refining, deodorization, and storage – on the content of tocopherols in vegetable oils has been studied. All the studies concluded that, with increased action of the external agent, the content of tocopherols decreased (Sayago et al. 2007). In the case of olive oil, the processing systems – extraction in two or three phases (Gimeno et al. 2002) – or destoning of the olive (Lavelli and Bondesan 2005) did not apparently affect the final tocopherol content. However, the state of maturation of the olive has a decisive influence on the tocopherol content of the oil, such that those obtained from green olives were richer in tocopherols than those obtained from ripe olives (Beltrán et al. 2005).

13.5.4 Chlorophylls and Derivatives

Chlorophylls and their derivatives are present in olive oils in variable amounts. The profile of VOO is determined by the green pigments that are initially found in the fruits and the derivatives formed during milling and paste beating. Chlorophylls a and b, originally found in the fruit, are converted into more stable pigments, pheophytins and subsequently pyropheophytins, which are the products of the removal of the carboxymethyl group (Gandul-Rojas et al. 2000; Roca and Mínguez-Mosquera 2001; Gallardo-Guerrero et al. 2005). These structural changes in the chromophore group of the chlorophylls affect the color of the oil, which changes from bright green to olive-brown and finally to yellow. The content of chlorophylls and their derivatives depends on the stage of maturity of the fruit, decreasing continuously from the beginning to the end of the olive harvesting period (Gutiérrez-Rosales et al. 1992).

Chlorophylls and pheophytins have a prooxidant action in the presence of light; they act as catalysts in the formation of singlet state oxygen, which can react directly with the double bonds of oleic, linoleic, or linolenic fatty acids, thereby generating reactive species of oxygen. Thus, chlorophylls and their derived pigments enhance the early phases of the process of autoxidation and generate allyl hydroperoxides (Giuliani et al. 2011).

Spanish olive oils contain from 0.4 to 45 ppm of total chlorophylls depending on the fruit cultivar, fruit maturity, processing system, and storage conditions, but the majority of these compounds were present in the form of degradation products such as pheophytins (Gandul-Rojas and Mínguez-Mosquera 1996; Criado et al. 2007). The oxidative stability of olive oil is greatly affected by the presence of chlorophylls and their derivatives, especially in the presence of light (Interesse et al. 1971). These compounds have the ability to transfer energy from light into chemical molecules (Bradley and Min 1992).

Several investigations have shown the prooxidative effect of chlorophylls and their derivatives in olive, soybean, and corn oils exposed to light. The amount of chlorophyll added to the oil proportionally reduced oxidative stability during storage in light. It has also been reported that during storage without light some chlorophyll derivatives act as antioxidants. These photosensitizers could show slight antioxidant effects on the oils in the dark, probably by donating hydrogen to break the free radical chain reactions (Endo et al. 1984; Gutiérrez-Rosales et al. 1992).

The relative prooxidant activity of pheophorbides, pheophytins, and chlorophylls was found to be 1.3:1.1:1, respectively, when measured by methyl linoleate oxidation (Endo et al. 1984). In soybean oil during storage in light, after 1 day of exposure, chlorophylls were destroyed, while their decomposition products were stable and present in the oil. Pheophytins were the most stable when present in oil and also had the highest activity as photosensitizers. These components must be removed from an oil to improve its oxidative stability (Usuki et al. 1984).

13.5.5 Carotenoids

Spanish olive oils contain from 0.8 to 18.3 mg/kg of carotenoids (Gandul-Rojas and Mínguez-Mosquera 1996; Criado et al. 2007) depending on the fruit cultivar, fruit maturity, processing system, and storage conditions. Carotenoids are naturally occurring hydrocarbon pigments possessing conjugated double bonds that have been found to be potent protectors against photosensitized oxidation by acting as singlet oxygen quenchers (Bradley and Min 1992). The physical quenching mechanism of carotenes is based on their low singlet energy state, which facilitates the acceptance of energy from the singlet oxygen. The carotenoid triplet formed transfers acquired energy as heat and returns to its original state (Foote and Denny 1970).

The rate of singlet oxygen quenching is related to the number of conjugated double bonds present in the polyene chain of carotenoid and increases when the number of these bonds increases. Canthaxanthin shows the greatest antioxidant activity, followed by β-carotene (Lee and Min 1990).

At levels of 5–20 ppm, β-carotene had the highest potency in protecting purified vegetable oils containing chlorophyll against deterioration under exposure to light, but the same protective effect was not observed during storage without light (Lee and Kim 1992). The presence of carotenoids in edible oils may also help to protect

against the formation of singlet oxygen by blocking light transmission through the oil. Due to their possible function as a natural light filter, carotenoids effectively absorb light with wavelengths between 400 and 500 nm (Frankel 1991).

Although carotenes are compounds efficient at protecting against photooxidation, their degradation by lipid peroxides is expected due to the oxidative susceptibility of hydroxy groups and the conjugated double-bond system in the carotenoid molecule (Haila and Heinonen 1994). Carotenoids either may act as singlet oxygen quenchers or are oxidized, thus protecting the oil until all of them are destroyed by oxidation (Kiritsakis and Dugan 1985).

Some studies suggest that β-carotene may act as an effective inhibitor of photooxidation in vegetable oils only when tocopherols are present (Warner and Frankel 1987). Furthermore, in the absence of light, carotenoids or their oxidation products may act as prooxidants in vegetable oils (Lee and Kim 1992).

The carotenoid fraction appears to have lower thermal stability than chlorophylls (Del Carlo et al. 2010).

13.5.6 Sterols

Plant sterols are collectively known as phytosterols, existing mainly as free sterols and sterol esters of fatty acids, with β-sitosterol, campesterol, brassicasterol, and stigmasterol as the major compounds identified in vegetable oils (Zambiazi 1997). The main olive oil sterols are β-sitosterol, Δ^5-avenasterol, and campesterol. A number of other sterols are also found, but they are present in smaller quantities. The total sterol content in olive oils is in the range of 180–265 mg/100 g (Mariani et al. 1993), while the ranges for each sterol in Spanish olive oils are described by Aparicio et al. (1996a).

Sterols show natural antioxidant activities in foods (Choe and Min 2009). They seem to be ineffective at low temperatures and under the conditions of accelerated storage tests, although their influence on oil stability at high temperatures, where they act as inhibitors of polymerization reactions, has been demonstrated (Velasco and Dobarganes 2002). There is evidence that some naturally occurring phytosterols may improve stability during heating of cooking oils. Unsaponifiable fractions from oat, olive, *Vernonia anthelmintic*, and corn oils, which are rich in sterols, were found to act as antioxidants when added to vegetable oils heated to frying temperatures. Among the sterols, vernosterol, Δ^7-avenasterol, and fucosterol were effective when added at a concentration of 0.2 %, while ergosterol, lanosterol, β-sitosterol, stigmasterol, and cholesterol were ineffective at the same concentration levels (White and Armstrong 1986).

It was found that Δ^5-avenasterol and fucosterol, when added at concentrations up to 0.1 %, retarded the oxidation of vegetable oils at elevated temperatures. The ethylidene group, which is characterized by a double bond between carbon atoms C_{20} and C_{24}, was expected to be responsible for this activity (Zambiazi 1997). The formation of an allylic free radical at carbon C_{29} followed by isomerization to a relatively

stable tertiary free radical (Gordon and Magos 1983) has been proposed as a mode of action of these phytosterols.

The prooxidant activity of some sterols has also been demonstrated. It was found that the addition of 5 % sitosterol caused a twofold increase in the oxidation rate of tristearin heated at 120 °C (Yanishleva and Schiller 1984). In olive oil, a reduction in sterol content was observed when oxidation of oil occurred (Leone et al. 1976).

It has been reported that some sterols were oxidized during storage of vegetable oil and at an accelerated rate during frying. During storage approximately 30–45 % of sterols were oxidized, while during frying almost all sterols were transformed into oxides and high molecular weight components (Rudzinska et al. 2009; Ryan et al. 2009). Sterol deterioration during frying was found to be affected by frying time, frying technique, and by the oils' unsaturation, being more extensive in polyunsaturated oils than in monounsaturated oils such as VOO (Salta et al. 2008).

13.5.7 Metals

Many reports have described the deleterious effect of trace amounts of metals on the oxidative stability of oils. The general chemical mechanism for metal catalysis of lipid oxidation may follow different pathways (Choe and Min 2006). Specifically, iron, copper, and nickel behave as direct initiators by electron transfer or indirect initiators by redox reactions. Another possible pathway is by initiation of fatty acid oxidation through the reaction of metals with triplet oxygen. The superoxide anion radical produced can either lose an electron to produce singlet oxygen or react with a proton of the lipid components to form a hydroperoxyl radical. Both radicals have high reactivity and may initiate autoxidation (Schaich 1992).

Metal reactions have proven to be complex and dependent on factors such as the specific metal complex, valence state of the metal, concentration of the metal, oxygen partial pressure in the system, types of lipids, and the presence of hydroperoxides (Schaich 1992).

Transition metals, which contain two or more valence states, have the highest oxidation potentials. These metals include iron, copper, and nickel, as well as others of minor importance such as cobalt, chromium, and aluminum (Labuza 1971).

Copper accelerates hydrogen peroxide decomposition 50 times faster than ferrous ion (Fe^{2+}), and ferrous ion acts 100 times faster than ferric ion (Fe^{3+}) (Choe and Min 2006). Metals also accelerate autoxidation of oil by decomposing hydroperoxides, and Fe^{2+} is much more active than Fe^{3+} in decomposing lipid hydroperoxides to catalyze autoxidation (Halliwell and Gutteridge 2001).

Crude oil contains transition metals such as iron and copper. However, refining reduces their contents. Edible oils manufactured without refining, such as extra virgin olive oil (EVOO), contain variable amounts of transition metals. Trace amounts of Fe and Cu may originate from the soil and fertilizers or from the contamination of the processing equipment and storage containers. Concentrations of Fe and Cu reported for VOO range between 0.5–3.0 and 0.001–0.2 mg/kg, respectively, and

other metals such as Cr, Mn, Sn, Ni, and Pb have also been reported in VOOs at very low concentrations (μg/kg) (Velasco and Dobarganes 2002).

Copper, iron, and nickel, in particular, greatly reduce the oxidative stability of unsaturated lipids and accelerate the development of rancidity in edible oils and fats when present at concentrations of a few parts per million (Angerosa and Di Giacinto 1993). Martín-Polvillo et al. (1994) set up a method for the direct determination of Al, Cr, Cu, Fe, Ni, and Pb by atomic absorption spectroscopy. They concluded that VOO showed lowered stability after storage in contact with a carbon-steel sheet than when stored in the absence of metals. In conclusion, metals affect not only the initiation but also the degree of chain reaction branching, secondary reactions, and the nature of termination reactions (Labuza 1971). Fe^{3+} also causes decomposition of phenolic compounds such as caffeic acid in olive oil and, thus, decreases oil oxidative stability (Keceli and Gordon 2002).

13.5.8 Volatile Compounds

The decomposition of primary oxidation products (hydroperoxides) produces secondary volatile oxidation products, which give rise to off-flavors in oils. The formation of volatile oxidation products in edible oils is a great concern for the edible oil industry as it leads to the development of undesirable flavors and odors in oils, which reduces their shelf life or makes them unfit for human consumption. However, in the case of VOO, enzymatic oxidation through the lipoxygenase pathway is the main producer for desirable green and fruity flavors (Morales et al. 1999), while chemical oxidation is the main cause of the spoilage of VOO (Morales et al. 1997; Luna et al. 2006).

Decomposition occurs through homolytic cleavage of hydroperoxides. Various reaction pathways for the development of these volatile products have been reviewed (Frankel 1985; Kochhar 1993; Choe and Min 2006). The dominance of a particular pathway depends on the oxidation state of the oil as well as on other factors such as oxygen pressure, temperature, and the presence of pro- and antioxidants. The main decomposition products include aldehydes, ketones, alcohols, acids, hydrocarbons, lactones, furans, and esters. Table 13.2 shows the volatile compounds identified in oxidized olive oil samples by different authors. Aldehydes are the most important volatile breakdown products and major contributors to unpleasant odors as they have very low odor thresholds. Aliphatic alcohols make a small contribution to off-flavors because their flavor thresholds are higher than those of the corresponding aldehydes. Saturated hydrocarbons have even higher threshold values, while several unsaturated hydrocarbons are moderately potent flavors. Aliphatic acids have high flavor thresholds, so they must be present in high concentrations to produce off-flavors. Furans and ketones also contribute to undesirable flavors in olive oils. The flavor response of these volatiles is affected by the position and geometry of the double bonds present in the molecule. Unsaturated aldehydes and ketones are

Table 13.2 Volatile compounds identified in oxidized olive oils by different authors

Volatile compounds	
Acetaldehyde	2,4-Heptadienal
Hexane	1,4-Heptadiene
Cyclohexane	1-Octen-3-ol
Propanal	2,5-Octadien-2-one
Octane	2-Nonenal
Tetrahydrofuran	Octanol
Pentanal	3,5-Octadien-2-one
Chloroform	Decanal
Methyl benzene	Undecanal
Hexanal	2-Decenal
4-Methyl-2,3-dihydrofuran	Nonanol
1-Penten-3-ol	Pyrrole
Heptanal	Butanoic acid
z-Methyl-x,y-dihydrofuran	2,6-nonadienal
2-Methyl-2-butenal	2,4-nonadienal
2-Pentenal	Terpenic hydrocarbons
2-Hexenal	2-Farnesene
1,3-Nonadiene	Pentanoic acid
Octanal	2-Undecenal
2-Heptenal	2,4-Decadienal
6-Methyl-5-hepten-2-one	Hexanoic acid
Hexanol	6-Methyl-5,9-undecadien-2-one
3-Hexenol	Heptanoic acid
Nonanal	Furan
2-Octenal	Octanoic acid
1,3-Dichlorobenzene	Nonanoic acid
Acetic acid	Methyl heptanoate

Source: Dobarganes et al. (1986), Solinas et al. (1987, 1988), Morales et al. (1997, 2005), Luna et al. (2006)

susceptible to further oxidation, and new off-flavor compounds are formed (Kochhar 1993). Sensory descriptions such as, for example, oxidized, rancid, fishy, soapy, painty, and tallowy have been used to describe the undesirable flavors developed in edible oils.

13.6 Thermoxidation: Frying

Deep-fat frying is one of the oldest and most popular food preparation methods. Frying is a process of immersing food in hot oil that is held at a temperature of 150–190 °C, where simultaneous heat and mass transfer occurs between the oil and fried food. High temperature, the presence of oxygen, and chemically active food and oil components form the desirable and unique qualities of fried foods. Fried foods are

Table 13.3 Fat content in fried products

Fried product	Oil (% w/w)
Potato chips	20–38
Corn chips	25–38
Tortilla chips	23–30
Doughnuts	20–25
French fries	6–20
Fried noodle	10–18

Adapted from Mehta and Swinburn (2001)

very popular among consumers due to their outstanding flavor, color, and crispy texture (Boskou et al. 2006). Frying oil acts as a cooking medium and contributes to the texture and flavor of fried food as a source of flavor precursors and taste components. The amount of oil absorbed by fried products (Table 13.3) is affected by time, food surface area, moisture in food, breading and coating of fried products, and type of frying oil (Mehta and Swinburn 2001). The absorbed oil during frying tends to accumulate on the surface of fried food and replaces evaporated water inside the product during cooling and draining (Moreira et al. 1997, 1999a). Olive oil has an advantage linked to its relatively low melting point, which means that it drains from the fried food easily, thus leading to a low content of oil in the fried food (Rossell 2001).

Complex reactions happen during deep-fat frying, causing the formation of pleasant or unfavorable flavors, as well as affecting the color, texture, and nutritional value of fried foods. Among these reactions, the most common are oxidation, hydrolysis, and oligomerization of oil and food components. A portion of the volatile compounds is steam-distilled into the frying facility atmosphere, often causing contamination. Volatile compounds formed from degradation undergo further chemical reactions to yield compounds with higher molecular weight. Nonvolatile compounds are another group of components formed during frying that change the physical and chemical properties of oil and fried foods. Deep-fat frying reactions decrease the content of unsaturated fatty acids and endogenous minor components such as phenolics and antioxidants in oil (Allouche et al. 2007). As a consequence of chemical changes, the following parameters increase in value: foaming, color, viscosity, density, specific heat, content of FFAs, polar materials, and oligomeric compounds (Fig. 13.6).

Physical parameters, which are usually selected for specific operations, such as temperature, time, type and freshness of frying oil, antioxidants, oxygen accessibility, endogenous oil and food components, and the design of a fryer, significantly affect the rate of oxidation, hydrolysis, and oligomerization occurring during frying.

13.6.1 Hydrolysis of Oil

When food is fried in heated oil, moisture moves from the food into the oil, where it is transformed into steam and evaporates with a bubbling action, significantly extending exposure to oxygen on the surface of the oil. Water attacks the ester bond

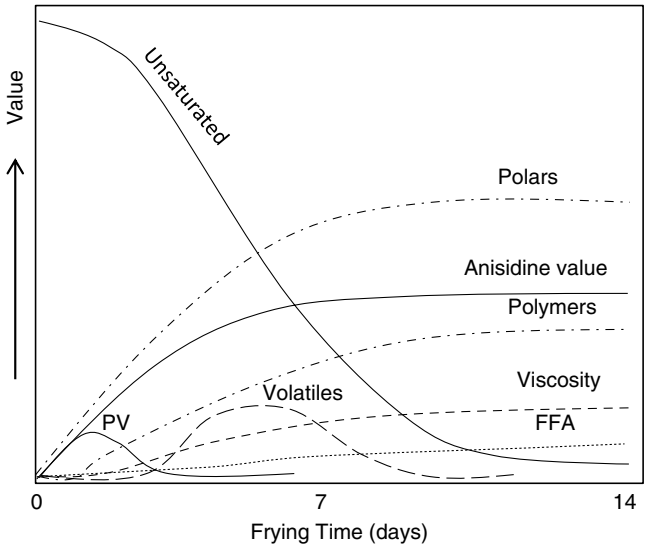

Fig. 13.6 Degradation of fatty acids and formation of products during frying

of triacylglycerols and produces di- and monoacylglycerols and FFAs (Fig. 13.4). The amount of FFAs formed in frying oil usually increases with the number of batches fried. However, in currently developed frying oils this increase is slow, as shown in Fig. 13.6 (Chung et al. 2004). Using FFA for frying oil quality monitoring is of minimal value due to the slow accumulation of these compounds. Hydrolysis occurs preferably in oils containing short and unsaturated fatty acids rather than in oils with long and saturated fatty acids and is driven by the solubility of fatty acids in water (Nawar 1969).

13.6.2 Thermal Oxidation of Oil

Oxygen reacts with oil faster under frying temperatures even when elevated temperatures lower its solubility in oil (Houhoula et al. 2003). The mechanism of thermal oxidation is essentially the same as for autoxidation. The rate of thermooxidative degradation of oils is thought to be higher than autoxidation at ambient conditions, but specific and detailed data are not available. The mechanism of thermal oxidation involves the initiation, propagation, and termination of the reaction, as described earlier.

The oil needs to be in a radical state to react with oxygen during the oxidation reaction, and the weakest point in the acid molecule is at the methylene-interrupted carbon located between double bonds. The energy required to break the

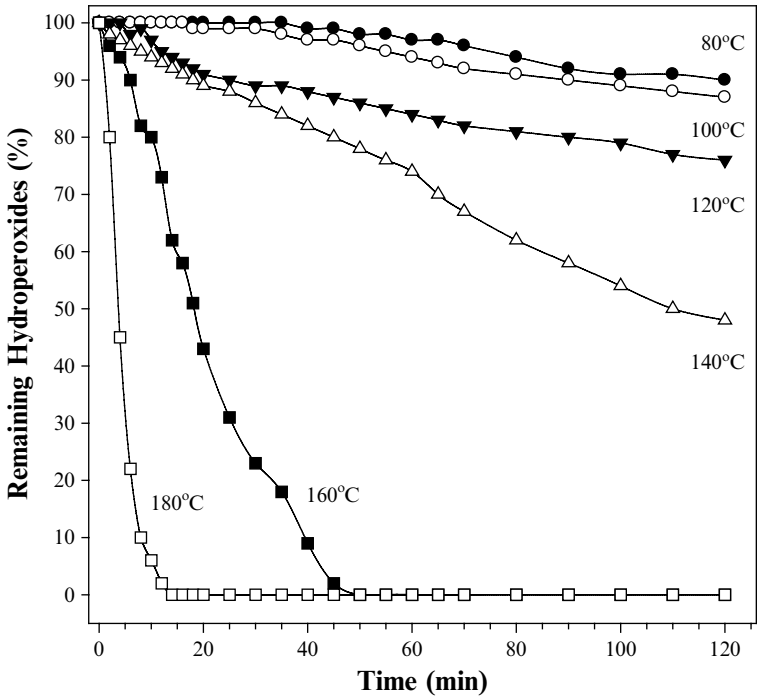

Fig. 13.7 Stability of hydroperoxides at different temperatures

carbon-hydrogen bond on carbon 11 of linoleic acid is 50 kcal/mol, while the hydrogen-carbon bond on saturated carbons without a neighboring double bond is approximately 100 kcal/mol (Min and Boff 2002). The various strengths of hydrogen-carbon bonds of fatty acids explain the differences in oxidation rates of stearic, oleic, linoleic, and linolenic acids during thermal oxidation or autoxidation.

Heat, light, metals, and reactive oxygen species facilitate radical formation in oils. Polyvalent metals such as Fe^{3+} and Cu^{2+} remove hydrogen protons from oils to form alkyl radicals by oxidation-reduction mechanisms with metals, even at low temperatures. The alkyl radicals formed are very reactive and interact with other alkyl, alkoxy, and peroxy radicals to form a variety of primary and secondary products.

The oxygen-oxygen bond formed in hydroperoxides is weak and requires only 44 kcal/mol of energy to break (Hiatt et al. 1968). The hydroperoxides are generally unstable and during frying decompose easily (Fig. 13.7). Hydroperoxides are decomposed into alkoxy radicals and hydroxy radicals by homolysis of the peroxide bond. Toschi et al. (1997) identified a variety of monooxygenated products formed during thermal oxidation of methyl linoleate. The alkoxy radicals react with each other or with other radicals to form new radicals and nonradical products, usually oligomers. The formation of nonradical volatile and nonvolatile compounds usually ends the oxidative degradation process.

Most volatiles are removed from frying oil by steam distillation during frying (May et al. 1983). Wu and Chen (1992) observed that when water was added to

the frying system, the amount of volatile compounds decreased significantly. The disappearance or loss of volatile compounds in frying oil is due to the combination of evaporation, decomposition, and the reaction of volatile compounds with other food components (Nawar 1985).

13.6.3 Oligomerization

The major decomposition products of frying oil are nonvolatile polar and nonpolar compounds, mainly oligomers of various radicals, triacylglycerol, and minor components such as sterols, tocopherols, and phenolics (Dobarganes et al. 2000). Dimers and polymers are large molecules and are formed by a combination of the following bonds: -C-C-, -C-O-C-, and -C-O-O-C- (Kim et al. 1999; Rudzinska et al. 2010). Hydroxy and keto dimers of linoleate, including the dimers of oleate, were found in soybean oil during frying (Christopoulou and Perkins 1989). Rudzinska et al. (2010) observed that phytosterols during thermooxidative degradation are mainly transformed into oligomers with small amounts of volatile and oxidized products formed. Oligomerization during frying follows the usual radical mechanism. Oils rich in linoleic and linolenic acids form oligomers more easily during frying than high oleic acid oils (Bastida and Sanchez-Muniz 2001).

Oligomers formed during frying are rich in oxygen and usually have ether or peroxide bridges. Yoon et al. (1988) reported that oxygen-rich oligomers accelerated the oxidation of oil and significantly increased the oil viscosity (Tseng et al. 1996). Furthermore, these compounds reduce heat transfer, increase foaming, stimulate absorption of oil by food, and cause changes in the color of fried foods. Oligomers are highly conjugated compounds forming a brown, resinlike residue on the walls of the fryer, at the contact area between oil and the air (Moreira et al. 1999b).

13.6.4 Degradation of Oil Components

High temperatures during frying stimulate the degradation of fatty acids, particularly unsaturated, into a variety of volatile and nonvolatile compounds, as discussed earlier. Among components formed are a variety of compounds that may be toxic to different extents. Uriarte and Guillén (2010) observed the formation of alkylbenzenes as products of linoleic and linolenic acid thermooxidative degradation. These components with gamma carbonyls are the primary contaminants in frying facilities, deteriorating air quality and causing continuous exposure of workers. It is well established that many minor components of oils degrade at similar rates to fatty acids during frying. Tocopherols, sterols, and phenolics are involved in oxidative degradation and form radical intermediates, which further interact to often produce oligomers. Andrikopoulos et al. (2002) observed that 85–90 % of tocopherols disappeared during multiple frying in olive oil. Other typical olive oil phenolics were more resistant to degradation compared to tocopherols. However, after multiple batch frying, only 20–30 % were left.

Rastrelli et al. (2002) reported that during storage, tocopherols were degraded at a faster rate than other phenolics in olive oil. However, during frying, tocopherols and olive oil phenolics disappeared at the same rate, indicating similar antioxidant potentials. Hydroxytyrosol, oleuropein, and derivatives of elenolic acid thermooxidatively degraded at a rate similar to that of tocopherols during food frying (Brenes et al. 2002; Gomez-Alonso et al. 2003). Most of the degradation of phenolics can be linked to the formation of oligomers by reactions between the same phenolic molecules or between different radical molecules. The most prone to oligomer formation are quinone-type phenolics (Robards et al. 1999). The effect of thermal oxidation on the minor components of VOO has been recently studied in depth. It was reported that the degradation of unsaturated fatty acids by the thermal oxidation process is related to the decreased concentration of *o*-diphenols and α-tocopherol. There is evidence that some phenolic compounds, such as tyrosol, lignans, and oxidized derivatives of secoiridoids, remain in the oil mostly in unchanged form, even when the level of polar components exceeds 25 %, whereas α-tocopherol is usually completely transformed into an oxidized form. Chlorophyll pigments, such as pheophorbides and pyropheophytins, are the most resistant to thermooxidative degradation (Tena 2010).

13.7 Measurement of Lipid Oxidation

Lipid oxidation leads to the formation of hydroperoxides, primary products that are very unstable, and, by decomposition, form secondary products. These secondary products may include hundreds of individual components that adversely affect food flavor. In many cases, these components are associated with oxidative rancidity or off-flavor formation.

The major importance of food quality control is the determination of the extent of oxidation in foods and food products. Analytical methods developed to measure the extent of oxidation are based on products formed or involved in this deteriorating process. Methods characterized below can be divided into two groups. The first measures oxygen (as the main cause of oxidation) and primary products, the second measures secondary products formed from the decomposition of hydroperoxides.

13.7.1 Primary Products, Substrates

13.7.1.1 Oxygen Consumption

Oxygen is one of the major causes of oxidation that can be measured to evaluate oil stability. Consumption of oxygen can be performed by gas chromatography (GC) with a thermal conductivity detector or by measuring the decrease in pressure when oxygen is consumed. GC measures directly the amount of oxygen present in headspace over the stored oil and can be performed under accelerated conditions with full control of parameters. The change in pressure is evaluated also under

controlled conditions and can be directly converted into oxygen consumption. Both methods are relatively simple and evaluate directly the amount of oxygen utilized for oxidation of the particular oil.

13.7.1.2 Conjugated Dienes and Trienes

During the formation of peroxy radicals and hydroperoxides a shift in double-bond configuration occurs, thus transforming the normal methylene-interrupted configuration into conjugated forms. Conjugated dienes (CDs) show absorption at 232 nm and trienes at 268 nm (Noor and Augustin 1984). By measuring CDs, the oxidative status of oil is estimated indirectly. Good correlations between sensory scores and CDs were reported for stored oils, and potato and tortilla chips (McMullen and Hawrysh 1991).

13.7.1.3 Peroxide Value

Measurement of peroxide values (PVs) is used as an indicator of the initial oxidation since it measures the hydroperoxide content. There are numerous procedures for the assessment of PV. The most common method is iodometric titration, although spectrometric methodologies have recently been established (Chap. 10). The second most common procedure used is based on the redox potential of iron combined with colorimetric measurement. An excellent discussion of the problems encountered with PV assessment can be found in Rossell (1987). A number of studies have shown a high correlation between PV and sensory scores for stored oils (Przybylski et al. 1993).

13.7.2 Secondary Decomposition Products

13.7.2.1 Thiobarbituric Acid Test

The thiobarbituric acid (TBA) test is based on the reaction between TBA and malondialdehyde. It is one of the most often used tests to assess rancidity in foods, particularly foods of animal origin. TBA produces a red color complex with an absorption maximum at 532 nm. This test is not specific to malondialdehyde, and there are reports stating that other oxidation aldehydes and peroxides also form a colored complex that could affect the evaluation. A good discussion of the limitations and modification of this procedure can be found in St. Angelo (1996).

13.7.2.2 Carbonyl Compounds

The oxidation of oils can be measured colorimetrically by determining the carbonyl compounds, which are formed as main products of hydroperoxide decomposition. With this method components directly responsible for off-flavor

are measured. Carbonyl compounds can be measured as 2,4-DNPH (2,4-dinitrophenylhydrazine) or hydroxylamine (oximes) derivatives (Henick et al. 1954; Przybylski and Hougen 1989). These compounds can be measured in either oils or food products. From another point of view, high correlations were found between the amount of unsaturated and saturated carbonyls and sensory scores (Eskin and Vaisey-Genser 1989).

13.7.2.3 Anisidine Value

The test is based on the reaction of *p*-anisidine with unsaturated carbonyl compounds present in oils where the colored reaction product is formed and measured by spectrophotometry. Anisidine values (AV) below 10 are recommended for freshly refined oils. A good correlation between AV and sensory scores has been found for oils and fried products (McMullen and Hawrysh 1991). A combination of the peroxide and anisidine values is often used as a complete description of oil oxidative status and is called the TOTOX index (Holm 1972).

13.7.3 Volatile Analysis by Gas Chromatography

Analysis of flavor components by GC is a direct measurement of compounds responsible for off-flavor formation and is, therefore, the most suitable for comparison with sensory evaluation. This methodology can also provide data on the origin of flavor compounds and their precursors (Frankel 1993).

Initially the most widely applied techniques for volatile analysis by GC were static headspace, dynamic headspace, and direct injection. Dynamic headspace techniques have been widely applied in the study of olive oil oxidation (Dobarganes et al. 1986; Solinas et al. 1987; Morales et al. 1997), although during recent years solid-phase microextraction (SPME) has been the most common sample preparation technique for the evaluation of volatile compounds in oxidized olive oil samples (Chap. 8).

13.7.4 Testing Resistance to Oxidative Stability

A number of accelerated tests have been developed to evaluate oil resistance to oxidation. According to Frankel (Frankel 1993), oxidative stability tests where a temperature above 85 °C is applied are not reliable due to the different mechanisms of oxidation involved. Furthermore, it has been found that results from Rancimat and the active oxygen method (AOM) did not agree with stability tests performed at 60 °C (Warner et al. 1989).

13.7.4.1 Active Oxygen Method

In the AOM or Swift test, air is bubbled through oil held at 98 °C. Samples of oil are taken at various intervals and the PV determined. By plotting PV against time, the induction period is established.

13.7.4.2 Oxygen absorption tests

Oxidative deterioration can also be monitored by holding the oil at elevated temperatures in a closed system and measuring oxygen absorption. Many variations of this procedure have been devised including ASTM oxygen bomb, the FIRA-Astell apparatus, the Sylvester test, and weight gain or gravimetric procedures.

13.7.4.3 Conductivity Tests

Conductivity tests are based on the decomposition of hydroperoxides and the formation of short-chain fatty acids, which change the conductivity of water. These acids are produced when oils are heated at 100 °C or higher temperatures and are mainly formic and acetic acids. Several automated instruments based on the conductivity principle have been developed, including Rancimat and OSI (Rossell 1987).

13.7.4.4 Accelerated Storage Tests

Accelerated storage tests have been used extensively to monitor the stability of vegetable oils using a modification of the Schaal oven test (Eskin et al. 1989). In this test, oxidation is accelerated by holding the sample of oil from 60 °C to 65 °C in the dark and samples are evaluated at defined intervals by measuring the extent of oxidation. Variations of the Schaal oven test conditions exist and include differences in the oil quantity used for storage, the size of storage containers, differences in ratios between the surface of the oil and its volume, and whether or not the containers are covered during storage. The ratio between surface area and volume of oil directly affects the rate of oxidation. The Schaal oven test is more reliable than tests carried out at elevated temperatures because it reproduces similar oxidative changes observed under actual shelf life conditions (De Man et al. 1987). By increasing the temperature of storage the oxidation rate is increased, so the whole test can be performed in a shorter time. One day of storage at this condition is equivalent to about 1 month at ambient temperature (Evans et al. 1973). This test is also performed with light exposure to evaluate any photooxidation effect on oil oxidative stability.

13.7.4.5 Ambient Storage Tests

These are performed similarly to the Schaal oven test but at ambient temperature. The tests must be performed for a long time due to the slow oxidation process; thus, they are costly and time consuming.

13.8 Flavor and Off-Flavor of Virgin Olive Oil

As was described in Chap. 8, VOO has a desirable and complex flavor mainly due to the presence of many volatile compounds. Many of them are produced through the lipoxygenase pathway and are always present in the headspace of VOOs, although at different concentrations depending on the olive cultivar. When oils are subjected to oxidation, new volatile compounds that were not initially present appear and the initially pleasant sensory characteristics of the oil eventually give way to unpleasant sensory attributes.

The changes in the chemical compounds responsible for the VOO flavor – mainly produced through biochemical pathways – and the formation of off-flavors, produced through (chemical) oxidative pathways (Frankel 1985; Dobarganes et al. 1986; Kochhar 1993), indicate the main differences found between VOO flavors and off-flavors. The volatile compounds identified in VOO flavor have been found to be quite different from those identified in VOO off-flavor. The explanation could be their different origins, mainly biochemical for flavors and chemical for off-flavors. The main differences that characterize off-flavors are the absence of C_6 aldehydes and alcohols (produced from linolenic acid), which contribute to the green flavor of VOO, the absence of esters contributing to fruity flavor, and the presence of many aldehydes with low odor thresholds contributing to the typical rancid odor of oxidized oils (Table 13.2). The profile of volatile compounds of a VOO sample responsible for its flavor, which can be considered a fingerprint of the oil, abruptly changes when the sample is deteriorated by oxidation. Figure 13.8 shows the profile of volatile compounds of various olive oil samples oxidized at various levels. Qualitative and quantitative changes in the volatile composition of the oil can be observed.

The change in the volatile composition of VOO along the oxidation process was studied using an accelerated process (Morales et al. 1997). In this experiment, the initial flavor disappeared in a few hours, and then the oxidation process started, producing a great amount of volatile compounds, only some of them being present in the initial flavor.

During the first hour the total volatiles decreased as they were stripped by the gas flow rate, aided by the rise in temperature. At 5 h, there were practically no volatiles in the oil. At this point, the oil could be considered refined oil, as the initial volatiles produced mainly through enzymatic pathways had completely disappeared. A remarkable point is the small amount of hexanal at 5 h, while it was one of the major peaks in the initial VOO flavor. From this time on, a further increase in new volatiles (breakdown products of lipid hydroperoxides) was observed while the oxidation

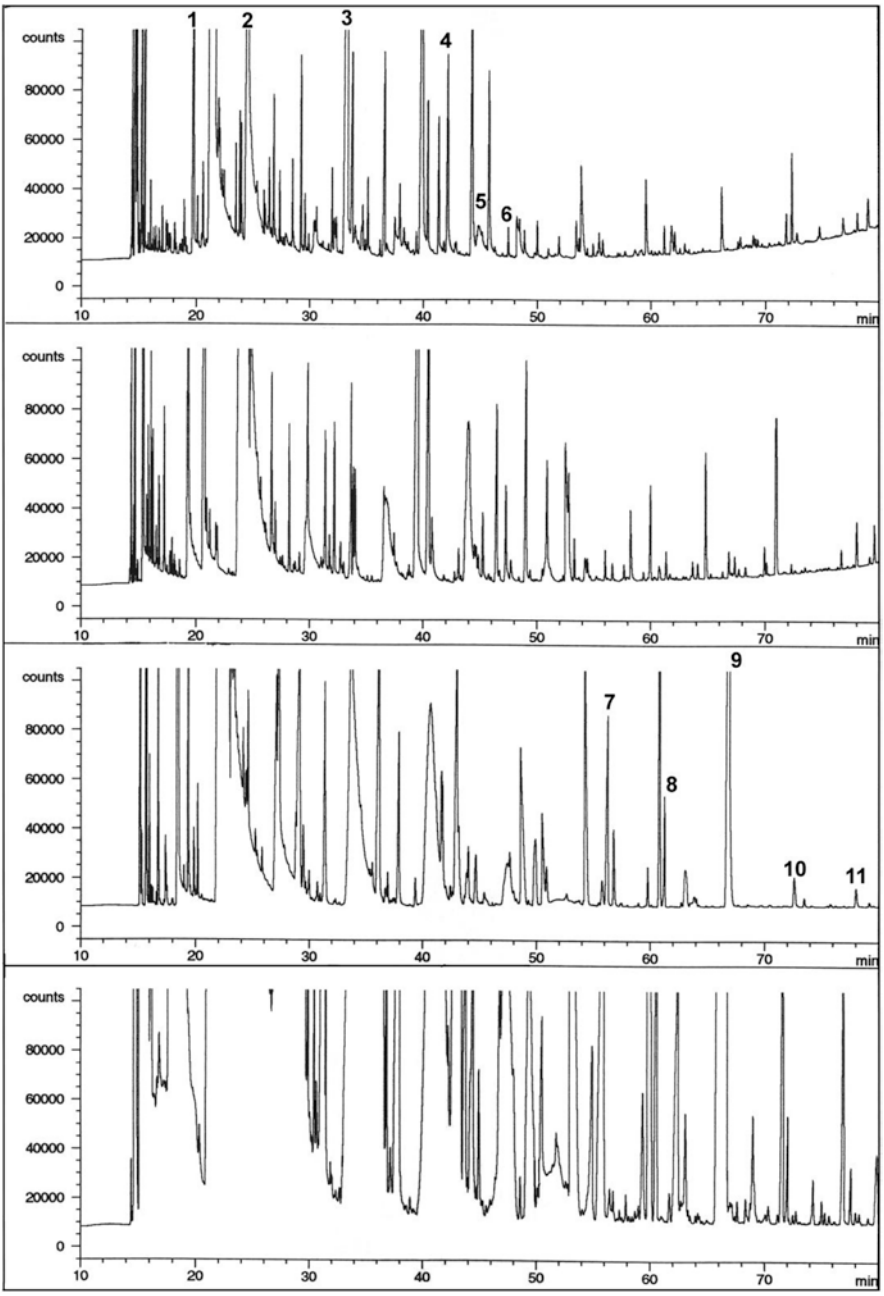

Fig. 13.8 Chromatograms of oxidized virgin olive oil samples. *Top* to *bottom*: increasing oxidation. Note:*1* pentanal, *2* hexanal, *3* (E)-2-hexenal, *4* hexan-1-ol, *5* nonanal, *6* acetic acid, *7* butanoic acid, *8* pentanoic acid, *9* hexanoic acid, *10* heptanoic acid, *11* octanoic acid. (Courtesy of SEXIA Group, Instituto de la Grasa, Sevilla, Spain)

Table 13.4 Threshold values and sensory properties of virgin olive oil aldehyde off-flavor components

Volatiles[a]	Sensory properties[b]	OT[c]
Hexanal	Fatty, powerful, oily, grassy	0.32
Nonanal	Fatty, waxy, painty, citrus	13.50
2-Heptenal	Oxidised, tallowy, pungent	–
2-Decenal	Painty, fishy, fatty	–
Pentanal	Woody, bitter, oily	0.24
2,4 Heptadienal	Fatty, rancid (hazelnut), cinnamon	3.60
Undecanal	Fatty, tallowy	–
Heptanal	Oily, fatty, heavy, woody, penetrating, nutty	3.20
Octanal	Fatty, sharp, citrus	0.32
2-Nonenal	Penetrating, fatty, waxy, beany, rancid	3.20
Decanal	Penetrating, sweet, waxy, painty	6.70
2,4-Decadienal	Powerful, fatty, citrus	2.15
2-Hexenal	Sweet, fragrant, almond, fruity, green, leafy	10.0
2-Octenal	Brown beans, herbaceous, spicy	–
2-Undecenal	Fresh, fruity, orange peel	–

OT odor threshold
[a]Chemical compounds identified and quantified by Morales et al. (1997)
[b]Sensory characteristics found in the literature (Kochhar 1993)
[c]Odor threshold values (mg/kg) in paraffin oil obtained from Meijboom (1964)

process continued. The great increase in the total volatile content, showing the progressive deterioration in the olive oil, corresponded to the induction time, determined by the Rancimat method as 46.4 ± 0.35 h. Once the initial volatiles disappeared, the concentration of certain volatiles increased, for instance, 2-farnesene greatly increased after 5 h oxidation. During subsequent hours the concentrations of several aldehydes increased, such as hexanal, produced by breakdown of linoleate 13-OOH, nonanal and 2-decenal, arising from oleate 9-OOH, and 2-heptenal, by decomposition of linoleate 12-OOH. Pentanal and heptanal originating from the decomposition of linoleate 13/11-OOH and octanal from oleate 11-OOH were also produced in the following hours. 2-Undecenal from oleate 8-OOH was also greatly increased. Almost all these volatiles are responsible for VOO off-flavors because their threshold levels for odor are very low, as can be seen in Table 13.4. This table also shows the aldehydes identified during the process and their odor thresholds (Meijboom 1964), which are low enough to contribute to olive oil off-flavor; in fact all of them are chemical compounds having sensory properties.

Hexanal/Nonanal Ratio. The advance of oxidation processes in refined vegetable oils is indicated by the increase in total volatiles and the concentration of some specific volatile compounds such as hexanal (Snyder et al. 1988; Warner et al. 1988). Some of the volatiles found in oxidized samples are also present in the initial flavor of VOO. This is true for hexanal, which is present in the initial VOO flavor because it is produced from linoleic acid through the lipoxygenase pathway. It has been demonstrated that hexanal is an important flavor compound of VOO and contributes

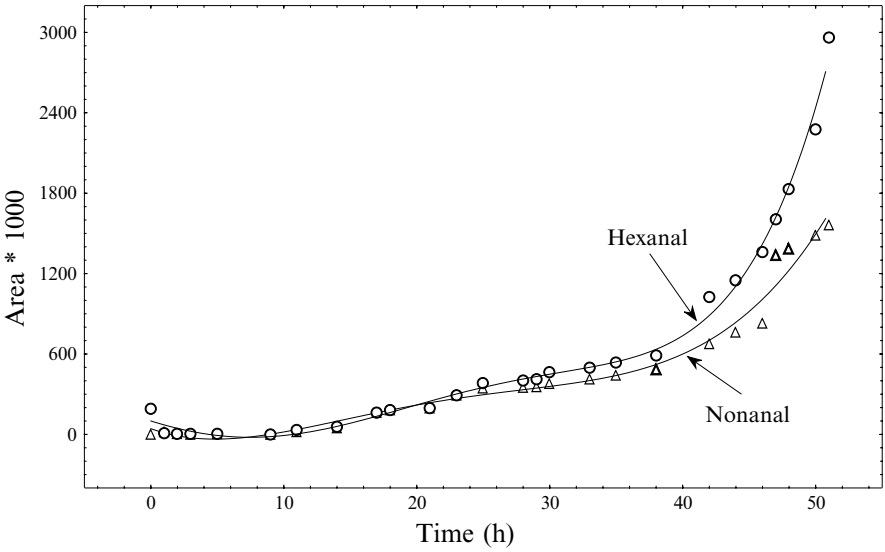

Fig. 13.9 Changes in hexanal and nonanal content of virgin olive oil during oxidative deterioration process (Courtesy of SEXIA Group, Instituto de la Grasa, Sevilla, Spain)

to the sweet perception (Morales et al. 1996; Aparicio et al. 1996b). On the other hand, it has been demonstrated that the hexanal content in VOO is positively correlated with the overall acceptability of potential and habitual consumers of VOO (McEwan 1994). In consequence, hexanal is not an adequate marker for the beginning of oxidation in the case of VOO, although it has been used with success in refined vegetable oils (Snyder et al. 1988; Warner et al. 1988).

Because the main biochemical pathway – the lipoxygenase pathway – promotes the formation of C_6 volatile compounds from linoleic and linolenic hydroperoxides rather than the C_9 compounds, nonanal was either not found or found only at trace levels in VOO. Nonanal and hexanal follow a similar behavior during VOO oxidation, and the shape of both curves, after a polynomial fit of their whole set of data, was similar (Fig. 13.9). In fact, both sets of data (nonanal and hexanal) have been found to be highly correlated ($R = 0.99$). A regression coefficient of $R = 0.89$ was found for nonanal and $R = 0.84$ for hexanal. But when a linear regression was applied to a partial set of data, from 9 to 46 h of oxidation, $R = 0.98$ was obtained for nonanal and $R = 0.94$ for hexanal. Thus, an appropriate way to detect the beginning of oxidation could be an early measurement of nonanal. In fact, there is a good correlation ($R = 0.98$) between the amount of this compound and the oxidation time from 5 h to the induction period, and an equation has been formulated for explaining the oxidation of a VOO until its induction period, with an acceptable predicted regression ($R = 0.88$) (Morales et al. 1997). Initially, hexanal was very high while nonanal was detected at trace levels, but in the course of the oxidation both compounds were present in similar amounts. Thus, the hexanal/nonanal ratio changes

abruptly from thousands, in gourmet oils, to lower than two in oxidized oils, whatever their levels of oxidation. Because the major part of hexanal does not result from the lipoxygenase cascade but from autoxidation, it would be possible to predict the level of oxidation of a VOO with a predicted regression of R = 0.84.

13.9 Sensory Characterization of Volatile Compounds Responsible for Off-Flavor

Although the sensory properties of VOO are well known (IOC 1996b), there is little information about the sensory characteristics of oxidized olive oils. Morales et al. (1997) trained assessors to evaluate VOO samples subjected to oxidation for several hours. Their results were compared with the evolution of volatiles during the process. Only a few volatiles appeared after 5 h of oxidation, and assessors characterized this oil as refined, fried, oxidized, despite its being well known that refined oils have no volatile compounds since they are deodorized. After 11 h of oxidation, the major volatile compounds were hexanal and nonanal, which smelled fatty and waxy. The assessors basically characterized this oil as rancid. The possible synergy between these compounds and other volatiles present at lower concentration could explain why the assessors characterized this oil as rancid. Hexanal (fatty), 2-heptenal (oxidized, tallowy), nonanal (fatty, waxy, painty), and decanal (penetrating, waxy) were the major volatiles at 21 h, and their sensory descriptors completely agreed with the sensory perceptions of the assessors for this oil – unpleasant, rancid, penetrating. After 21 h of oxidation, several aliphatic acids, such as hexanoic, nonanoic, octanoic, and heptanoic acids, appeared, perhaps having been formed by further oxidation of their corresponding aldehydes. Aliphatic ketones formed by autoxidation of unsaturated fatty acids also contributed to the undesirable flavors of the VOO because they have low threshold values (Kochhar 1993), e.g., 5-hepten-2-methyl-6-one and 3,5-octadien-2-one, characterized as having fatty, fruity odor notes, were found in samples. 1-3-nonadienes arising from linoleate 9-OOH, described as rancid and buttery (Evans et al. 1971), and furans and alcohols such as 1-penten-3-ol, 2-pentanol, 1-octen-3-ol, and octanol were also found in oxidized samples. Aliphatic alcohols make a small contribution to off-flavors because their flavor thresholds are significantly higher (Kochhar 1993) than those of their aldehyde counterparts. The transformation into an alcohol of even a small amount of potent off-flavor aldehyde above the detection level will bring about considerable odor reduction (Eriksson et al. 1977; Kochhar 1993).

Morales et al. (1997) describe that the initially pleasant sensory characteristics of the oil (pleasant, green banana) changed abruptly after 30 min oxidation. All their trained assessors agreed on the initial sensory description of the oil, its overall acceptability being given as 7.1, which that means EVOO according to EC regulations (EC 1995). But this description got progressively worse during olive oil oxidation. After 30 min the initial value decreased to 4.1, which means lampante VOO according to EC regulations (EC 1995), and the assessors agreed that the oil did not smell like a VOO due to the loss of the fresh VOO volatiles. Surprisingly, the PV, at 3.2, had not changed at this point of

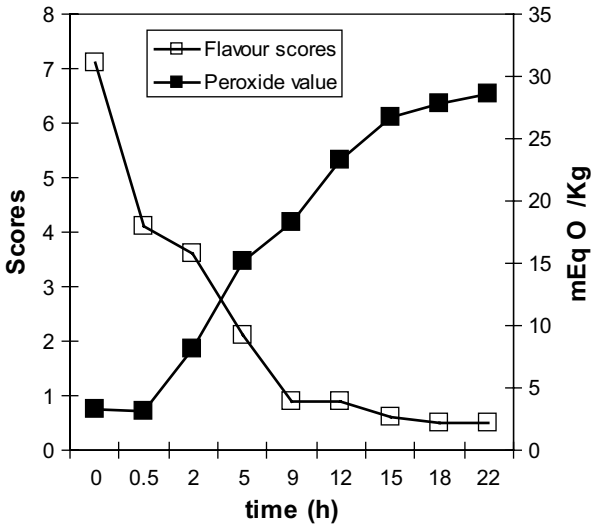

Fig. 13.10 Changes in peroxide value and flavor score of a virgin olive oil sample during oxidative deterioration process (Courtesy of SEXIA Group, Instituto de la Grasa, Sevilla, Spain)

the process (Fig. 13.10). The authors detected an apparent disagreement between sensory evaluation and PV. Thus the odor was described as rancid by assessors after 9 h of oxidation, while the PV remained at 18.3, an accepted value for a VOO in EC regulations (EC 1995, 2011); its maximum is 20. To check this disagreement, the authors carried out a hedonic evaluation that showed that the initially acceptable VOO was rejected in the initial steps of oxidative deterioration. After 12 h of oxidation, assessors stated that they would never have tried the oil. A good correlation ($R=-0.85$) was detected between the flavor scores and the amount of nonanal from 0 to 12 h, although the coefficient abruptly dropped to $R=-0.51$ when the oxidation process was analyzed from 0 to 22 h. The latter result has a logical explanation since the amount of nonanal rose exponentially when the flavor score had reached its minimum.

Morales et al. (1997) also studied the evolution of the total volatile compounds responsible for VOO flavor during the oxidation process. A regression coefficient of $R=0.91$ was obtained by the polynomial fit of the whole set of total volatile compounds versus time. When the linear regression was applied to a selected set of data, from 9 to 46 h (induction time), the linear regression coefficient was $R=0.98$.

13.10 Sensors and Oxidation

An alternative to the analysis of volatile compounds by GC to detect the presence of oxidation secondary products in olive oil has been the use of different kind of sensors, usually arranged as a sensor array and connected to a pattern recognition procedure to discriminate between high-quality and oxidized/rancid oils.

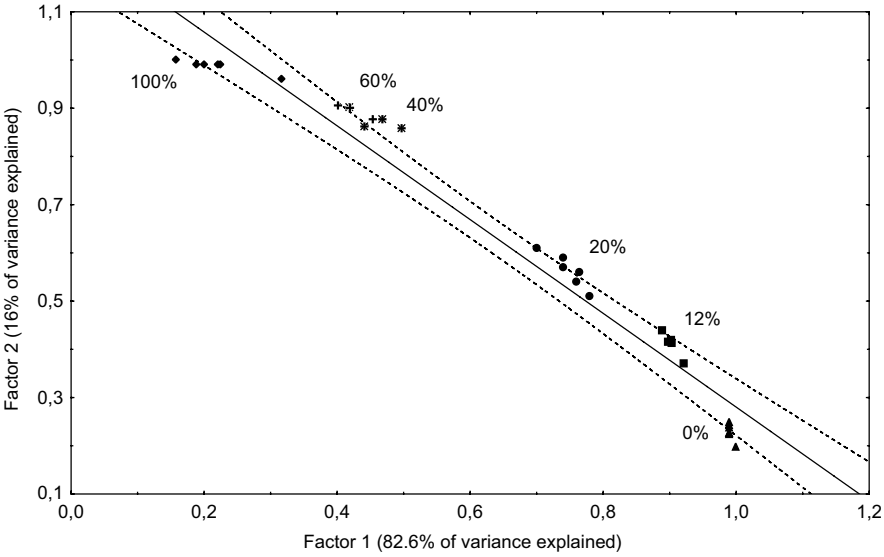

Fig. 13.11 Result of regression on principal components. The codes of each group represent the measures carried out with each sample (blends of virgin olive oil with 0 % (▲), 12 % (■), 20 % (•), 40 % (*), 60 % (+), and 100 % (♦) of the rancid standard) on different days and at different times (*Source*: Aparicio et al. (2000), with permission of American Chemical Society)

Sensors based on semiconductive organic polymers (Kreiss-Rogers 1997; Hodgins 1997) were initially applied to ascertain the quality of VOO and to evaluate rancidity in VOOs (Morales et al. 2000; Aparicio et al. 2000). A selected set of sensors was correlated with the main volatile compounds responsible for the rancid perception using a standard olive oil that is used by the International Oil Council for training assessors. The results (Fig. 13.11) show the utility of this kind of sensor, not only in terms of their linear response but also in detecting low percentages of rancid sensory attributes up to levels that fully trained assessors are barely able to detect properly.

Later, metal oxide semiconductor (MOS) sensors were used to classify olive oils of various qualities (García-González and Aparicio 2004), to evaluate olive oil oxidation during storage (Buratti et al. 2005), and to monitor the oxidative status of VOOs with different phenolic contents (Lerma-García et al. 2009). MOS sensors have also been used as a chromatographic detector by coupling to GC, demonstrating the sensor sensitivity to alcohols, aldehydes, and compounds responsible for the rancid sensory defect in VOO (García-González and Aparicio 2010).

Other kinds of sensors have also been employed to discriminate rancid from good-quality oils, an electronic nose based on SPME coupled with a surface acoustic wave sensor array was used to analyze different quality VOOs (García-González et al. 2004), and recently an artificial olfactory system, based on quartz crystal microbalance sensors using chromatographic stationary phases as sensing films of the sensors has provided promising perspectives to differentiate edible from lampante VOOs (Escuderos et al. 2010).

13.11 Future Trends

The finding of safe and accurate oxidation markers for olive oil, the implementation of efficient sample preparation protocols and powerful analytical separation procedures (using new or improved detectors), the unification of analytical methods for the establishment of antioxidant activity, the measurement of oxidative stability, and the prediction of performance during frying, as well as absorption and bioavailability to clarify the nutritional benefits of VOO, will be some of the aspects related to olive oil oxidation of interest in the near future.

References

Allouche Y, Jimenez A, Gaforio JJ, Uceda M, Beltrán G (2007) How heating affects extra virgin olive oil quality indexes and chemical composition. J Agric Food Chem 55:9646–9654

Alter M, Gutfinger T (1982) Phospholipids in several vegetable oils. Riv Ital Sost Grasse 59:14–18

Andrikopoulos NK, Dedoussis GVZ, Falirea A, Kalogeropoulos N, Hatzinikola HS (2002) Deterioration of natural antioxidant species of vegetable edible oils during the domestic deep-frying and pan-frying of potatoes. Int J Food Sci Nutr 53:351–363

Angerosa F, di Giacinto L (1993) Oxidation of virgin olive oil by metals: Mn and Ni. Rev Fran Corps Gras 40:41–48

Aparicio R, Morales MT (1998) Characterization of olives ripeness by green aroma compounds of virgin olive oil. J Agric Food Chem 46:1116–1122

Aparicio R, Alonso V, Morales MT (1996a) Developments in olive oil authentication. In: Food authenticity: methods for the measurement of food authenticity and adulteration. Royal Society of Chemistry, Norwich, pp 84–122

Aparicio R, Morales MT, Alonso MV (1996b) Relationship between volatile compounds and sensory attributes of olive oils by the sensory wheel. J Am Oil Chem Soc 73:1253–1264

Aparicio R, Roda L, Albi MA, Gutiérrez F (1999) Effect of various compounds on virgin olive oil stability measured by Rancimat. J Agric Food Chem 47:4150–4155

Aparicio R, Rocha SM, Delgadillo I, Morales MT (2000) Detection of rancid defect in virgin olive oil by the electronic nose. J Agric Food Chem 48:853–860

Bastida S, Sanchez-Muniz FJ (2001) Thermal oxidation of olive oil, sunflower oil and a mix of both oils during forty discontinuous domestic fryings of different foods. Food Sci Technol Int 7:15–21

Baur C, Grosch W, Wieser H, Jugel H (1977) Enzymatic oxidation of linoleic acid: formation of bitter tasting fatty acids. Z Lebensm Unters Forsch 164:171–176

Bellus D (1978) Quenchers of singlet oxygen – a critical review. In: Ranby B, Rabek JF (eds) Singlet oxygen reactions with organic compounds and polymers. Wiley, New York, pp 61–110

Beltrán G, Aguilera MP, del Rio C, Sánchez S, Martinez L (2005) Influence of fruit ripening on the natural antioxidant content of Hojiblanca virgin olive oils. Food Chem 89:207–215

Bendini A, Cerretani L, Carrasco-Pancorbo A, Gómez-Caravaca AM, Segura-Carretero A, Fernández-Gutiérrez A, Lercker G (2007) Phenolic molecules in virgin olive oils: a survey of their sensory properties, health effects, antioxidant activity and analytical methods. An overview of the last decade. Molecules 12:1679–1719

Biermann V, Wittmann A, Grosch W (1980) Occurrence of bitter hydroxy fatty acids in oat and wheat. Fette Seifen Anstrichm 82:236–240

Boskou D, Blekas G, Tsimidou M (2005) Phenolic compounds in olive oil and olives. Curr Top Nutraceut R 3:125–136

Boskou G, Salta FN, Chiou A, Troullidou E, Andrikopoulos NK (2006) Content of trans, tans-2,4-decadienal in deep-fried and pan-fried potatoes. Eur J Lipid Sci Technol 108:109–115

Bradley DG, Min DB (1992) Singlet oxygen oxidation of foods. Crit Rev Food Sci Nutr 31:211–236
Brenes M, Garcia A, Dobarganes MC, Velasco J, Romero C (2002) Influence of thermal treatments simulating cooking processes on the polyphenol content in virgin olive oil. J Agric Food Chem 50:5962–5967
Buratti S, Benedetti S, Cosio MS (2005) An electronic nose to evaluate olive oil oxidation during storage. Ital J Food Sci 2:203–221
Carlsson DJ, Suprunchuk W, Wiles DM (1976) Photooxidation of unsaturated oils: effects of singlet oxygen quenchers. J Am Oil Chem Soc 53:656–659
Casal S, Malheiro R, Sendas A, Oliveira BPP, Pereira JA (2010) Olive oil stability under deep – frying conditions. Food Chem Toxicol 48:2972–2979
Choe E, Min DB (2006) Mechanisms and factors for edible oil oxidation. Compr Rev Food Sci F 5:169–186
Choe E, Min DB (2009) Mechanisms of antioxidants in the oxidation of foods. Compr Rev Food Sci F 8:345–358
Christopher JP, Pistorius EK, Regnier FE, Axelrod B (1972) Factors influencing the positional specificity of soybean lipoxygenase. Biochem Biophys Acta 289:82–87
Christopoulou CN, Perkins EG (1989) Isolation and characterization of dimers formed in used soybean oil. J Am Oil Chem Soc 66:1360–1370
Chung J, Lee J, Choe E (2004) Oxidative stability of soybean and sesame oil mixture during frying of flour dough. J Food Sci 69:574–578
Clark JP, Hunsicker JC, Megremis CJ (1990) Tocopherols: natures antioxidants. Food Austr 42:262–263
Criado MN, Motilva MJ, Goñi M, Romero MP (2007) Comparative study of the maturation process of the olive fruit on the chlorophylls and carotenoid fractions of drupes and virgin oils from Arbequina and Farga cultivars. Food Chem 100:748–755
Cuvelier ME, Richard H, Berset C (1992) Comparison of the antioxidative activity of some acid-phenols: structure-activity relationship. Biosci Biotech Biochem 56:324–325
Daniels DG, Martin HF (1961) Isolation of a new antioxidant from oat. Nature 191:1302–1303
De Man JM, Tie F, De Man L (1987) Formation of short chain volatile organic acids in the automated AOM method. J Am Oil Chem Soc 64:993–995
Del Carlo M, Cerretani L, Bendini A, Cichelli A, Compagnone D (2010) Changes of pigment composition of virgin olive during frying process. Riv Ital Sostanze Grasse 87:3–13
Dobarganes MC, Márquez-Ruiz G, Velasco J (2000) Interactions between fat and food during deep-frying. European Journal of Lipid Science and Technology 102:521–528
Dobarganes MC, Perez-Camino MC (1988) Fatty acid composition: a useful tool for the determination of alteration level in heated fats. Rev Franç Corps Gras 35:67–70
Dobarganes MC, Rios JJ, Pérez-Camino MC (1986) Relationship between the composition of vegetable oils and the volatile components produced in their thermoxidation. Grasas Aceites 37:61–67
Dziedzic SZ, Hudson BJE (1984) Phenolic acids and related compounds as antioxidants for edible oils. Food Chem 14:45–51
EC (1995) Official Journal of the Commission of the European Communities, Regulation No. 656/95, L69, Mar 28
EC (1997) Official Journal of the Commission of the European Communities, Regulation No. 2472/97, L341, Dec 11
EC (2007) Official Journal of the Commission of the European Communities, Regulation No. 702/2007, L 161, June 21
EC (2008) Official Journal of the Commission of the European Communities, Regulation No. 640/2008, L 178, July 4
EC (2011) Official Journal of the European Union, Regulation No. 61/2011, L23, January 24
El Riachy M, Priego-Capote F, León L, Rallo L, Luque de Castro MD (2011) Hydrophilic antioxidants of virgin olive oil. Part 1: hydrophilic phenols: a key factor for virgin olive oil quality. Eur J Lipid Sci Tech 113:678–691

Endo Y, Usuki R, Keneda T (1984) Prooxidant activities of chlorophylls and their decomposition products on the photooxidation. J Am Oil Chem Soc 61:781–784
Eriksson CE, Qvist JA, Vallentin K (1977) Conversion of aldehydes to alcohols in liquid foods by alcohol dehydrogenase. In: Ory RL, St. Angelo AJ (eds) Enzymes in food and beverage processing. American Oil Chemists' Society, Champaign, pp 132–142
Escuderos ME, Sánchez S, Jiménez A (2010) Virgin olive oil sensory evaluation by an artificial olfactory system, based on quartz crystal microbalance (QCM) sensors. Sens Act B: Chemical 147:159–164
Eskin NAM, Vaisey-Genser M (1989) Applications for genetically modified oils. J Am Oil Chem Soc 66:1058–1063
Eskin NAM, Vaisey-Genser M, Durance–Tod S, Przybylski R (1989) Stability of low linolenic acid canola oil to frying temperatures. J Am Oil Chem Soc 66:1081–1084
Evans CD, Moser HA, List GR (1971) Odour and flavour responses to additives in edible oils. J Am Oil Chem Soc 48:495–498
Evans CD, List GR, Moser HA, Conan IC (1973) Long term storage of soybean and cottonseed salad oils. J Am Oil Chem Soc 50:218–220
Feussner I, Kuhn H, Wasternack C (2001) Lipoxygenase-dependent degradation of storage lipids. Trends Plant Sci 6:268–273
Feussner I, Wasternack C (2002) The lipoxygenase pathway. Annu Rev Plant Biol 53:275–297
Foote CS (1968) Mechanism of photosensitized oxidation. Science 162:963–970
Foote CS (1976) Photosensitized oxidation and singlet oxygen: consequences in biological system. In: Pryor WA (ed) Free radicals in biology, vol 2. Academic, New York, pp 85–97
Foote CS, Denny RW (1970) Chemistry of singlet oxygen. J Am Oil Chem Soc 92:5216–5218
Frankel EN (1962) Hydroperoxides. In: Schultz HW, Day EA, Sinnhuber RO (eds) Symposium on foods: lipids and their oxidation. AVI Publishing Co., Westport, pp 51–78
Frankel EN (1983) Volatile lipid oxidation-products. Prog Lipid Res 22:1–33
Frankel EN (1985) Chemistry of autoxidation: mechanism, products and flavor significance. In: Min DB, Smouse TH (eds) Flavor chemistry of fats and oils. AOCS, Champaign, pp 1–37
Frankel EN (1989) The antioxidant and nutritional effects of tocopherols, ascorbic acid and β-carotene in relation to processing of edible oils. Bibl Nutr Dieta 43:297–312
Frankel EN (1991) Recent advances in lipid oxidation. J Sci Food Agric 54:495–511
Frankel EN (1993) In search of better methods to evaluate natural antioxidants and oxidative stability in food lipids. Trends Food Sci Technol 4:220–223
Frankel EN (1996) Antioxidants in lipid foods and their impact on food quality. Food Chem 57:51–55
Frankel EN (2005) Lipid oxidation, 2nd edn. Oily Press, Bridgwater
Frega N, Mozzon M, Lercker G (1999) Effects of free fatty acids on oxidative stability of vegetable oil. J Am Oil Chem Soc 76:325–329
Gallardo-Guerrero L, Gandul-Rojas B, Roca M, Mínguez-Mosquera MI (2005) Effect of storage on the original pigment profile of Spanish virgin olive oil. J Am Oil Chem Soc 82:33–39
Gallina-Toschi T, Cerretani L, Bendini A, Bonoli-Carbognin M, Lercker G (2005) Oxidative stability and phenolic content of virgin olive oil: An analytical approach by traditional and high resolution techniques. Journal of Separation Science 28:859–870
Gandul-Rojas B, Mínguez-Mosquera MI (1996) Chlorophyll and carotenoid composition in virgin olive oils from various Spanish olive varieties. J Sci Food Agric 72:31–39
Gandul-Rojas B, Cepero MR, Mínguez-Mosquera MI (2000) Use of chlorophyll and carotenoid composition to determine authenticity of virgin olive oil. J Am Oil Chem Soc 77:853–858
García-González DL, Aparicio R (2004) Classification of different quality virgin olive oils by metal-oxide sensors. Eur Food ResTechno 218:484–487
García-González DL, Aparicio R (2010) Coupling MOS sensors and gas chromatography to interpret the sensor responses to complex food aroma application to virgin olive oil. Food Chem 120:572–579

García-González DL, Barié N, Rapp M, Aparicio R (2004) Analysis of virgin olive oil volatiles by a novel electronic nose based on a miniaturized SAW sensor array coupled with SPME enhanced headspace enrichment. J Agric Food Chem 52:7475–7479

Gardner HW (1987) Reactions of hydroperoxides-products of high molecular weight. In: Chan HW-S (ed) Autoxidation of unsaturated lipids. Academic, London

Gimeno E, Castellote AI, Lamuela-Raventós RM, de la Torre MC, López-Sabater MC (2002) The effects of harvest and extraction methods on the antioxidant content phenolics, α-tocopherol, and β-carotene in virgin olive oil. Food Chem 78:207–211

Giuliani A, Cerretani L, Cichelli A (2011) Chlorophylls in olive and in olive oil: chemistry and occurrences. Crit Rev Food Sci Nutr 51:678–690

Gomez-Alonso S, Fregapane G, Salvador MD, Gordon MH (2003) Changes in phenolic composition and antioxidant activity of virgin olive oil during frying. J Agric Food Chem 51:667–672

Gordon MH, Magos P (1983) The effect of sterols on the oxidation of edible oils. Food Chem 10:141–147

Guichardant M, Lagarde M (2009) Analysis of biomarkers from lipid peroxidation: a comparative study. Eur J Lipid Sci Technol 11:175–182

Gutiérrez-Rosales F, Garrido-Fernández J, Gallardo-Guerrero L, Gandul-Rojas B, Mínguez-Mosquera MI (1992) Action of chlorophylls on the stability of virgin olive oil. J Am Oil Chem Soc 69:866–871

Haila K, Heinonen M (1994) Action of β-carotene on purified rapeseed oil during light storage. Lebensm Wiss Technol 27:573–577

Halliwell B, Gutteridge JMC (2001) Free radicals in biology and medicine, 3rd edn. Oxford University Press, New York, p 297

Heath HB, Reineccius GA (1986) Flavor chemistry and technology. AVI Publishing, Westport, pp 112–141

Henick AS, Benca MF, Mitchell JH (1954) Estimation of carbonyl compounds in rancid fats and foods. J Am Oil Chem Soc 31:881–883

Hiatt R, Mill T, Irwin KC, Mayo TR, Gould CW, Castleman JK (1968) Homolytic decompositions of hydroperoxides. I. Summary and implications for autoxidation. J Org Chem 33:1416–1441

Ho CT, Shahidi F (2005) Flavor components of fats and oils. In: Shahidi F (ed) Bailey's industrial oil and fat products, 6th edn. Wiley, Hoboken, pp 387–411

Hodgins D (1997) The electronic nose: sensor array-based instruments that emulate the human nose. In: Marsili R (ed) Techniques for analyzing food aroma. Marcel Dekker, New York, pp 331–371

Holm U (1972) Quality criteria for edible fats. SIK-Report, No 448. SIK Institute, Göteborg, Sweden, pp 79–88

Houhoula DP, Oreopoulou V, Tzia C (2003) The effect of process time and temperature on the accumulation of polar compounds in cottonseed oil during deep-fat frying. J Sci Food Agric 83:314–319

Huang SW, Frankel EN, German JB (1994) Antioxidant activity of α-and γ-tocopherols in bulk oils and in oil-in-water emulsions. J Agric Food Chem 42:2108–2114

Hudson BJF, Ghovami M (1984) Phospholipids as antioxidant synergists for tocopherols in the autoxidation of edible oils. Lebensm Wiss Technol 17:191–194

Husain SR, Terao J, Matsushita S (1986) Effect of browning reaction products of phospholipids on autoxidation of methyl linoleate. J Am Oil Chem Soc 63:1457–1460

Ingold KU (1969) Peroxy radicals. Accounts Chem Res 2:1–14

Interesse F, Ruggiero S, Vitagliano M (1971) Autoxidation of olive oil: influence of chlorophyll pigments. Ind Agric 9:318–323

International Olive Oil Council (IOC) (1996a) Trade standard applying to olive oil and olive pomace oil. COI/T.15/Doc. nº 2/Rev. 5, Madrid, 20 Nov

International Olive Oil Council (IOC) (1996b) Organoleptic evaluation of virgin olive oil. COI/T.20/Doc. nº 15/Rev.1, Madrid, 20 Nov

Jawad IM, Kochhar SP, Hudson BJF (1984) The physical refining of edible oils. II. Effect on unsaponifiable components. Lebensm Wiss Technol 17:155–159

Jung MY, Min DB (1991) α-, γ-, δ-Tocopherols effects on chlorophyll photosensitized oxidation of soybean oil. J Food Sci 56:807–815
Jung MY, Yoon SH, Min DB (1989) Effects of processing steps on the contents of minor compounds and oxidation of soybean oil. J Am Oil Chem Soc 66:118–120
Kasai H, Kawai K (2008) 4-oxo-2-hexenal, a mutagen formed by omega-3 fat peroxidation: occurrence, detection and adduct formation. Mutat Res-Rev Mutat 659:56–59
Keceli T, Gordon MH (2002) Ferric ions reduce the antioxidant activity of the phenolic fraction of virgin olive oil. J Food Sci 67:943–947
Kim IH, Kim CJ, Kim DH. 1999. Physicochemical properties of methyl linoleate oxidized at various temperatures. Korean J Food Sci Technol 31:600–605
Kiritsakis A (1990) Olive oil. American Oil Chemists' Society, Champaign, pp 104–127
Kiritsakis A, Dugan LR (1985) Studies in photooxidation of olive oil. J Am Oil Chem Soc 62:892–896
Kiritsakis A, Tsipeli A (1992) Relationship of the acidity of olive oil to its resistance to oxidation. Riv Ital Sost Grasse 69:513–515
Knothe G, Kenar JA, Gunstone FD (2007) Chemical properties. Chapter 8. In: Harwood JL, Gunstone FD, Dijkstra AJ (eds) The lipid handbook with CD-ROM, 3rd edn. CRC Press, Boca-Ratón, pp 535–589
Kochhar SP (1993) Oxidative pathways to the formation of off-flavours. In: Saxby MJ (ed) Food taints and off-flavours. Blackie Academic & Professional, London, pp 150–201
Kochhar SP (2000) Stable and healthful oils for the 21st century. Inform 11:642–647
Kreiss-Rogers E (1997) Biosensors and electronic noses for practical applications. In: Kress-Rogers E (ed) Handbook of biosensors and electronic noses. CRC Press, Boca-Ratón, pp 3–39
Labuza TP (1971) Kinetics of lipid oxidation in foods. Crit Rev Food Sci Nutr 2:355–395
Lavelli V, Bondesan L (2005) Secoiridoids, tocopherols, and antioxidant activity of monovarietal extra virgin olive oils extracted from destoned fruits. J Agric Food Chem 53:1102–1107
Lee SH, Kim DH (1992) Effects of β-carotene on the stability of soybean oil subject to autoxidation and photosensitized oxidation. Food Biotechnol 1:1–7
Lee SH, Min DB (1990) Effects, quenching mechanism, and kinetics of carotenoids in chlorophyll-sensitized photooxidation of soybean oil. J Agric Food Chem 38:1630–1634
Leone AM, LaNotte E, Lamparelli F (1976) Analytical significance of sterol fraction of virgin olive oil. Riv Techol Alim Nutr 6:205–209
Lerma-García MJ, Simó-Alfonso EF, Bendini A, Cerretani L (2009) Metal oxide semiconductor sensors for monitoring of oxidative status evolution and sensory analysis of virgin olive oils with different phenolic content. Food Chem 117:608–614
Luna G, Morales MT, Aparicio R (2006) Changes induced by UV radiation during virgin olive oil storage. J Agric Food Chem 54:4790–4794
Mariani CS, Venturini P, Fedeli E (1993) Determination of hydrocarbons and free and esterified minor components in olive oil of different classes. Riv Ital Sost Grasse 70:321–327
Martín-Polvillo M, Albi T, Guinda A (1994) Determination of trace elements in edible vegetable oils by atomic absorption spectrophotometry. J Am Oil Chem Soc 71:347–353
Matthaeus B (2010) Oxidation of edible oils. In: Foods and beverages and antioxidant applications, vol 2, Woodhead Publishing series in food science, technology and nutrition oxidation. Woodhead Publishing, Oxford/Philadelphia, pp 183–238
May WA, Peterson RJ, Chang SS (1983) Chemical-reactions involved in the deep-fat frying of foods. 9. Identification of the volatile decomposition products of triolein. J Am Oil Chem Soc 60:990–995
McEwan JA (1994) Consumer attitudes and olive oil acceptance: the potential consumer. Grasas Aceites 45:9–15
McMullen LM, Hawrysh ZJ (1991) Ascorbyl palmitate efficacy in enhancing the accelerated storage stability of canola oil. J Food Sci 56:1651–1654
Mehta U, Swinburn B (2001) A review of factors affecting fat absorption in hot chips. Crit Rev Food Sci Nutr 41:133–154

Meijboom PW (1964) Relationship between molecular structure and flavour perceptibility of aliphatic aldehydes. J Am Oil Chem Soc 41:326–328
Min DB, Boff JM (2002) Lipid oxidation of edible oil. In: Akoh CC, Min DB (eds) Food lipids. Marcel Dekker, New York, p 344
Mistry BS, Min DB (1987) Effects of fatty acids on the oxidative stability of soybean oil. J Food Sci 52:831–832
Miyashita K, Takagi T (1986) Study on the oxidative rate and prooxidant activity of free fatty acids. J Am Oil Chem Soc 63:1380–1384
Morales MT, Aparicio R, Aparicio-Ruiz R, García-González DL (2000) Detection of defects in virgin olive oil by the electronic nose. In: Flavour and Fragrance Chemistry. Dordrecht: Kluwer, pp 151–161
Morales MT, Aparicio R, Calvente JJ (1996) Influence of olive ripeness on the concentration of green aroma compounds in virgin olive oil. Flavour Fragr J 11:171–178
Morales MT, Rios JJ, Aparicio R (1997) Changes in the volatile composition of virgin olive oil during oxidation: flavors and off-flavors. J Agric Food Chem 45:2666–2673
Morales MT, Angerosa F, Aparicio R (1999) Effect of the extraction conditions of virgin olive oil on the lipoxygenase cascade: chemical and sensory implications. Grasas Aceites 50:114–121
Morales MT, Luna G, Aparicio R (2005) Comparative study of virgin olive oil sensory defects. Food Chem 91:293–301
Moreira RG, Sun X, Chen Y (1997) Factors affecting oil uptake in tortilla chips in deep-fat frying. J Food Eng 31:485–498
Moreira RG, Castell-Perez ME, Barrufet MA (1999a) Oil absorption in fried foods. In: Deep-fat frying; fundamentals and applications. Chapman & Hall Food Science Book, Gaithersburg, pp 179–221
Moreira RG, Castell-Perez ME, Barrufet MA (1999b) Frying oil characteristics. In: Deep-fat frying; fundamentals and applications. Chapman & Hall, Gaithersburg, pp 33–74
Nawar WW (1969) Thermal degradation of lipids. A review. J Agric Food Chem 17:18–21
Nawar WW (1985) Chemistry of thermal oxidation. In: Min DB, Smouse TH (eds) Flavor chemistry of fats and oils. American Oil Chemists Society, Champaign, pp 39–60
Noor N, Augustin MA (1984) Effectiveness of antioxidants on the stability of banana chips. J Sci Food Agric 35:805–808
Papadopoulos G, Boskou D (1991) Antioxidant effect of natural phenols on olive oil. J Am Oil Chem Soc 68:669–671
Perrin JL (1992) Minor constituents and natural antioxidants of olives and olive oil. Rev Franç Corps Gras 39:25–32
Pokorny J (1990) Influence of lipid alteration on taste and flavour of foods. Nahrung 34:887–897
Pokorny J (1991) Natural antioxidants for food use. Trends Food Sci Technol 2:223–227
Przybylski R, Eskin NAM (1988) A comparative-study on the effectiveness of nitrogen or carbon-dioxide flushing in preventing oxidation during the heating of oil. J Am Oil Chem Soc 65:629–633
Przybylski R, Eskin NAM (1995) Methods to measure volatile compounds and the flavor significance of volatile compounds. In: Methods to assess quality and stability of oils and fat-containing foods. AOCS Press, Champaign, pp 107–133
Przybylski R, Hougen FW (1989) Simple method for estimation of volatile carbonyl compounds in edible oils and fried potato chips. J Am Oil Chem Soc 66:1456–1458
Przybylski R, Malcolmson LJ, Eskin NAM, Durance-Tod S, Mickle J, Carr R (1993) Stability of low linolenic acid canola oil to accelerated storage at 60 °C. Lebensm Wiss Technol 26:205–209
Rastrelli L, Passi S, Ippolito F, Vacca G, De Simone F (2002) Rate of degradation of alpha-tocopherol, squalene, phenolics, and polyunsaturated fatty acids in olive oil during different storage conditions. J Agric Food Chem 50:5566–5570
Rawls HR, Van Santen PJ (1970) A possible role for singlet oxygen in the initiation of fatty acid autoxidation. J Am Oil Chem Soc 47:121–124

Robards K, Prenzler PD, Tucker G, Swatsitang P, Glover W (1999) Phenolic compounds and their role in oxidative processes in fruits. Food Chem 66:401–436
Roca M, Mínguez-Mosquera MI (2001) Changes in the natural ratio between chlorophylls and carotenoids in olive fruit during processing for virgin olive oil. J Am Oil Chem Soc 78:133–138
Rontani JF, Jameson I, Christodoulou S, Volkman JK (2007) Free radical oxidation (autoxidation) of alkenones and other lipids in cells of *Emiliania huxleyi*. Phytochemistry 68:913–924
Rossell JB (1987) Measurement of rancidity. In: Allen JC, Hamilton RJ (eds) Rancidity in foods. Applied Science Publishers, London, pp 21–45
Rossell JB (2001) Factors affecting the quality of frying oils and fats. In: Rossell JB (ed) Frying. Improving quality. Woodhead Publishing Ltd., Cambridge, pp 116–164
Rudzinska M, Przybylski R, Wasowicz E (2009) Products formed during thermo-oxidative degradation of phytosterols. J Am Oil Chem Soc 86:651–662
Rudzinska M, Przybylski R, Zhao YY, Curtis JM (2010) Sitosterol thermo-oxidative degradation leads to the formation of dimers, trimers and oligomers: a study using combined size exclusion chromatography/mass spectrometry. Lipids 45:549–558
Ruiz-Lopez M, Artacho R, Pineda MAF, Garcia HL, Martinez MCL (1995) Stability of α-tocopherol in virgin olive oil during microwave heating. Lebensm Wiss Technol 28:644–646
Ryan E, McCarthy FO, Maguire AR, O'Brien NM (2009) Phytosterol oxidation products: their formation, occurrence, and biological effects. Food Rev Int 25:157–174
Salta FN, Kalogeropoulos N, Karavanou N, Andrikopoulos NK (2008) Distribution and retention of phytosterols in frying oils and fried potatoes during repeated deep and pan frying. Eur Food Res Technol 227:391–400
Sanders TAB (1983) Nutritional significance of rancidity. In: Allen JC, Hamilton RJ (eds) Rancidity in foods. Applied Science Publishers, London, pp 59–66
Sayago A, Marín MI, Aparicio R, Morales MT (2007) Vitamin E and vegetable oils. Grasas Aceites 58:74–86
Schaich KM (1992) Metals and lipid oxidation. Contemporary issues. Lipids 27:209–218
Schieberle P, Grosch W (1981) Model experiments about the formation of volatile carbonyl compounds. J Am Oil Chem Soc 58:602–607
Servili M, Selvaggini R, Esposto S, Taticchi A, Montedoro G, Morozzi G (2004) Health and sensory properties of virgin olive oil hydrophilic phenols: agronomic and technological aspects of production that affect their occurrence in the oil. J Chromatogr A 1054:113–127
Shahidi F, Wanasundra PD (1992) Phenolic antioxidants. Crit Revs Food Sci Nutr 32:67–103
Siedow JN (1991) Plant lipoxygenase – structure and function. Ann Rev Plant Physiol Plant Mol Biol 42:145–188
Simic MG, Jovanovic SV, Niki E (1992) Mechanisms of lipid oxidative process and their inhibition. In: St. Angelo AJ (ed) Lipid oxidation in food. American Chemical Society, Washington, DC, pp 33–54
Snyder JM, Frankel EN, Selke E, Warner K (1988) Comparison of gas chromatographic methods for volatile lipid oxidation compounds in soybean oil. J Am Oil Chem Soc 65:1617–1620
Solinas M, Angerosa F, Camera L (1988) Evoluzione ossidativa di oli vegetali durante la frittura: determinazione dei componenti volatili mediante HRGC e HPLC. Riv Ital Sost Grasse 65:567–574
Solinas M, Angerosa F, Cucurachi A (1987) Connessione tra prodotti di neoformazione ossidativa delle sostanze grasse e insorgenza del difetto di rancidità all' esame organolettico. Nota 2. Determinazione quantitativa. Riv Ital Sost Grasse 64:137–145
St. Angelo A (1996) Lipid oxidation in foods. Crit Rev Food Sci Nutr 36:175–224
Tena N (2010) Evolution of major and minor components of thermoxidised olive oils: implementation of spectroscopic and chromatographic methods. Ph.D. thesis, University of Seville
Toschi TG, Costa A, Lercker G (1997) Gas chromatographic study on high-temperature thermal degradation products of methyl linoleate hydroperoxides. J Am Oil Chem Soc 74:387–391

Tseng YC, Moreira RG, Sun X (1996) Total frying-use time effects on soybean-oil deterioration and on tortilla chip quality. Intl J Food Sci Technol 31:287–294
Uriarte PS, Guillén MD (2010) Formation of toxic alkylbenzenes in edible oils submitted to frying temperature. Influence of oil composition in main components and heating time. Food Res Internat 43:2161–2170
Usuki R, Endo Y, Kaneda T (1984) Prooxidant activities of chlorophylls, and pheophytins on the photooxidation of edible oils. Agric Biol Chem 48:991–994
Velasco J, Dobarganes C (2002) Oxidative stability of virgin olive oil. Eur J Lipid Sci Technol 104:661–676
Vercellotti JR, St. Angelo AJ, Spanier AM (1992) Lipid oxidation in food: an overview. In: St. Angelo AJ (ed) Lipid oxidation in foods. American Chemical Society, Washington, DC, pp 1–11
Wang T, Hammond EG (2010) Lipoxygenase and lipid oxidation in foods. In: Decker EA, Elias RJ, McClements DJ (eds) Oxidation in foods and beverages and antioxidant applications, vol 1, Understanding mechanisms of oxidation and antioxidant activity. Woodhead Publishing, Cambridge, pp 105–121
Warner K, Frankel EN (1987) Effect of β-carotene on light stability of soybean oil. J Am Oil Chem Soc 64:213–218
Warner K, Frankel EN, Moulton KJ (1988) Flavor evaluation of crude oil to predict the quality of soybean oil. J Am Oil Chem Soc 65:386–391
Warner K, Frankel EN, Mounts TL (1989) Flavor and oxidative stability of soybean, sunflower and low erucic rapeseed oils. J Am Oil Chem Soc 66:558–562
White PJ, Armstrong LS (1986) Effect of selected oat sterols on the deterioration of heated soybean oil. J Am Oil Chem Soc 63:525–529
WHO (2003) Diet, nutrition and the prevention of chronic diseases. Technical report series 916. World Health Organization, Geneva
Wu CM, Chen SY (1992) Volatile compounds in oils after deep frying or stir frying and subsequent storage. J Am Oil Chem Soc 69:858–865
Wu H, Weng X (2001) Antioxidant activity of natural tocopherol. Ziran Kexueban 7:142–146
Yamaguchi R, Matsushita S (1977) Quenching effect of tocopherols on methyl linoleate photooxidation and their oxidation products. Agric Biol Chem 41:1425–1430
Yanishleva N, Schiller H (1984) Effect of sitosterol on autoxidation rate and product composition in a model lipid system. J Sci Food Agric 35:219–224
Yoon SH, Jung MY, Min DB (1988) Effects of thermally oxidized triglycerides on the oxidative stability of soybean oil. J Am Oil Chem Soc 65:1652–1656
Zambiazi RC (1997) The role of endogenous lipid components on vegetable oil stability. Ph.D. thesis, University of Manitoba, Canada

Chapter 14
Sensory Quality: Methodologies and Applications

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Contents

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties*,
DOI 10.1007/978-1-4614-7777-8_14, © Springer Science+Business Media New York 2013

14.1 Introduction

Generally, the sensory quality of a food influences its acceptability and desirability. It is determined by a set of positive characteristics evaluated through sense organs. Color, odor, taste, and tactile sensations have a true physiological function as they stimulate the appetite and gratification effect and make foods acceptable.

Stimulation of the sense organs from volatile and nonvolatile compounds gives rise to a spectrum of perceptions that are memorized as characteristic of a given product and allows its correct identification when next experienced.

Quality attributes that can be detected through the sense organs are appearance, size and shape, color, viscosity, kinesthetic sensations (e.g., hardness, fibrousness, palatability, crispness), tactile sensations, chemesthetic sensations (the combined response to cutaneous, thermal, and painful stimuli elicited by some irritants), odor, and taste. The number of attributes is drastically reduced for olive oils because some, such as size, shape, and kinesthetic sensations, have no significance for liquid materials, while others, such as cloudiness and viscosity, can be easily modified or measured using suitable instruments. Therefore, only color, odor, taste, sensations such as oiliness and fluidity, and chemesthetic sensations (e.g., pungent and astringent) have importance in the evaluation of the sensory quality of olive oils.

Analytical methods have been investigated to identify and quantify chemical volatile and nonvolatile compounds responsible for flavor (Chaps. 8 and 9). However, sometimes the search for relationships is made difficult by the fact that these connections may exist between a single odor note and the sum of volatiles, rather than between a single odor note and a single compound (Piggott 1990). Thus, the possible relationships between detected components and the sensory perceptions have been pointed out by the application of multivariate statistical procedures (Aparicio et al. 1996; Morales and Aparicio 1993; Morales et al. 1995). For example, the close relationship between volatiles and desirable and undesirable sensory perceptions has been considered by several researchers to be a useful tool to describe and quantify oil flavor (Angerosa et al. 1996a; Aparicio and Morales 1994).

In addition, the qualitative and quantitative determinations of volatiles, even if carried out by means of concentration systems for oil aroma, do not provide information about flavor because they cannot render an account of the interactions occurring in the olfactory system or between taste and smell. Because of the chemical complexity of olive oil, it is very difficult to know the kinds of mixed interactions involved (Breslin 1996; Lawless 1986). It is likely that there are interactions within volatile compounds; interactions of odor with taste, of color with flavor and taste, and of taste with viscosity; interactions between oral trigeminal irritations from some compounds such as secoiridoid compounds and taste and odor. The result of all these complex interactions is that the strength of a few sensations elicited by some compounds could be suppressed, whereas the sensations of other stimuli could be enhanced. Moreover, new qualities can be produced by the perceptual fusion and blending of taste and odor sensations (Erickson and Covey 1980). Even more, it should be taken into account that high concentrations of a volatile compound do not always result in a strong odor response.

Thus, in spite of the progress made in the analytical field, some problems have not been resolved completely using only instrumental analyses. Therefore, sensory analysis is still the most effective means to evaluate differences, qualities, and quantities of the sensory stimuli elicited by foods and to determine whether they are liked or preferred.

14.2 Quality Parameters of Olive Oils

Color, taste, and aroma are the main parameters for the definition of the quality of olive oils. These attributes are ascribable to certain compounds retained in olive oil as the result of its mechanical extraction from the fruit of the olive tree (*Olea europaea* L.) and to the fact that it usually can be consumed without further treatment.

14.2.1 Color

Color is perhaps the most important sensory characteristic of foodstuffs, especially if it is related to some other aspects of quality.

The color of virgin olive oils (VOOs), when just extracted, ranges between light yellow and a more or less deep green depending on the content of liposoluble pigments (chlorophylls and carotenoids) naturally occurring in the fruits. Chlorophylls give oils their yellow/green color, whereas carotenoids determine shades between yellow and red. The level of these pigments is related to genetic factors, to the ripening degree of olives, and to the conditions adopted for oil extraction. Such pigments decrease in concentration as the fruit ripens and disappear at the moment of complete maturity. For more information see Chap. 7.

14.2.2 Taste

Taste is the sensation perceived when the gustatory papillae are stimulated by some soluble substances. The four basic distinctive tastes are sweet, salty, acid, and bitter. The sweet sensation is elicited by substances such as sucrose, salty by sodium chloride, acid by the pH of some acids (weak acids give rise to weaker stimuli), and bitter by such substances as caffeine and quinine. Olive oil contains no sugars or sodium chloride, and, moreover, acid sensations cannot be stimulated by the free fatty acids responsible for its free acidity because of their very low dissociation constant. Secoiridoid compounds are, perhaps, the only substances responsible for true gustatory perception in VOOs.

Aglycons of the various glycosides naturally occurring in olive fruits (Gariboldi et al. 1986; Kubo and Matsumoto 1984), all of which contain tyrosol or hydroxytyrosol in their molecular structure (Angerosa et al. 1995, 1996b; Montedoro et al. 1993), are mainly responsible for bitterness (Chap. 9). Bitter is the most important

taste perception of VOOs; it is due to an interaction between polar molecules and the lipid portion of taste papillae membranes (Bate-Smith 1973).

The level of secoiridoid compounds in VOOs is heavily influenced by genetic factors, the ripening stage of fruits, and the technological process of oil extraction. Secoiridoid concentration increases with fruit growth until a maximum is reached that is generally observed as the olives turn to purple; from this stage on, their polyphenol content decreases (Montedoro et al. 1992).

The kind of crushing affects bitter taste (Angerosa and Di Giacinto 1995), long periods and high temperatures of malaxation weaken the bitter intensity, while extraction systems in which partition phenomena between water and oil and the converse are reduced, such as in pressing or when using two-phase centrifugal decanters, provide oils that have a stronger bitterness (Angerosa and Di Giovacchino 1996; Di Giovacchino et al. 1994).

The same compounds mentioned previously could also be responsible for the astringency that is sometimes perceived in VOOs. Astringency, a chemically induced tactile sensation (Lee and Lawless 1991), can be defined as the drying, puckering sensation in the mouth elicited, for example, by quince fruits. There is wide agreement on attributing this sensation to precipitation in the oral cavity of salivary proteins because of their nonspecific hydrogen bondings with *o*-diphenolic groups with a concomitant loss of their lubricating power and production of an unpalatable dry sensation (Lee and Lawless 1991; Naish et al. 1993).

In addition, secoiridoid compounds give rise to a pungent perception through the stimulation of the fibers carrying thermic, tactile, and painful sensations (nociceptive fibers) from the trigeminal nerve. Nociceptive fibers are associated with taste buds in the fungiform papillae (Withehead et al. 1985).

14.2.3 Aroma

The delicate fragrant aroma of VOOs is the sum of sensations perceived when several chemicals, transported by air streams during inhalation and expiration actions, reach and stimulate the odor receptors located in neurons of the olfactory epithelium (García-González et al. 2011; Royet and Plailly 2004).

Odorants are compounds with a low molecular weight (<300), volatile enough so that a suitable number of molecules reach the olfactory epithelium and can dissolve into the mucus that covers the sensitive olfactory cells, forming bonds with the specialized receptors (Moruzzi 1977; Rolls 2006). The mechanism that allows humans to identify the intensity and quality of odorous stimuli correctly is still not completely understood (Faurion 2006; García-González et al. 2011).

Individual differences in sensitivity have been observed in “normal” human subjects when they have been tested for the determination of thresholds of some odorants. Such significant individual differences in the ability to smell among different groups of subjects in the world would seem to be related to genetic, cultural, and environmental factors.

The odor strength seems to be related more to chemical factors, such as the volatility and hydrophobic character, and to the stereochemical structure of odorants rather than to their concentration. The size, shape, conformation, type, and position of functional groups of odorants have been shown to be important elements in establishing their suitability to bond to olfactory receptor proteins present in the olfactory epithelium and giving rise to perceptions. Odor quality is also influenced by *cis*/*trans* isomerism. Bedoukian (1971) studied the olfactory characteristics of seven primary hexenols and found appreciable differences between the *cis* and the *trans* isomers of each alcohol, the *cis* forms being sharper and greener and *trans* isomers more fatty. In addition, changes in the position of the double bond have resulted in different shades of green odor and in different degrees of pleasantness. The kind of functional group is more related to odor strength than to odor quality. Hatanaka and co-workers (1992) found that the odor intensity of C_6 unsaturated aldehydes is 10–1,000 times stronger than that of the corresponding hexenols having the same position and geometry of the double bond, but the odor quality was practically the same in both classes of compounds.

These findings are of particular importance for VOOs because they contain more than 100 volatiles identified by gas chromatography-mass spectrometry (GCMS), which give rise to the special aroma of VOOs. The characterization from a sensory point of view as established by sniffing techniques for most identified compounds has proven that no substance is able to give rise to all the sensations and shades that form the olive oil aroma but that each one contributes to it; see Chap. 8 for more information.

The ripening degree of fruits at harvesting affects considerably the oil aroma (Chap. 12). In fact, the strongest balanced aroma is presented when the maximum content of volatiles is achieved (Angerosa and Basti 2001). This is reached at various times according to the olive variety, though the processing system and the conditions adopted during oil extraction are also responsible for changes in volatile composition and, thus, in the quality and strength of aroma. The severe crushing of fruits performed by metallic crushers and long periods and high temperatures of malaxation have negative effects on the aroma of resulting oils (Angerosa 2002). Further, extraction systems in which the partition phenomena between water and oil and vice versa are reduced as much as possible, such as in pressing or in two-phase centrifugal decanters, provide oils with stronger aromas.

14.3 Specific Vocabulary for Virgin Olive Oils

A specific vocabulary has been developed to describe flavors of VOOs, that is, oils obtained from fruits by purely mechanical means and which can be consumed without any further treatment. Extracting oil from olives that have suffered damage from diseases or adverse weather conditions or alterations owing to fermentation and catabolic phenomena results in lampante VOO. Because of the disgusting tastes and odors, these oils must undergo a set of technological treatments, known as the refining process, to make them edible.

The refined oils obtained from these technological treatments, which include neutralizing, bleaching, and deodorizing as essential steps of the refining process (Chap. 19), do not contain substances that are able to stimulate the olfactory epithelium, the gustative buds, and the free endings of the trigeminal nerve; therefore, they are flavorless. The lack of natural antioxidants in refined oils (Boskou 2006) also promotes their autoxidation and, hence, the production of short-chain aldehydes that are responsible for rancid perception (Chap. 8).

Generally, descriptions of the fragrant and delicate aromas of VOOs fall into two main groups: (1) *olive fruity*, the sensation that recalls olive fruit, and (2) *green notes*, which are reminiscent of leaves or green fruits. Six-carbon aldehydes, alcohols, and esters, having odor qualities reminiscent of leaves, green fruits, or vegetables and smelling of different shades of freshly cut grass, are considered responsible for the so-called green notes (Morales et al. 1994). Therefore, because of the usually high concentration of the C_6 compounds, green odor notes play an important role in producing the fragrant and delicate aroma of high-quality VOOs that is obtained through the balance of green and fruity attributes (Chap. 8).

The sensations perceived when a VOO is consumed, however, are due to the combined stimulation of olfactory and gustatory receptors. The combination of sensations elicited by the senses of smell and taste is commonly called flavor. Therefore, besides the separate sensations arising from the stimulation of the aforementioned senses, interactions of taste–taste and odor–odor stimuli at the receptor level, as well as interactions of taste–aroma at the central cognitive process level, contribute to flavor (Verhagen and Engelen 2006). Such interactions can considerably modify the information that could be normally obtained by the simple sum of the separate sensations arising from taste, smell, and touch. The flavor description, performed by dissecting all possible elicited sensations, represents the sensory profile of foods and beverages.

The basic positive attribute of VOOs is fruity, the typical sensation that is reminiscent of healthy, fresh fruits harvested at the right ripening stage. It is accompanied by other sensations that differ in relation to the ripening degree of olives when processed. The flavor of oils obtained from unripe olives is generally characterized by green sensations that recall freshly cut grass (grass attributes) or leaves (leaf attributes), a more or less bitter taste, and rather intense pungency ascribable to a high content of both C_6 components and secoiridoid compounds. The green sensations are stronger when oils are extracted from unripe fruits or from fruits processed with their leaves. In this case, bitterness and pungency are quite intense and a chemically induced tactile sensation of astringency also appears. These oils are not commonly accepted by consumers for direct consumption and must be suitably modified by blending. Nevertheless, their bitterness, pungency, and astringency, being characteristic attributes of oils obtained from unripe healthy fresh fruits, must always be considered positive attributes. These attributes become weaker during oil preservation owing to the drop in the concentration of secoiridoids (Angerosa et al. 1993).

Green attributes do not describe the flavor of oils extracted from ripe olives or from those preserved for some time because of the lower accumulation of compounds arising from the lipoxygenase pathway. Also, they are characterized by weak intensities of bitter and pungent sensations that are due to decreasing amounts of secoiridoid compounds during the ripening of fruits. These oils are called sweet.

Table 14.1 Organoleptic evaluation and flavor characteristics of oils obtained from fruits at different storage times

Storage days	Fruity intensity	Main defects
0	2.8 ± 0.3	Absent
2	2.3 ± 0,2	Absent
4	2.0 ± 0.0	Fusty
6	1.3 ± 0.4	Fusty, vinegary, musty
8	0.9 ± 0.2	Fusty, vinegary, musty

Source: Angerosa et al. (1996c), with permission of Grasas y Aceites

Sometimes oils are not reminiscent of olive fruity notes but, depending on the variety of olives and their ripening degree, other fruit attributes, such as apple, tomato, almond, artichoke, or wild flowers may be detectable.

Often, the basic sensory profile of VOO is considerably modified because of the presence of defects. These defects are mainly ascribable to the deterioration of fruits that results from lengthy storage of the olives and from the fermentation or oxidizing processes to which the oils are subjected during their preservation. Descriptions of these defective flavors require a further number of attributes.

- *Fusty*: This flavor is typical of oils obtained from fruits that have been stored in jute sacks or in larger, deep piles for long periods before their extraction. It is a common defect. Small processing plants and the lack of sufficient storage areas for large quantities of fruit often result in olives being stored in unsuitable conditions for a long time. This leads to their deterioration, with a noteworthy warming of bulk, and to the appearance of this characteristic (fusty) defect in the resulting oils (Angerosa et al. 1990; García-González and Aparicio 2010; Morales et al. 2005). It must be underlined that the fusty defect, ascribable to *Pseudomonas* and *Clostridium* genera, is perceived after only 4 days of olive preservation, and its strength increases during subsequent storage days, as shown in the Table 14.1. Moreover, after 6 days vinegary and musty attributes are strongly perceived because of the considerable development of both acetic acid bacteria ascribable to *Acetobacter* genus, which transform the ethanol produced by yeasts, and of molds (Angerosa et al. 1999).
- *Winey*: Sometimes the conditions of olive storage facilitate the development of a greater amount of yeasts and result in only a poor growth of *Acetobacter*. In this case, ethanol, ethyl acetate, and, in a lower proportion, acetic acid are the main metabolites, and the resulting oil suffers from the winey defect whose intensity is linearly dependent on metabolite concentrations (Morales et al. 2000).
- *Musty*: Relatively low temperatures and high humidity promote mold development and the emergence of the musty sensory defect. Among those species naturally occurring on the olive cuticle, numerous *Aspergillus* and *Penicillium* genera have been identified (Marsilio and Spotti 1987). Instead of C_6 and C_5 metabolites produced by the normal plant hydroperoxide lyase cascade, molds produce C_8 primary and secondary alcohols and C_8 ketones that have an earthy mushroomlike odor quality and give the resulting oils their characteristic sensory musty defect (Angerosa et al. 1999; García-González and Aparicio 2010; Morales et al. 2005).
- *Rough*: This thick, pasty mouthfeel sensation is sometimes perceived in olive oils.

- *Metallic*: This flavor is reminiscent of metal and occurs in oils obtained from processing plants used at the beginning of the olive crop and, especially when any apparatus involved in olive oil processing is made of iron.
- *Muddy sediment*: This aroma is typical of oils stored for a long time on their sediments (Angerosa et al. 2004; García-González and Aparicio 2010; Morales et al. 2005).
- *Rancid*: During its preservation, oil suffers an oxidizing process characterized by two steps. During the first step, oxygen reacts with unsaturated fatty acids, giving rise to odorless and flavorless hydroperoxides that do not result in any special sensory characteristics. At the same time, secoiridoid compounds sharply decrease because of their antioxidant activity (Chap. 9). Oils in this step are characterized, from the sensory point of view, by a weakening of fruity, green, bitter, and pungent attributes. During the second stage, a fragmentation of hydroperoxides occurs. The accumulation of products of this fragmentation, mainly formed by disgusting odor aldehydes, causes the appearance of rancid defect and its subsequent increase (Chaps. 8 and 13).
- *Hearty*: This flavor is typical of oils obtained from muddy ground picked olives that have spontaneously fallen from trees and have been processed without washing. Sometimes, more or less strong musty shades may also contribute to this flavor.
- *Pressing mat*: This flavor is characteristic of oils obtained from olive pastes pressed with unclean pressing mats.

14.4 Sensory Testing

Various sensory tests have been developed to provide the evaluation of the organoleptic characteristics of foods, including oils. Some of these tests are designed to give information about overall acceptability of a product or to determine the preference of assessors or consumers between two products or among samples of the same food (preference tests). Other tests are used to evaluate qualitative and quantitative sensory differences between two products (paired tests, triangle tests, duo-trio tests, ranking tests), others to identify, describe, and quantify each sensory quality perceived in the assessment (descriptive tests). Several tests work well in determining an individual's sensory acuity, and they are used to screen candidates who wish to become assessors. The most common tests used in the sensory analysis of olive oils are tests for consumers, qualitative discriminant tests, qualitative-quantitative discriminant tests, and descriptive tests.

14.4.1 Consumer Tests

14.4.1.1 Acceptability Tests

The acceptability tests are generally performed to evaluate the sensory characteristics of a given product during its preservation. Assessors are requested to give evaluations by means of rating scales for those organoleptic properties that make it acceptable.

Preference Tests

Preference tests are usually carried out by a large number of persons who are generally chosen from habitual consumers of the product to be assessed. These tests aim to measure the rate of preference granted by consumers to a given product or their overall general liking for the product. There is evidence that the pattern of overall preferences differs between trained and untrained assessors, thus demonstrating the inappropriateness of using panelists to provide measures of preference or acceptance (Shepherd et al. 1988). In fact, it is unlikely that a limited number of assessors could represent all the consumers of a product in an effective way because assessors might differ from the consumer population in socioeconomic status, sex, culture, region of residence, or familiarity with the product tested. In addition, the preference behavior of any individual or of any group is going to differ in some aspects from that of other individuals and groups.

An example of the application of this kind of test to VOOs is provided by McEwan (1994). A number of judges, totaling more than 100, all having familiarity with the product to be evaluated and differing in age, socioeconomic status, and culture, evaluated the acceptability of 8 VOOs extracted from varieties cultivated in Greece, Spain, and Italy. The overall grading was evaluated using nine-point hedonic scales that ranged from "like extremely" (9) to "dislike extremely" (1) (Peryam and Pilgrim 1957). The calculation of the median showed that consumers preferred Greek oils with medium fruity and slightly bitter and pungent attributes, and they disliked oils, such as Italian oils from the Coratina variety, characterized by a strong odor and taste and chemically induced tactile sensations.

14.4.1.2 Qualitative Discriminant Tests

Paired difference, paired preference, triangle, and duo-trio tests are quite popular because they are easily carried out and results can be interpreted immediately. In these tests, assessors evaluate samples differing in only one characteristic.

Paired Tests

In paired tests, assessors are requested to indicate the existence of a possible difference or to express a preference between two suitably labeled samples. Generally, more replications of the evaluation of the same samples are carried out. In paired difference tests, assessors can provide only right or wrong identifications; thus, these tests are unimodal. In contrast, paired preference tests are bimodals because judges can prefer a sample with a greater or a lesser intensity of a given characteristic. There are different probabilities that an assessor could provide, at random, correct identifications (null hypothesis) for paired difference or preference tests. Roessler et al. (1978) calculated, listed, and published for either difference or preference tests the different critical values, at various levels of significance. These values are needed to establish whether or not the differences detected by assessors between

two samples are statistically significant. Samples are significantly different if the number of right identifications is greater than the corresponding critical value for the number of total identifications at a given level of significance.

Triangle Test

The triangle test is the test most used for screening and training assessors and for evaluating foods. Two identical samples and one slightly different sample, suitably labeled, are presented to each assessor, who is required to indicate the odd or different sample. Great attention is required in planning the test, in randomizing, and in counterbalancing the order of presentation of stimuli in the triad because there is better discrimination when the odd sample shows the strongest stimulus (O'Mahony 1995). Samples will be statistically different, at a given level of significance, if the observed number of right identifications is greater than the critical value calculated for the total number of identifications and listed in special tables (Roessler et al. 1978). This test, besides pointing out sensory differences among similar samples of olive oils, also has been used to evaluate the threshold concentration of the most important odorants in VOOs (Guth and Grosh 1993).

Duo-Trio Test

Three quite similar samples are involved in this test, with one differing from the other two. One of the two identical samples, identified as a reference, is assessed first by each assessor. Next, the other samples, suitably labeled (blind samples), are assessed. Assessors are requested to identify the sample that is identical to the one used as a reference. The same statistical procedure adopted for paired difference tests is applied to data provided by the assessors, with 0.5 being the probability of choosing samples at random.

14.4.1.3 Qualitative-Quantitative Discriminant Test

The arrangement or ranking test is widely used in the screening of subjects who will become assessors of VOOs. They are requested to arrange a series of oil samples according to the increasing or decreasing strength of a specific quality (e.g., flavor, bitterness, rancidity). Assessors can be required to arrange samples in accordance with their overall general liking. In this case, a great number of assessors or consumers will carry out the test.

14.5 Scales

Some sensory tests use various kinds of scales for the quantification of the strength of different attributes. Some advantages result from the use of scales. They provide more information about samples than difference or ranking tests because their

use points out not merely the difference between two samples but also the size of the difference. Generally, scales are easy to use for both experts and inexperienced subjects and can be applied to a large number of different stimuli. Moreover, data derived from the application of scales to the evaluation of sensory characteristics are quite useful as they can be statistically processed.

The most common scales applied to the sensory evaluation of olive oils are interval structured or unstructured scales. In structured scales, the amplitude of the attribute intensity is divided into equal intervals, generally ranging from 6 to 9, so that the end effects due to the reluctance of assessors to use the extreme portions of the scales can be limited (Lawless 1991). Figures and descriptive words are usually associated with each step of the scale to make the choice easier for the assessors and to allow the application of statistical procedures to the results in order to verify the existence of significant differences among the attributes or the scores of the samples.

This kind of scale can also be used to measure the degree of pleasantness attributed to a given product. In this case, it is termed a *hedonic scale*, and the rating of the product is committed to verbal categories (e.g., like extremely, like very much, like moderately, like slightly, neither like nor dislike, dislike slightly, dislike moderately, dislike very much, dislike extremely). Each category can be associated with a score, as in the scale developed by Peryam and Pilgrim (1957). Another scale often used, especially with children or with people who speak different languages, is the face scale that is formed by a series of facial expressions (look) ranging between repulsion and maximum pleasantness (Kroll 1990).

Improvements in discrimination accuracy can be obtained by adopting continuous unstructured scales simply formed with a line defined by the lower and higher intensities of the attribute to be evaluated. The assessor draws a vertical line at the point that, in his or her opinion, represents the strength of the perceived sensation. The distance from the starting point, expressed as centimeters or millimeters, is regarded as the measure of the intensity of the attribute. This kind of unstructured scale, recently adopted in the sensory evaluation of VOOs, has the advantage of allowing the assessor to evaluate freely the strength of the various attributes along a continuum, rather than according to predetermined steps, such as in structured scales. In addition, the adoption of an unstructured scale eliminates possible discrepancies that could take place in the evaluation of the amplitude of the intervals between each step of the structured scale and, consequently, produce incorrect results.

14.6 Descriptive Analysis Tests

The most valuable approach to the sensory evaluation of foods and beverages, including olive oil, has been found in the descriptive analysis. This sensory methodology uses a group of selected and trained persons, called a panel, who identify and measure the strength of different sensations perceived by their sense organs in standardized environmental conditions. In this method, the panel represents a sensory instrument.

The panel is termed *analytical* if the sensory evaluation is performed by a small number of selected and well-trained subjects called assessors, judges, or panelists; the panel is termed *consumer* when a larger number of persons, randomly chosen among regular consumers of the product to be assessed, provide the sensory data. In the latter type, the inexperience of the assessors is outweighed by their increased number in order to avoid any loss of the test's significance.

The descriptive analysis has been developed in many forms, including the flavor profile method, quantitative descriptive analysis (QDA), free-choice profile, and some grading techniques. Flavor profile and QDA are the most common forms used in the sensory analysis of oils. The application of QDA to the sensory evaluation of olive oils is usually known as a panel test. Common features of all descriptive techniques are the selection of assessors, their training, and the maintenance of the panel under the supervision of a sensory analysis scientist, who is the panel leader. Descriptive tests require established protocols for the sample preparation, scales for the quantification of attributes or for the evaluation of overall general liking, and techniques for the development of a vocabulary. In QDA, application of statistical procedures to data provided by the assessors produces results that can be considered to have a degree of reliability similar, because of their significance levels, to that of other methods usually adopted in scientific fields.

14.6.1 Flavor Profile

A quite descriptive technique, the flavor profile, was developed by Caincross and Sjöström (1950). It is based on the identification and strength evaluation of each characteristic of aroma, flavor, and aftertaste of the product to be tested by selected and trained assessors who work in a group.

Assessor screening uses suitable fixed discrimination tests of odor and flavor differences, and the training of assessors includes the teaching of olfaction and tasting techniques, the use of references for the identification of each sensory characteristic, and the calibration of their intensities.

Assessors are requested to develop an ad hoc vocabulary and to list the sensory attributes in the order in which they occur in the tasting. Several panel sessions are usually needed so that the panelists understand the meaning of each descriptive term and adopt a common terminology. Every effort must be made to encourage complete freedom of expression during discussions and to avoid pressure and domination.

The strength of each sensory characteristic is generally rated by means of an arbitrary scale with the following designations: not detectable, barely detectable, slightly strong, moderately strong, and strong. The overall flavor is evaluated with the same scale and is represented by the sum of the intensities of all characteristics.

The flavor profile is finally obtained through group consensus for each attribute after discussion of individual results, in which all the members of the panel, including the panel leader, take part. Statistical procedures cannot be applied to data from the flavor profile method. Because only one profile is obtained for each sample as a consensus of the group, the method is not usually applied to the sensory evaluation of olive oils.

14.6.2 Quantitative Descriptive Analysis

QDA is a technique for quantitative characterization of sensory attributes according to their order of occurrence. It was developed because of the need to submit the responses of assessors working in a group to statistical procedures (Stone et al. 1974, 1980). QDA is a valuable means of obtaining information about the appearance, texture, aroma, and flavor of foods. It is used to check the quality of a product, to study its shelf life, to improve its quality, to develop new formulations, and to verify the influence of technology in treated foods.

The technique, like all quantitative descriptive analyses, has five essential features: (1) screening of panelists, (2) training panelists, (3) setting objectivity conditions, (4) maintaining the skill of panelists, and (5) determining scores. The QDA has proved to be a valuable and effective tool in evaluating the sensory quality of VOOs. In fact, it is the descriptive method most often applied to olive oils. The most common sensory tests used in the screening and training of assessors and the vocabulary specifically developed for VOOs have been extensively described by the Commission of the European Communities (EC) in its Regulation 2568/91 (EC 1991).

14.6.2.1 Screening of Panelists

Assessors are selected from a group of individuals (at least double the number are needed) who should have some familiarity with the product to be assessed. Obviously, sensory acuity plays an important role in the selection, but it is not the only screening criterion. Certain personal characteristics of the candidates, such as ability to memorize odors, patience, persistence, motivation, availability, interest in sensory analysis, cooperation, verbal ability to describe different sensations, and ability to deal with abstract concepts, must be considered as even more important prerequisites.

Identification and arrangement tests are carried out to verify the ability of the candidate to recognize and describe odors, to identify bitter tastes, and to determine off-odor characteristics, such as rancid, winey-vinegary, or fusty sensations and certain oral chemesthetic sensations.

14.6.2.2 Training

The training of the selected subjects is the most critical step of the QDA. It begins with an orientation, during which the selected subjects learn to work in a group, to identify and describe attributes, and to quantify differences of sensation intensity. After this initial step, the real training follows. Panel members learn tasting techniques and familiarize themselves with the sensory methodology and the multiple stimuli elicited by a specific food. They also learn to dissect, identify, and define

each sensory characteristic of olive oils, and in this way they develop an ad hoc vocabulary that allows them to communicate each sensation that they experience. In addition, they learn to recognize and record the attributes in the same order in which they occur.

Different concepts representing the various sensory characteristics should be defined through the presentation of suitable standards. The use of reference standards during the training of panelists plays a key role in developing appropriate descriptive terminology and in evaluating intensities (Raney 1986). It is desirable to have many references of various intensities for each attribute. It has been proven that the presentation of more than one standard (which can define sensations falling outside, as well as within, a given concept) is quite useful in producing a concept that can be agreed upon by panelists (Civille and Lawless 1986; Ishii and O'Mahony 1991; O'Mahony 1991) in a more precise way than that obtained by giving to panelists only one standard (Ishii and O'Mahony 1987). The sensory profile is described by the average strengths of all characteristics perceived by panelists.

14.6.2.3 Conditions for Test Objectivity

Because panelists play the role of a measurement instrument, it is very important to minimize all the effects that can trouble them or disturb the experiments during their execution. It is known that the mental or physical state of a panelist can influence the sensory evaluation of foods because mental or physical stress can modify taste perception (Nakagawa et al. 1996). Some sensory facilities must be adapted to minimize sensory fatigue and bias that result from the test environment. For this reason, noise, room temperature, hygrometric conditions, uniform and diffuse lighting, comfortable booths constructed of appropriate materials that are easily cleaned, glasses, and temperature are generally standardized. In addition, the sensory laboratory must be organized in a suitable way so that the panelist's work is facilitated and the repeatability and reproducibility of results are ensured.

14.6.2.4 Maintenance of the Panel

Maintenance of the panel is provided by continuous training throughout its duration of the same panel, checks to determine the sensory acuity of the panelists, and exercises to allow the measurement of panel performance. The sensory testing experience seems to have only small effects on the performance of assessors (Chambers and Smith 1993; Clapperton and Piggott 1979; McBride and Finley 1989); improvements in reproducibility by the panelists would be ascribable more to the training than to experience (Chambers and Smith 1993). The performance of new panelists, after a period of training, should be compared with that of more experienced panelists through mathematical procedures (Aparicio et al. 1991).

14.6.2.5 Scale and Score Sheet

Assessors are requested to record and measure the intensity of each attribute, according to the order of its occurrence, on structured or unstructured scales. This allows for the application of statistical procedures to the responses of each panelist. Panelists often use score sheets for the evaluation of sensory characteristics; in this case, they cannot provide information on the order of attribute occurrence. This limitation can be serious because considerable changes in some attributes are not indicated.

14.6.3 Free-Choice Profile

A free-choice profile is a descriptive sensory analysis developed some years ago and applied to a number of foods and beverages, including olive oil (Beal and Mottram 1993; Servili et al. 1995). This technique assumes that assessors perceive the sensory qualities of a product in the same way but describe them in different ways. In free-choice profiling, each assessor is requested to (1) describe freely all or some defined sensory characteristics in his or her own words in accordance with their occurrence and (2) score each quality according to an intensity scale in a similar way to flavor profiling. It is not necessary that all assessors consider the intensity scale in the same way. The number of descriptors depends on the sensory acuity of the assessors and their ability to develop vocabularies; the latter point is related to their experience and familiarity with the product to be assessed.

Assessors are selected on a number of criteria, the most important being their objectivity, their willingness to participate, their ability to recognize and describe odors, and their availability.

The free-choice profile is faster than other conventional descriptive techniques because it does not require extensive training of assessors and because the development of a language for the product being assessed and the consensus of all the assessors about the meaning of the terms they use are not required. Because of their lack of training, assessors are asked to profile only a limited number of samples (two or three), generally presented after randomization during each session. Three or four replication evaluations are usually carried out (Beal and Mottram 1993). Because assessors use individual vocabularies and regard intensity scales in different ways, it is not possible to describe the sensory characteristics of a product as a result of the average intensities of each attribute perceived, such as in the QDA. Free-choice-profile data are usually processed by generalized Procrustes analysis (GPA) to eliminate the effects resulting from the different scales and to remove the differences in the scoring range used by different assessors. GPA allows for the calculation of a consensus configuration as the average of the individual configurations of assessors. Generally, principal component analysis (PCA) is applied to the consensus configuration to reduce the number of its dimensions (Oreskovich et al. 1991).

14.7 Sensory Evaluation of Virgin Olive Oils

Performed with the initial aim of establishing a basic quality of VOOs to be commercialized, the sensory evaluation represents a powerful tool to get additional information of great interest for producers and dealers. Thus, the sensory evaluation of VOOs can be performed for any of these six reasons: (1) to establish a basic quality of the commodity by verifying the absence or the presence and strength of defects; (2) to give help in the identification of particular sensory attributes that make typical VOOs produced in certain geographical areas to which the EU has granted Protected Designation of Origin (POD) or Protected Geographical Indication (PGI) designations as well as to verify that their productions fit into their disciplinary bodies; (3) to reveal possible modifications of sensory profiles in relation to the variety, geographical origin, technology, and shelf life of the product; (4) to find critical sensory characteristics for consumer preferences; (5) to produce uniform sensory profiles by blending VOOs from diverse cultivars, producing orchards and extraction systems; or (6) to evaluate, in sensory terms, the differences in preference between habitual and potential consumers.

The first point can be well solved using the sensory assessment, also called the IOC panel test, which has proven to be a fine sensory instrument to distinguish between oils with defects and those without defects, and therefore to classify the various categories of VOOs defined by IOC (Chap. 1). The panel test is the result of collaborative international studies encouraged and supported by IOC that were undertaken during the 1970s and 1980s in an attempt to achieve a quantitative descriptive analysis of VOOs. Although most of the producer countries took part in developing the methodology, Spain (Gutiérrez et al. 1974, 1984; Gutiérrez 1989) and Italy (Solinas 1987) offered the most important contributions.

The method was based on the application of the QDA adapted to VOOs with the use of an agreed vocabulary of sensory attributes specifically developed for VOOs, a uniform testing technique, and environmental standardization. The carefully selected ad hoc vocabulary, including positive and negative attributes recognized in VOOs, are evaluated in an agreed scale by persons suitably screened and trained. Completely defined after many years of work, the test was adopted as trade standard by IOC in June 1987 for evaluating the sensory characteristics of VOOs. The test was also incorporated into EU regulations (EC 1991) with the main aim of detecting defects and their intensity for the classification of various grades of VOOs.

In the last 20 years, the test has undergone several changes by IOC experts that were adopted by the Commission of the European Communities in subsequent regulations. However, the documents about the screening tests of assessors, the indication about their training, the testing technique, and the environmental standardization (IOC 2007a, b, c; 2011a, b) are basically those described in the first draft of IOC method and, therefore, in EC Regulation 2568/91 (EC 1991). The only differences concern (1) the introduction of wet wood, among other negative attributes; (2) the temperature of the testing room (actually 20–25 °C instead of 20–22 °C); (3) the elimination of information about the hygrometry of the testing room; and (4) a more

detailed description of the knowledge, competences, duties, and responsibilities of the panel leader, recognized as the key figure for good work by the panel.

The removal of the hygrometry of the testing room could hide the wish to meet the difficulties of some countries, especially those of subtropical zones, in reproducing and keeping the relative humidity (RH) between 60 % and 70 %, as suggested in the original method. A study aimed at investigating the question of whether the olfactory function changes in relation to air pressure and humidity showed that the odor threshold for butanol, for example, is lower in humid atmospheres than in drier conditions (Kuehn et al. 2008). Excessive dryness and excessive humidity of the mucous membrane reduce or suppress smell (Cavazzani and Uras 2000). It was shown that at 25 °C it is necessary to keep the RH above 30 % to avoid dryness of the eyes and skin and above 10 % to avoid dryness of the nasal mucous membrane (Sunwoo et al. 2006a, b).

A review of the relevant health literature indicates that at room temperature, the lowest level of risks to human health is achieved when the RH ranges from 30 % to 60 % (Sterling et al. 1985). This range of RH provides comfort for room occupants. IOC stipulations comply with this range when the methodology stipulates that assessors must perform the sensory tests in comfortable rooms (IOC 2007c). Also, the increased range of room temperature between 20 °C and 25 °C is aimed at assuring comfort to assessors and, consequently, of assuring their complete attention to the sensory test.

In sensory analysis, generally, panelists independently evaluate the smell, taste, aroma, mouthfeel, and other traits of each sensation that they perceive. In the IOC test methodology, however, panelists measure each perception resulting from the entire olfactory–gustatory–tactile sensation. On the other hand, it has been shown that the sensory characterization of olive oils is not improved by the independent evaluation of all sensations that form the flavor (Aparicio et al. 1994). Moreover, all olfactory–gustatory–tactile sensations perceived when oil is in the mouth are enhanced and more precisely identified because of the higher concentration of odorants in the retronasal pathway and trigeminal stimulation (Voirol and Daget 1986).

In forming a panel for the sensory evaluation of VOOs, identification tests are used to verify the ability of each candidate to recognize and describe odors. The candidate must be able to identify bitter taste, certain undesirable odors such as rancid, winey-vinegary, and fusty/muddy sediment sensations, and some kinesthetic sensations. The methodology establishes that for the formation of a panel, first of all, the determination of the mean threshold concentration for fusty/muddy sediment, winey-vinegary, rancid, and bitter attributes should be performed. In a second step, the identification and arrangement tests, to verify the ability of each candidate to recognize and describe odors and an oral sensation, should be carried out.

Determination of the mean threshold is performed using dilution techniques of reference oils with known high intensities of fusty/muddy sediment, winey, rancid, and bitter attributes, respectively. The mean threshold concentration, not being linked to any habit, should be similar for different groups of assessors and therefore may be used to integrate various panels by their olfactory gustatory sensitivity. On the basis of the mean threshold concentration, a series of 12 samples is prepared

for each attribute in such a way that the mean threshold concentration holds the tenth place in this scale. The screening is carried out by asking each candidate to arrange some prefixed samples in the series. The statistical procedures allow the selection of only those candidates showing sufficient discriminatory power for the intensities of the stimuli used in the selection (IOC 2011a). The number of errors made by candidates evaluating samples during the test must fall within the limits described in the methodology.

In screening candidates, particular attention must be given to the choice of subjects who do not show bitter taste blindness. It has been proven that bitter taste blindness (very high thresholds for bitter attributes) is associated with genetic and anatomic differences among human subjects. Genetic studies led by Kalmus (1971) to ascribe bitter taste blindness to subjects having two recessive *tt* alleles in their genetic store and the ability to perceive bitter taste to subjects with at least one dominant and one recessive *Tt* allele (low thresholds). Other research (Bartoshuk et al. 1992; Jones and McLachlan 1991) confirmed three different distributions of thresholds relating to bitter taste and divided subjects into nontasters, tasters, and supertasters. Nontasters are weak at perceiving bitterness, whereas supertasters strongly taste this attribute (Bartoshuk 1993). This subdivision seems to relate to the number of recessive alleles that a human subject has in his or her genetic store (Bartoshuk 1993).

In addition, large anatomic differences in the number and size of fungiform *papillae* on the tongues of nontasters, tasters, and supertasters have been observed (Bartoshuk 1993; Miller and Reedy 1990). Supertasters have the greatest number of fungiform *papillae* and, therefore, perceive the strongest bitter intensity. Obviously, different proportions of nontasters, tasters, and supertasters on the panels have repercussions for the evaluation of the bitter attributes in VOOs. This gustative note, which gives rise to pleasant or disgusting sensations according to its low or high concentration, can be perceived by assessors, in the same way of other attributes, as a quality factor or as a reason for aversion in relation to its intensity. Moreover, the aforementioned distribution of subjects also affects the rating of the strength of pungency. Supertasters, having more taste buds that are associated in fungiform *papillae* with nociceptive fibers from the trigeminal nerve (Withehead et al. 1985), might perceive the pungent attributes (another chemically induced tactile sensation in VOOs) as stronger than do tasters and nontasters. For the same reason, they experience a stronger sensation of burning from capsaicin (Bartoshuk 1993).

The training of assessors is an important duty and responsibility of the panel leader and is essential for having repeatable and reproducible data. Panel leaders must be experts in sensory analysis as well as in the different kinds of oils they will come across during their work. They must have sensory ability, skill, and patience in planning tests, meticulousness in their preparation, organization, and execution. In addition to the selection, training, and checking of adequate assessors, they are responsible for organization of the panel, including the reception and storage of samples, their preparation, coding, and presentation to the assessors, the compiling and processing of the data, and the keeping of records of panel activity.

During their training, assessors must be guided by the panel leader to familiarize themselves with the methodology and to increase their own ability in recognizing, identifying, memorizing, and quantifying the different sensory notes that can be

perceived when VOOs are tasted. The training degree achieved after a number of sessions is evaluated on the basis of the increasing percentage of correct judgments if discriminatory trials are used, or by analyzing the variance in the average individual values of the panel if tests based on scales are implemented. One of the most common procedures for verifying whether assessors have acquired or are keeping up with their skills is to include one or more reference samples among those to be assessed and to study the variance of each assessor. Study of the variance allows an assessment about whether or not an assessor works well.

In the training of panelists, the difficulty of finding a set of reference standards capable of defining each possible sensory characteristic of VOOs is a big handicap because, unfortunately, only a few such standards are available. Initially, many panels were formed through the IOC in the producer countries and subsequently in the European countries because of the inclusion of the IOC test in EC regulations (EC 1991) for the classification of olive oils in relation to quality. Until now the IOC has made a great effort to provide some reference standards to all the panels selected and trained according to the procedure detailed in the initial and subsequent modifications (EC 1991, 2008). Despite this effort, the number of reference standards available from the IOC is not sufficient for all possible attributes of VOOs. Usually each year (because of the stability of the oil over time) during the time of oil extraction from fruits, panel leaders, who have a profound knowledge of olive oil from sensory, chemical, and technological points of view, look for oils with some dominant attributes to be used as references.

Unfortunately, it is not always easy to find oils that show a particular dominant defect that is present at high intensity. This is often the case for oils characterized by fusty or winey defective sensory attributes. Often VOOs, because of poor fruit preservation before oil extraction (Angerosa et al. 1996c, 1999; García-González and Aparicio 2004, 2010; Morales et al. 2000, 2005), elicit fusty or winey sensory notes, but their intensities are not usually high enough to be used as reference materials. For this reason, the reference material for the winey sensory defect is often replaced by vinegary, which can be more easily prepared by adding small amounts of acetic acid to VOOs. Unfortunately, however, it is not easy to reproduce the defective fusty sensory attribute since it results from a complex mixture of chemical compounds; see Chap. 8 for more information.

The sensory perception of the qualities of olive oil samples is evaluated in booths (Fig. 14.1) of an environmentally isolated sensory panel room at a temperature between 20 °C and 25 °C by 8 to 12 assessors. All facilities adopted for the evaluation of VOOs are extensively described in IOC trade standards (IOC 2007b, c). The oil samples (15 mL each) are presented in covered blue glasses (Fig. 14.2) (diameter 70 mm; capacity 130 mL) and warmed at 28 ± 2 °C. The cover is removed from the warmed glass, and the sample is smelled and then tasted by each assessor to judge its flavor.

In the first draft of the method (EC 1991) panelists noted on the score sheet (Fig. 14.3) the intensity of each attribute on a structured scale of 0–5. They also rated the overall grading for the characteristics of the oil on a 9-point scale (9 for exceptional extra virgin olive oils [EVOOs], 1 for the worst). The grading had to agree with the absence or presence of defective flavors, as well as with the intensity

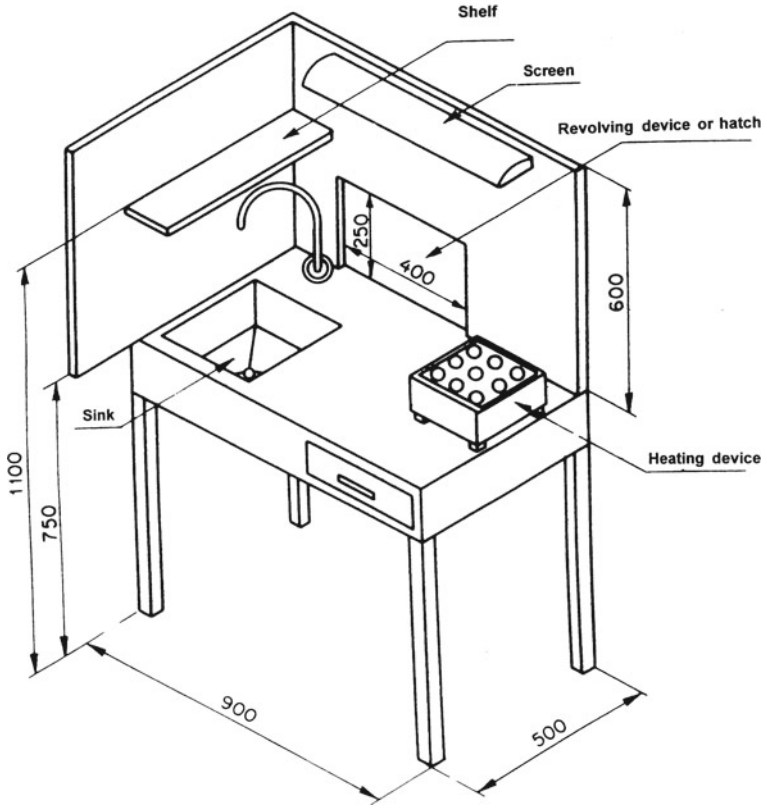

Fig. 14.1 Arrangement of booth for olive oil sensory analysis according to Commission Regulation (EEC) 2568/91 (EC 1991) (*Source*: IOC (2007a), with permission of the International Olive Council)

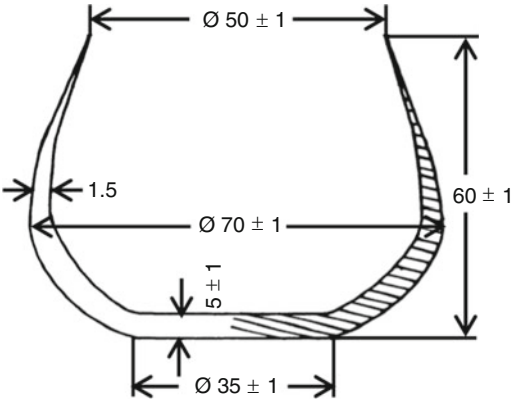

Fig. 14.2 Glass used for olive oil sensory analysis according to Commission Regulation (EEC) 2568/91 (EC 1991) (*Source*: IOC (2007a), with permission of the International Olive Council)

VIRGIN OLIVE OIL

PROFILE SHEET

OLFACTORY-GUSTATORY-TACTILE NOTES

	0	1	2	3	4	5
Olive fruity (ripe and green)						
Apple						
Other ripe fruit						
Green (leaves, grass)						
Bitter						
Pungent						
Sweet						
Other allowable attribute(s) (Specify.....)						
Sour/Winey/Vinegary/Acid						
Rough						
Metallic						
Mustiness/humidity						
Muddy sediment						
Fusty ("Atrojado")						
Rancid						
Other unallowable attribute(s) (Specify.....)						

1 Barely perceptible
2 Slight perceptible
3 Average
4 Great
5 Extreme

GRADING TABLE

Defects	Characteristics	Overall Mark: Points
None	Olive Fruity Olive fruity and fruitness of other fresh fruit	**9** **8** **7**
Barely perceptible	Weak fruitness of any type	**6**
Slight perceptible	Rather imperfect fruitness, anomalous odours and tastes	**5**
Considerable, on the border of acceptability	Clearly imperfect, unpleasant odours and tastes	**4**
Great and/or serious, clearly perceptible	Totally inadmissible odours and tastes for consumption	**3** **2** **1**

REMARKS.....
.....
NAME OF ASSESSOR.....
LEGEND OF SAMPLE.....
DATE.....

Fig. 14.3 Profile sheet and grading table of IOC old sensory assessment according to Commission Regulation (EEC) 2568/91 (EC 1991) (*Source*: IOC (2007a), with permission of the International Olive Council)

of the flavors. This overall grading was considered a measure of the quality of the oil and identified the grades of olive oil in relation to their quality.

The minimum score to classify an olive oil as extra virgin (6.5) was established in the initial EC regulations (EC 1991), but it has been modified several times by subsequent EC regulations that granted an allowance for oils that had obtained scores of at least 5.0 on the IOC test. The allowance was one point from 1995 (EC 1995) to 2002 (EC 2001). Thus, the minimum score to classify any oil as EVOO was 5.5, and oils with slight but perceptible defects were included among oils of very high quality.

When defective flavors were absent, panelists verified the presence and strength of the positive attributes, particularly the fruity sensation that is defined as the whole of olfactory–gustatory perceptions that recall healthy and fresh fruit at the correct ripening degree. The lack of fruity notes in an oil sample excluded it from extra virgin and virgin grades, whereas a very strong intensity of fruity – often associated with extreme bitter and pungent sensations – made the oil rather disagreeable for direct consumption, thus suggesting that it should be mixed with VOOs characterized by weak strengths of these sensory attributes.

On the other hand, medium intensities of positive notes, such as fruity, that the methodology ascribed to high-quality VOOs did not always reflect consumer requirements (Lyon and Watson 1994). A study carried out in 1994 showed, generally speaking, that people living in northern regions preferred oils with weak sensory attributes, whereas people in living in southern regions preferred oils characterized by strong aromas and more pronounced bitter and pungent sensations (Pagliarini et al. 1994). The consumption of olive oils with delicate aromas was also accepted among nonhabitual consumers living in countries where olive oil was not yet well known (McEwan 1994).

The first draft of the IOC method, adopted by the EC in 1991, as shown by the results of tests performed by panels in many countries working in agreement with IOC directives (Fedeli 1993), yielded a poor reproducibility of the overall grading scores (Ranzani 1994). The poor reproducibility seemed mainly related to panelists' using different portions of the intensity scale in their evaluation of oil samples with barely or slightly perceptible defects because of ineffective training. It could also have been due to differences in the evaluations of the positive quality attributes because assessors of panels might have diverse cultural and food habits.

At the request of some national members, the IOC attempted to overcome these problems by revising the method of organoleptic evaluation of VOO, and a new methodology was developed after reevaluation of the criteria needed to assess flavor characteristics (IOC 1996). The new profile sheet (Fig. 14.4), agreed on by producer countries after numerous sessions of work, mainly points out the negative attributes (e.g., fusty, musty, winey-vinegary, muddy sediment, metallic, and rancid) that can usually be detected in VOOs (EC 2002). Other defects, such as heated or burnt, hay-wood, greasy, vegetable water, brine, earthy, pressing mat, rough, cucumber (the flavor sometimes produced in oils hermetically packed for too long), grubby (the flavor of oils obtained from olives attacked by the olive fly), carefully described in the specific vocabulary for VOO, can be used as negative attributes for the designation "others." Among positive notes, only fruity, bitter, and pungent sensations are considered.

The strength of each attribute is rated on an unstructured scale 100 mm long, and the limits are linked up to the lowest and the highest intensities of each attribute. This kind of scale gives assessors the opportunity to freely evaluate the strength of the attributes continuously and not according to prefixed intervals. In this way, problems arising from different interpretations of the terms used in the structured scale with clear intervals, and possibly ignoring the assumption that all the sensory intervals of the scale are equal, are overcome. In fact it is very difficult to be sure that the distance between "barely perceptible" and "slightly perceptible" in Fig. 14.3 is the same as the distance between "average" and "extreme."

Assessors place a vertical mark on the scale that better describes their response to the attribute that is being evaluated. Intensity data, expressed as centimeters, are statistically processed to calculate the median of each negative and positive attribute. When at least 50 % of panelists report a negative attribute as "others," the panel leader must calculate the median of this attribute and the corresponding classification.

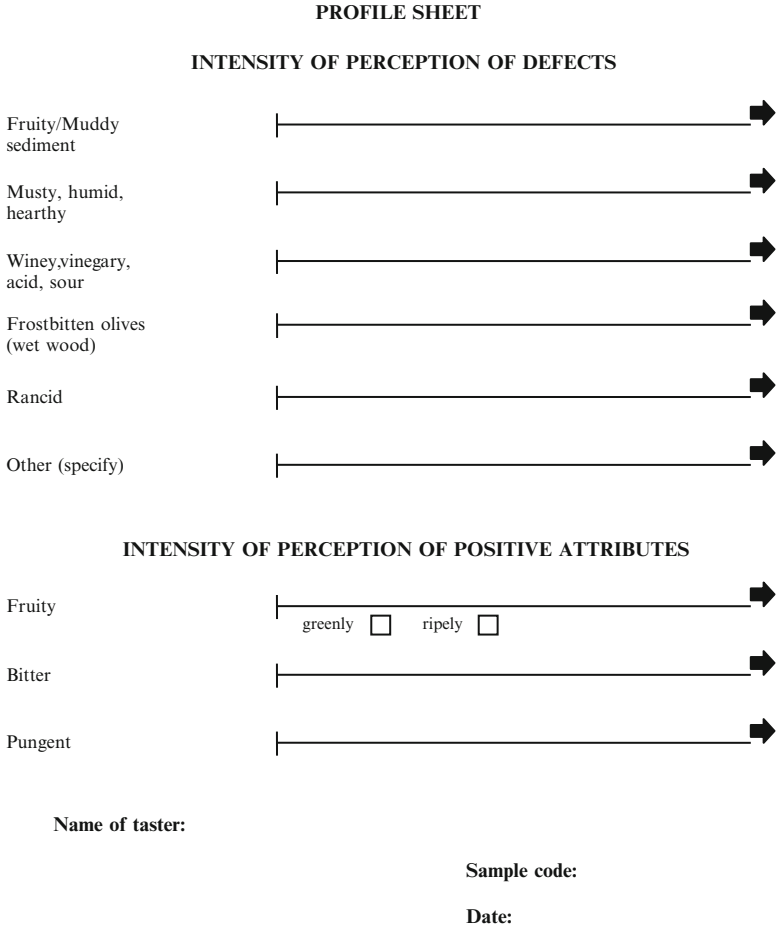

Fig. 14.4 Profile sheet and grading table of IOC current sensory assessment according to Commission Regulation (EC) 640/2008 (EC 2008) (*Source*: IOC (2007c), with permission of the International Olive Council)

Some years later, IOC experts reported some problems when the revised method was used in collaborative tests. These concerned both the robust variation coefficient, exceeding the limit value established by the methodology, and the reproducibility of the classification of defective VOOs. Thus, the analysis of data provided by the different panels participating in collaborative trials revealed some confusion between two negative sensory notes, i.e., muddy sediment and fusty attributes. The wrong identification of the defective note by some panels often implied different classifications of the same VOO. Consequently, this had repercussions for the reproducibility of methodology. The lack of reproducibility in classifying some defective VOOs showed an ineffective training of assessors, who proved to have an unsatisfactory knowledge of defects and, consequently, a poor precision in recognizing them.

In 2007, IOC experts adopted revised versions of the methodologies related to the organoleptic assessment of VOOs (IOC 2007a, b, c, 2011a) in order to improve the reproducibility of panels from different countries and to meet requests of both producers and dealers, who asked to have the possibility of adding some information about sensory characteristics in the oil labels. The latest revised methodology (IOC 2011b) was exclusively designed for the classification or labeling of VOOs according to the intensity of fruity and sensory defects perceived by a panel of assessors suitably selected and trained (IOC 2011b; EC 2011). To overcome problems related to reproducibility, the revised methodology unified fusty and muddy sediment sensory descriptors, though the origin of these defects is very different (Morales et al. 2005). The fusty defect arises from improper preservation of olive fruits (Angerosa et al. 1996c; García-González and Aparicio 2010), whereas muddy sediment arises as a result of incorrect preservation of oil (Angerosa et al. 2004), so that information for producers and oil mill workers is lost by using the suggested profile sheet for the VOO sensory assessment. The new methodology shows, in addition to the merging of fusty and muddy sediment defects, three other changes when compared with EC Regulation 796/02 (EC 2002): (1) a new maximum limit value of defect perception (EC 2011; IOC 2011b) (Table 16.8) for VOO category (3.5) to minimize any problem deriving from a possible poor harmonization among different panels; (2) the differentiation between green and ripe fruity sensory perceptions; (3) the possibility for panel leaders to certify that oils comply with the definitions (mild oil, well balanced) and ranges that correspond to some adjectives (light, medium, intense) according to the intensity and perception of the attributes as indicated by EC regulations and the IOC Trade Standard.

Neither the current IOC trade standard for sensory assessment (IOC 2011c) nor EC regulations (EC 2011) take into account color, though it could positively or negatively affect a consumer's decision to purchase. In fact, the flavor of appropriately colored foods is proven, in general, to be perceived with a stronger intensity and much better identified than those with inappropriate coloring (Clydesdale 1993).

The color of just-extracted VOOs ranges from light yellow to deep green, depending on the content of pigments naturally occurring in the fruits. It is known that oils from fruits that are green or turning purple appear to be more or less green and are characterized by stronger flavor and more bitter and pungent attributes than oils obtained from black olives, which are light yellow or yellow-green and elicit delicate aromatic notes and a weak bitter perception. Therefore, colors of VOOs often provide suggestions about their flavor to consumers and direct their preferences toward oils specifically colored in accordance with familial, cultural and socioeconomic, and behavioral factors relating to their food habits. Moreover, the color of olive oil is perceived by consumers as a mark of freshness. One must remember that oil is obtained from olives within a period of a few months but is consumed over a period of at least a year. During its storage, it is possible to see a progressive lightening of green color and deepening of yellow color because of the slow degradation of chlorophylls (Chap. 7).

14.8 PDO and PGI Designations

Today consumers choose foods on the basis of new criteria such as nutritional and healthy aspects, freshness, and genuineness, in addition to sensory quality. The choice of foods is often linked to a particular territory where producers continue using traditional methods of production of genuine and high-quality food products with specific and unique sensory characteristics, which obviously are typical of that production zone. The warranty label of quality and genuineness is perceived by consumers as an important assurance against adulteration and fraud.

Great effort has been made by the EC in the promotion of food products having particular quality and typical characteristics linked to specific geographical zones since 1992 when the EU promulgated the strict and detailed rigorous regulations EC Regulation 2081/92 (EC 1992a) to protect the reputation of regional food products (e.g., VOO) clustered inside the word typical. The aim of this regulation was also to avoid unfair competition by nongenuine products, whose inferior quality could mislead consumers. The regulation provides producers with exclusive trade labels – Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI) – for their food products.

The PDO designation covers agricultural products and foodstuffs produced, processed, and prepared in a given geographical area using recognized procedures whose quality or characteristics are expected to be tied, essentially or completely, to the geographic area of origin. PGI, however, is used to denote agricultural products and foods closely linked to the geographical area where they are produced, processed, or prepared. Furthermore, there are Agencies of Protection (PDO and PGI Authorities), independent of the producers, that assure the enforcement and respecting of rules (Article 10.1 of Regulation 510/2006) (EC 2006). These agencies encourage the diversification of agricultural production, protect product names from misuse and imitation, and help consumers by providing information on the specific characteristics of the products. The list of registered names can be found on the DOOR database: http://ec.europa.eu/agriculture/quality/database/index_en.htm. The current designations are shown in Table 14.2. For more information on PDO and PGI see http://ec.europa.eu/agriculture/quality/schemes/index_en.htm.

The physicochemical characteristics of PDO oils reported to disciplinary bodies are not always able to make an unequivocal differentiation because geographical

Table 14.2 Number of PDO and PGI designations registered for various European countries until 2010

Country	PDO	PGI
Spain	20	–
Italy	37	1
Greece	15	11
Portugal	6	–
France	7	–
Slovenia	1	1

Source: Authors

Table 14.3 List of possible descriptors for DO extra virgin olive oils according to information in IOC trade standard COI/T.20/Doc.No. 22

Direct or retronasal aromatic olfactory sensations		
Almond	Apple	Artichoke
Camomile	Citrus fruit	Eucalyptus
Exotic fruit[a]	Fig leaf	Flowers
Grass	Green pepper	Green
Greenly fruity	Herbs	Olive leaf
Pear	Pine kernel	Ripely fruity
Soft fruit	Sweet pepper	Tomato
Vanilla	Walnut	
Gustatory sensations		
Bitter	Sweet	
Tactile or kinaesthetic sensations		
Fluidity	Pungent	
Qualitative retronasal sensation		
Retronasal persistance		

Source: IOC (2005), with permission of the International Olive Council
[a]Pineapple, banana, passion fruit, mango, papaya

regions are sometimes very close to each other. Thus, a working group of sensory experts was created by the IOC with the responsibility for drawing up a method to assess the sensory attributes of EVOOs characteristic of a geographical origin, within the policy of product quality differentiation.

The designation of origin (DO) – PDO or PGI – authority shall select the sensory descriptors of its DO and incorporate them into a profile sheet, together with the maximum and minimum limits of the median for each descriptor. Table 14.3 shows the direct or retronasal aromatic olfactory sensations and gustatory and tactile sensations described by the IOC in its trade standard for designations of origin (IOC 2005). Some of these sensory descriptors from Table 14.3 are often perceived in VOOs (e.g., apple, almond, artichoke, grass, and tomato), while other descriptors are less frequently detected such as fig leaf and chamomile. Some of these attributes are defined in an unequivocal way (e.g., almond, chamomile, vanilla, artichoke), although others – such as citrus fruit, exotic fruit, and soft fruit – include a plurality of different sensations.

Obviously, not all the descriptors can be evaluated by assessors at the same time because of their very large number. Thus, the DO authority shall select the characteristic descriptors of the designation of origin (ten at the most) from those defined in Table 14.3 and incorporate them into the profile sheet (Fig. 14.5). This means that the authorities for the indications of origin need to collect a large number of VOO samples that include all the kinds of VOO produced within the geographical area. In addition, the authorities need to determine the characteristic of the sensory profile by analyzing the results with the accompanying software of the methodology that allows the determination of the median, the confidence intervals of the individual descriptors from the median, the robust percentage coefficients of variation, the related minimum and maximum limits, and the intervals of the attributes used.

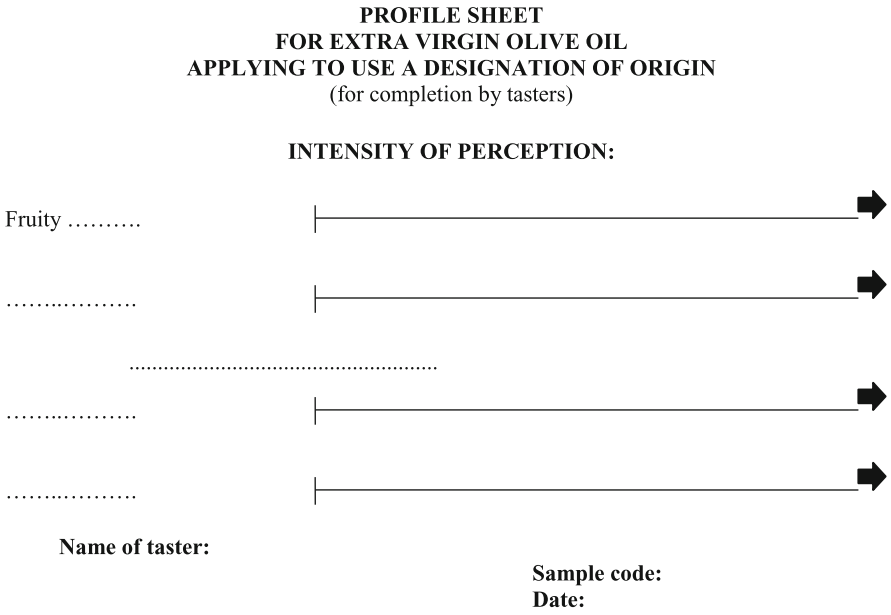

Fig. 14.5 Profile sheet adopted for designation of origin of virgin olive. Information collected from IOC Document COI/T.20/Doc. No. 22 (*Source*: IOC (2005), with permission of the International Olive Council)

Analysis of the regulations of PDO and PGI designations approved by the Commission of the European Communities frequently reveals the inadequacy of the number and kind of sensory descriptors used as evidence for the various sensory features of each PDO or PGI. Most of the regulations only report indications about the presence and intensity of the main sensory descriptors of VOOs of good quality (e.g., fruity, bitter, and pungent). Attributes such as grass, leaf, almond, and tomato are cited in approximately 50 % of the regulations only, while other descriptors shown in Table 14.3 – such as chamomile, walnut, pear, citrus fruit, and fig leaf – do not appear at all. However, recently approved PDOs (e.g., Colline Pontine, Terre Aurunche, and Tergeste) already report the limit values of the intensities of each sensory descriptor as recommended by the IOC in its "Guide for Determining the Characteristic Profile of D.O. Extra Virgin Olive Oils" (IOC 2005). Therefore, it has been suggested that the PDO and PGI regulations approved some years ago should be revised.

The verification of compliance with a given PDO or PGI designation from a sensory point of view includes the preliminary evaluation of the olive oil grade and then checking of the sensory features reported in the regulation. The oil classification must be performed according to regulations in force and therefore by applying the revised IOC method for the sensory evaluation of VOOs (EC 1992b and subsequent modifications) that establishes the intensity of the sensory descriptors on the basis of the median of intensities perceived by each panelist quantified on a continuous scale 100 mm long; EVOOs must have no organoleptic

defects (medians of negative attributes = 0) or median of fruity greater than 0. However, many PDOs were approved by the EU before the adoption of the revised IOC method by the EU when the grading score for the sensory evaluation of VOOs was carried out by means of a profile sheet with a structured scale from 0 to 5. The two methodologies are different not only because of the kind of scale (structured versus unstructured) but because of the mathematical algorithm applied to determine the intensity of each descriptor.

The large number of requests and grants of PDO and PGI designations from the EU emphasizes the need to develop methods for assessing the sensory attributes of these EVOOs in order to have harmonized procedures of evaluation.

14.9 Other Regulations

The US Department of Agriculture (USDA) revised the old US grade standards for olive oils and olive-pomace oils to conform to current industry standards commonly accepted in the USA and abroad by request of the California Olive Oil Council (COOC), a trade association of olive oil producers. These revised standards (USDA 2010), which are organized and structured similarly to the most recent IOC trade standards, provide a common language for trade and provide consumers more assurance of the quality of the olive oil that they purchase. The revised standards include objective criteria for the various grades of olive oil and olive-pomace oil, including definitions of EVOO, VOO, olive oil, refined olive oil, and olive-pomace oil. This revision affects importers of olive oil and more than 500 domestic olive oil producers and growers.

The North American Olive Oil Association (NAOOA) – a trade association of marketers, packagers, and importers of olive oil in the USA, Canada, and their respective suppliers abroad – was established in 1989. The association strives to foster a better understanding of olive oil and its taste, versatility, and health benefits. A condition of membership in the NAOOA is that all members must agree to abide by the IOC standard and agree that the testing of EVOO samples will include organoleptic testing by an IOC-certified panel.

The COOC is the only North American certified quality control program that exceeds strict international standards for EVOO with respect to the percentage of free fatty acids (0.5 vs. 0.08 of the IOC). The COOC is dedicated to promoting quality EVOOs made in California, and it represents over 90 % of all olive oil production in California, which is estimated to be around 1.2 million gallons in the 2010–2011 harvest, with 30,000 acres planted with over 50 different cultivars.

The blending of olive oil with other edible oils, if clearly labeled as a blended vegetable oil and if the content and proportion of the blend are prominently displayed on the container's label, is not prohibited. California Senate Bill (SB) 634 (CSB 2007) defines flavored olive oil to reflect market practices (e.g., flavored oils, such as lemon olive oil or jalapeño olive oil, are becoming increasingly popular with consumers) but also requires that olive oil be labeled consistently with the updated

food grade definitions. State law, however, does not define olive oil grades, and as a result the grades commonly seen on olive oil bottles – such as extra virgin – do not need to meet any standards. SB 634 helps consumers make informed choices based on consistent standards of quality.

The Australian Olive Association (AOA) was created to underpin and enhance the quality of all Australian olive products and associated production processes. The association developed the Australian Olive Industry Code of Practices (AOA 2009) that address olive oil quality, food safety, ethical marketing, environmental standards, and other issues. The oil must meet most of the same chemical standards as the IOC and be certified free of sensory defects and possessing some olive fruitiness by three AOA-accredited tasters. The association has the "Australian Certified Extra Virgin" seal that can only be found on AOA member olive oils produced in Australia.

Olives New Zealand (ONZ) was established to create an environment for the New Zealand olive industry to produce premium-quality EVOOs and olive products and to market them successfully nationally and internationally. Its certification program, which is open to nonmembers as well as members, includes bottling and labeling standards as well as chemical and sensory criteria in accordance with IOC trade standards (IOC 2011c). The ONZ certification also requires that the oil be bottled in dark glass, or if in clear glass that it be in a box or other light-excluding package, together with the pressing date.

14.10 Importance of Sensory Profile in Virgin Olive Oil Analysis

The current official IOC profile sheet of flavor description and quality grading, even if it allows an oil to be placed in one given category rather than in another, does not provide any description of flavors because the panelists mainly evaluate defects and only three sensory descriptors related to quality characteristics of VOOs (fruity, bitter, and pungent). Therefore, if one wanted to monitor small but meaningful changes in oil sensory profiles ascribable to genetic factors, ripening stages of fruits, or technological processing of oil extraction, it would be necessary to use a larger number of descriptors on the score sheet and have suitably trained assessors to detect the various flavor shades. An example could be represented by the profile sheet shown in Fig. 14.6, which was developed in the course of a project carried out within the framework of Agro-Industrial Research (AIR) (AIR 1999) with the aim of differentiating subtle changes in the green attributes of VOOs resulting from unripe fruits (Angerosa et al. 1997).

QDA was the sensory methodology used. Twelve assessors, eight females and four males, ranging in age from 22 to 50 years, were requested to dissect and freely describe, in their own words, the different sensations they perceived during the evaluation of VOOs. The oils were extracted from fruits of diverse olive varieties at different ripening stages. Descriptors used by each assessor were different, as former experiences associated with the perceived sensations of each assessor were obviously not the same. Several sessions were needed for extensive discussion among

VIRGIN OLIVE OIL
PROFILE SHEET
OLFACTORY-GUSTATORY-TACTILE NOTES

Descriptors	0	1	2	3	4	5
Cut green lawn						
Green leaf or twig						
Green olives						
Wild flowers						
Green banana						
Green tomato						
Almond						
Artichoke						
Apple						
Walnut husk						
Green hay						
Butter/Cream						
Bitter						
Pungent						

1 Barely perceptible
2 Slight perceptible
3 Average
4 Great
5 Extreme

REMARKS.....
.....
NAME OF ASSESSORS.....
LEGEND OF SAMPLE.....
DATE.....

Fig. 14.6 Profile sheet developed to differentiate shades of green attributes in virgin olive oils (*Source*: Authors)

assessors to determine the meaning of each descriptor and to discard redundant descriptors. The descriptors finally agreed on by consensus were selected and included in the evaluation profile sheet. Assessors were selected and trained according to the EC (1991) regulation.

After suitable training to familiarize assessors with the developed vocabulary, the methodology was applied to the evaluation, in triplicate, of oils obtained from unripe fruits of different varieties and of changes occurring during olive paste malaxation in relation to temperature and time. The oils were presented according to an experimental design that minimized any possible bias and carryover effects. Figures 14.7–14.10 display the results of the experiments. Thus, the increase from 25 °C to 35 °C in the malaxation temperature (Fig. 14.7) causes a general weakening of the oil flavor. Green banana and almond notes disappear, and other significant losses are recorded for walnut husk, green tomato, and particularly for bitterness and pungency. Malaxation time influences the flavor of the resulting oils; prolonged

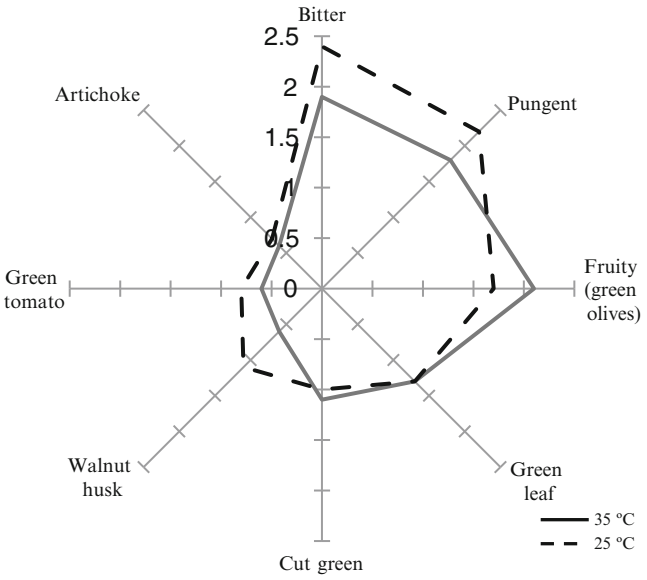

Fig. 14.7 Changes in green attributes of oils from Frantoio variety in relation to malaxation temperature (*Source*: Authors)

periods (Fig. 14.8) reduce typical green notes such as green leaf, cut green lawn, and walnut husk, whereas they do not seem to affect the intensity of fruity sensations.

The evaluation of oils extracted from unripe olives of Greek (Koroneiki) and Spanish (Picual) varieties harvested in the production countries at the same ripening degree showed evidence of different flavor profiles for the two varieties (Fig. 14.9). In addition, the profile sheet was able to differentiate oils from the same Greek variety in relation to geographic origin (Fig. 14.10).

14.11 Statistical Procedures

Sensory data, as obtained by assessors through the use of evaluations, represent the sensory profile of VOOs; nevertheless, their examination does not give complete information. Information regarding possible correlations among attributes, the performance of panels and individuals, and possible discrepancies among assessors in the meaning of descriptors or in the use of scales can be obtained from sensory data by the application of statistical procedures. These last-named methodologies can also identify sensory attributes that can differentiate samples of a product, to reveal how many differences could exist in the work of various panels, to compare different descriptive analytical methodologies, and to predict consumer acceptance from descriptive panel profiles. Although mathematical procedures are profusely described in Chap. 12, Table 14.4 briefly summarizes the most common procedures applied in sensory analysis and their corresponding purposes.

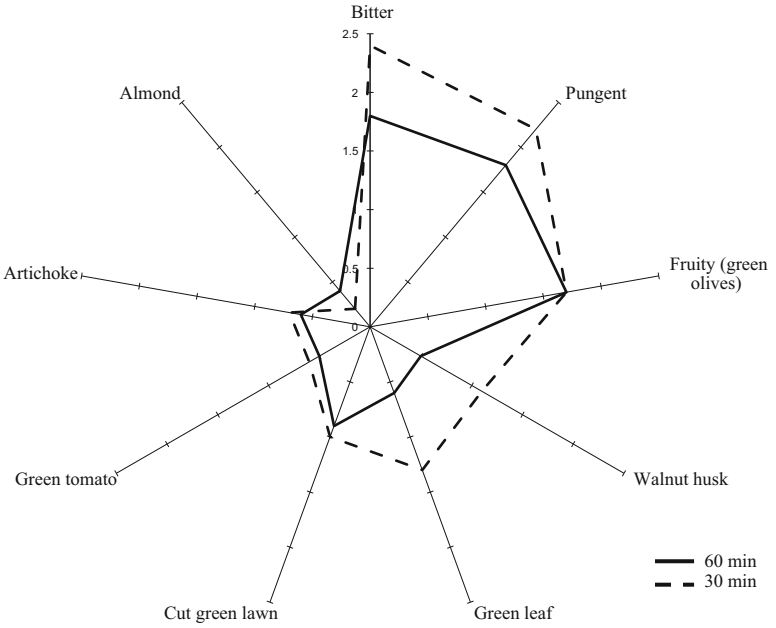

Fig. 14.8 Influence of malaxation time on the sensory profile of oils from Frantoio variety (*Source*: Authors)

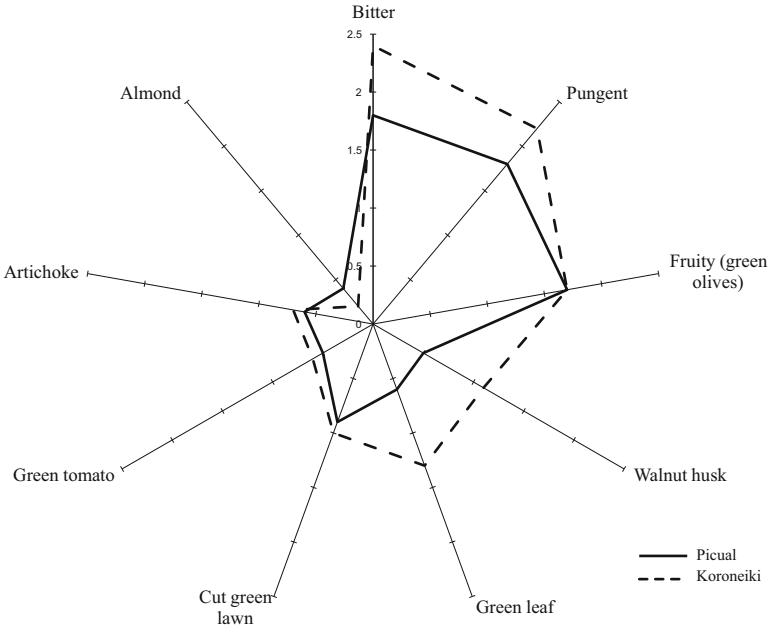

Fig. 14.9 Free profiles of Spanish (Picual) and Greek (Koroneiki) varieties harvested at same ripening degree (*Source*: Authors)

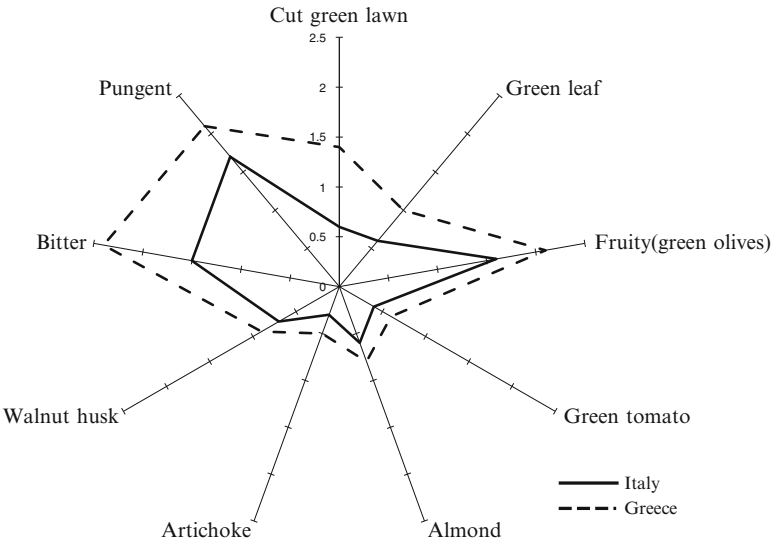

Fig. 14.10 Sensory differentiation of oils from same variety (Koroneiki), harvested at same ripening degree, in relation to their geographic origins (*Source*: Authors)

Table 14.4 Classic statistical procedures applied in sensory analysis

Procedure	Purposes
Analysis of variance (one-two way)	To test the arrangement test reliability
Student's *t*	To test individual and panel performance
Principal component analysis (PCA)	To test individual and panel performance
	To find primary sensory variables and eliminate redundant data
Factor analysis (FA)	To reveal relationships between sensory attributes
Correlation coefficients	To test degree of association between mean intensities and overall grading
	To verify possible discrepancies in meanings of attributes among different panels
	To point out relationships between panels or different scales
Cluster analysis	To group descriptors used by different panels in sensory evaluation
	To verify whether or not two panels work in the same way
Multiple regression	To predict consumer acceptance from descriptive panel profiles
	To provide information about panel performance and identify discrepancies among panelists in the meaning of descriptors and in the use of scales
Generalized procrustes Analysis (GPA)	To identify sensory attributes that allow a clear differentiation of samples
	To use consumers for descriptive purposes
	To compare different descriptive, analytic methodologies

Source: Authors

References

Agro-Industry Research (AIR) (1999) Olive oil flavour and aroma: biochemistry and chemistry of sensory factors affecting consumer appreciation and their analysis by artificial intelligence. Final report of European Research Project AIR3-CT94-1967, The Commission of the European Communities

Angerosa F (2002) Influence of volatile compounds on virgin olive oil quality evaluated by analytical approaches and sensor panels. Eur J Lipid Sci Technol 104:639–660

Angerosa F, Basti C (2001) Olive oil volatile compounds from the lipoxygenase pathway in relation to fruit ripeness. Ital J Food Sci 13:421–428

Angerosa F, Di Giovacchino L (1996) Natural antioxidants of virgin olive oil obtained by two and tri-phase centrifugal decanters. Grasas Aceites 47:247–254

Angerosa F, Di Giacinto L (1995) Crushing influence on the quality characteristics of virgin olive oil. Riv Ital Sost Grasse 72:1–4

Angerosa F, Di Giacinto L, Solinas M (1990) Influence of olive mass storage on the aroma of resulting oils: evaluation of "fusty" defect by means of HPLC and GLC analyses of volatile compounds. Riv Merceol 29:275–294

Angerosa F, Di Giacinto L, De Mattia G (1993) Shelf-life of bottled extra virgin olive oils. Riv Merceol 32:243–259

Angerosa F et al (1995) GC-MS evaluation of phenolic compounds in virgin olive oil. J Agric Food Chem 43:1802–1807

Angerosa F et al (1996a) Sensory evaluation of virgin olive oils by artificial neural network processing of dynamic head-space gas chromatographic data. J Sci Food Agric 72:323–328

Angerosa F et al (1996b) Characterization of phenolic and secoiridoid aglycons present in virgin olive oil by gas chromatography-chemical ionization mass spectrometry. J Chromatogr A 736:195–203

Angerosa F, Lanza B, Marsilio V (1996c) Biogenesis of "fusty" defect in virgin olive oils. Grasas Aceites 47:142–150

Angerosa F, Di Giacinto L, d'Alessandro N (1997) Relationship between aroma components and malaxation time of olive paste. J High Resol Chromatogr 20:507–510

Angerosa F et al (1999) Olive oil off-odour compounds produced by *Aspergillus* and *Penicillium*. Acta Hortic 474:695–699

Angerosa F, Servili M, Selvaggini R, Taticchi A, Esposto S, Montedoro GF (2004) Volatile compounds in virgin olive oil: occurrence and their relationship with the quality. J Chromatogr A 1054:17–31

Aparicio R, Morales MT (1994) Optimization of a dynamic headspace technique for quantifying virgin olive oil volatiles. Relationship among sensory attributes and volatile peaks. Food Qual Pref 5:109–114

Aparicio R, Gutiérrez F, Rodríguez J (1991) A chemometrics? study of analytical panels in virgin olive oil. An approach for evaluating panels in training. Grasas Aceites 42:202–210

Aparicio R, Alonso MV, Morales MT, Calvente JJ (1994) Relationship between the COI test, and other sensory profiles by statistical procedures. Grasas Aceites 45:26–41

Aparicio R, Morales MT, Alonso MV (1996) Relationship between volatile compounds and sensory attributes by statistical sensory wheel. J Am Oil Chem Soc 73:1253–1264

Australian Olive Association (AOA) (2009) Australian Olive Association code of practices for olive oil. Including AOA determination. June, Pendie Hill, NSW

Bartoshuk LM (1993) The biological basis of food perception and acceptance. Food Qual Pref 4:21–32

Bartoshuk LM et al (1992) PROP supertasters and the perception of sweetness and bitterness. Chem Sens 17:594–612

Bate-Smith EC (1973) Haemananalysis of tannins: the concept of relative astringency. Phytochem 12:907–912

Beal AD, Mottram DS (1993) An evaluation of the aroma characteristics of malted barley by free-choice profiling. J Sci Food Agric 61:17–22

Bedoukian PZ (1971) The seven primary hexenols and their olfactory characteristics. J Agric Food Chem 19:1111–1114
Boskou D (2006) Olive oil composition. In: Boskou D (ed) Olive oil chemistry and technology, 2nd edn. AOCS Press, Champaign, pp 44–45
Breslin PAS (1996) Interactions among salty, sour and bitter compounds. Trend Food Sci Technol 71:390–399
Caincross SE, Sjöström LB (1950) Flavor profiles - a new approach to flavor problems. Food Technol 4:308–311
California Senate Bill 634 (CSB) (2007) and AB 2824, 1 Jan 2009
Cavazzani M, Uras P (2000) Le flogosi rino-faringo-sinusali e le loro complicanze. Piccin Nuova Libraria S.p.A, Padova
Chambers E, Smith EA (1993) Effects of testing experience on performance of trained sensory panelists. J Sens Stud 8:156–166
Civille GC, Lawless HT (1986) The importance of language in describing perceptions. J Sens Stud 7:203–215
Clapperton JF, Piggott JR (1979) Flavor characterization by trained and untrained assessors. J Inst Brew 85:275–277
Clydesdale FM (1993) Color as a factor in food choice. Crit Rev Food Sci Nutr 33:83–101
Di Giovacchino L, Solinas M, Miccoli M (1994) Effect of extraction systems on the quality of virgin olive oil. J Am Oil Chem Soc 71:1189–1194
Erickson RP, Covey E (1980) On the singularity of taste sensations: what is a taste primary? Physiol Behav 25:527–533
European Communities (EC) (1991) Official journal of the commission of the European communities. Regulation No. 2568/91, L248, 5 Sept 1991
European Communities (EC) (1992a) Official journal of the commission of the European communities. Regulation No. 2081/92, L 208, 14 July 1992
European Communities (EC) (1992b) Official journal of the commission of the European communities. Regulation No 796/02, L 128/8, 6 May 2002
European Communities (EC) (1995) Official journal of the commission of the European communities. Regulation No. 2527/95, L 258, 28 Oct 1995
European Communities (EC) (2001) Official journal of the commission of the European communities. Regulation No. 2042/01, L276, 19 Oct 2001
European Communities (EC) (2002) Official journal of the commission of the European communities Regulation No. 796/02, L 128/8, 6 May 2002
European Communities (EC) (2006) Official journal of the European Union. Regulation No. 510/06, L93/12, 31 Mar 2006
European Communities (EC) (2008) Official journal of the European Union. Regulation No. 640/08, L148/11, 4 July 2008
European Communities (EC) (2011) Official journal of the European Union. Regulation No. 61/2011, L23/1, 24 Jan 2011
Faurion A (2006) Sensory interactions through neural pathways. Physiol Behav 89:44–46
Fedeli E (1993) Organoleptic evaluation of virgin olive oils. Riv Ital Sost Grasse 70:81–85
Garcia-Gonzales DL, Vivancos J, Aparicio R (2011) Mapping brain activity induced by olfaction of virgin olive oil aroma. J Agric Food Chem 59:10200–10210
García-González DL, Aparicio R (2004) Classification of different quality virgin olive oils by metal-oxide sensors. Eur Food Res Technol 218:484–487
García-González DL, Aparicio R (2010) Coupling MOS sensors and gas chromatography to interpret the sensor responses to complex food aroma: application to virgin olive oil. Food Chem 120:572–579
Gariboldi P, Jommi G, Verrotta L (1986) Secoiridoids from *Olea europaea*. Phytochemistry 25:865–869
Guth H, Grosh W (1993) Quantitation of potent odorants of virgin olive oil by stable isotope dilution assay. J Am Oil Chem Soc 70:513–518
Gutiérrez R (1989) Methodologie d'évaluation des qualités organoleptiques de l'huile d'olive vierge. In: Proceedings of international congress "Chevreul", Angers, pp 534–541

Gutiérrez F, Cabrera J, Gutiérrez R (1974) Considerations on the "Panel test" advantages for the organoleptic quality evaluation of foods. Application to virgin olive oil. Grasas Aceites 25:111–116
Gutiérrez F, Alba JM, Gutiérrez R (1984) The selection of judges by rating of intensity method. Grasas Aceites 35:310–314
Hatanaka A et al (1992) Odor-structure relationships in n-hexanols and n-hexenales. Z Naturforsch 47:183–189
International Olive Council (IOC) (1996) Document COI/T.20/Document No. 15/Rev.1. Revised method for the evaluation of virgin olive oil, Madrid
International Olive Council (IOC) (2005) Document COI/T.20/Doc. No. 22. Method for the organoleptic assessment of extra virgin olive oil applying to use a designation of origin, Madrid
International Olive Council (IOC) (2007a) Document COI/T.20/Doc No. 4/Rev.1. Sensory analysis of olive oil standard. Sensory analysis: general basic vocabulary. Madrid
International Olive Council (IOC) (2007b) Document COI/T.20/Doc.No 5/Rev.5 Sensory analysis of olive oil standard. Glass for oil tasting, Madrid
International Olive Council (IOC) (2007c) Document COI/T.20/Doc. No 6/Rev.1. Sensory analysis of olive oil: standard guide for the installation of a test room, Madrid
International Olive Council (IOC) (2011a) Document COI/T.20/Doc. No 14/ Rev. 3. Sensory analysis of olive oil standard: guide for the selection, training and monitoring of skilled virgin olive oil tasters, Madrid
International Olive Council (IOC) (2011b) Document COI/T.20/Doc. No 15/Rev. 4. Sensory analysis of olive oil: method for the organoleptic assessment of virgin olive oil, Madrid
International Olive Council (IOC) (2011c) COI/T.15/NC No 3/Rev.6.Trade standard applying to olive oils and olive-pomace oils, Madrid
Ishii R, O'Mahony M (1987) Defining a taste by a single standard: Aspects of salty and umami tastes. J Food Sci 52:1405–1409
Ishii R, O'Mahony M (1991) Use of a multiple standards to define sensory characteristics for descriptive analysis: aspects of concept formation. J Food Sci 56:838–842
Jones PN, McLachlan GJ (1991) Fitting mixture distributions to phenylthiocarbamide (PTC) sensitivity. Am J Hum Genet 48:117–120
Kalmus H (1971) The genetics of taste. In: Beilder LM (ed) Taste. Springer, New York, pp 165–178
Kroll BJ (1990) Evaluating rating scales for sensory testing with children. Food Technol 44:82–88
Kubo I, Matsumoto A (1984) Molluscicides from *Olea europaea* and their efficient isolation by countercurrent chromatographies. J Agric Food Chem 32:687–688
Kuehn M et al (2008) Changes of pressure and humidity affect olfactory function. Eur Arch Otorhinolaryngol 265:299–302
Lawless HT (1986) Sensory interactions in mixtures. J Sens Stud 1:259–274
Lawless HT (1991) Bridging the gap between sensory science and product evaluation. In: Sensory science theory and applications in foods. Marcel Dekker, New York, pp 353–393
Lee CB, Lawless HT (1991) Time-course of astringent sensations. Chem Senses 16:225–238
Lyon DH, Watson MP (1994) Sensory profiling: a method for describing the sensory characteristics of virgin olive oil. Grasas Aceites 45:20–25
Marsilio V, Spotti E (1987) Investigation into mould contamination of dried black table olives. Ind Conserve 62:287–291
McBride RL, Finley DC (1989) Perception of taste mixtures by experienced and novice assessors. J Sens Stud 3:237–248
McEwan JA (1994) Consumer attitudes and olive oil acceptance: the potential consumer. Grasas Aceites 45:9–15
Miller IJ Jr, Reedy FE Jr (1990) Quantification of fungiform papillae and taste pores in living human subjects. Chem Senses 15:281–294
Montedoro GF, Baldioli M, Servili M (1992) Sensory and nutritional relevance of phenolic compounds in olive oil. Giornale Ital Nutriz Clin Preven 1:19–32

Montedoro GF et al (1993) Simple and hydrolyzable compounds in virgin olive oil. 3. Spectroscopic characterizations of secoiridoid derivatives. J Agric Food Chem 41:2228–2234
Morales MT, Aparicio R (1993) Characterizing some European olive oil varieties by volatiles using statistical tools. Grasas Aceites 44:113–115
Morales MT, Aparicio R, Rios JJ (1994) Dynamic headspace gas chromatographic method for determining volatiles in virgin olive oil. J Chromatogr A 668:455–462
Morales MT, Alonso MV, Rios JJ, Aparicio R (1995) Virgin olive oil aroma: relationship between volatile compounds and sensory attributes by chemometrics. J Agric Food Chem 43:2925–2931
Morales MT, Luna G, Aparicio R (2000) Sensory and chemical evaluation of winey-vinegary defect in virgin olive oils. Eur Food Res Technol 211:222–228
Morales MT, Luna G, Aparicio R (2005) Comparative study of virgin olive oil sensory defects. Food Chem 91:293–301
Moruzzi G (1977) L'olfatto. In: Fisiologia della vita di relazione. UTET, Torino, pp 373–381
Naish M, Clifford MN, Birch GG (1993) Sensory astringency of 5-O-caffeoylquinic acid, tannic acid and grape-seed tannin by a time-intensity procedure. J Sci Food Agric 61:57–64
Nakagawa M, Mizuma K, Inui T (1996) Changes in taste perception following mental or physical stress. Chem Senses 21:195–200
O'Mahony M (1991) Descriptive analysis and concept alignment. In: Sensory science theory and applications in food. Marcel Decker, New York, pp 233–267
O'Mahony M (1995) Who told you the triangle test was simple? Food Qual Pref 6:227–238
Oreskovich DC, Klein BP, Sutherland JW (1991) Procrustes analysis and its applications to free-choice and other sensory profiling. In: Sensory science theory and applications in foods. Marcel Dekker, New York, pp 353–393
Pagliarini E, Bertuccioli M, Abbà S (1994) Consumer attitudes and olive oil acceptance: the traditional consumer. Grasas Aceites 45:16–19
Peryam DR, Pilgrim FJ (1957) Hedonic scale method of measuring food preferences. Food Technol 11:9–14
Piggott JR (1990) Relating sensory and chemical data to understand flavor. J Sens Stud 4:261–272
Raney BA (1986) Importance of reference standards in training panelists. J Sens Stud 1:149–154
Ranzani C (1994) Olive oil quality and EEC regulations. Grasas Aceites 45:1–4
Roessler EB et al (1978) Expanded statistical tables for estimating significance in paired-preference, paired-difference, duo-trio and triangle tests. J Food Sci 43:940–947
Rolls ET (2006) Brain mechanisms underlying flavour and appetite. Phil Trans R Soc B 361:1123–1136
Royet JP, Plailly J (2004) Lateralization of olfactory processes. Chem Senses 29:731–745
Servili M et al (1995) Sensory characterization of virgin olive oil and relationship with headspace composition. J Sci Food Agric 67:61–70
Shepherd R, Griffiths NM, Smith K (1988) The relationship between consumer preferences and trained panel responses. J Sens Stud 3:19–35
Solinas M (1987) Evaluation of the sensory characteristics of virgin olive oil. Ann Ist Sper Elaiot 10:unpaginated
Sterling EM, Arundel A, Sterling TD (1985) Criteria for human exposure to humidity in occupied buildings. ASHRAE Trans 91:611–622
Stone H et al (1974) Sensory evaluation by quantitative descriptive analysis. Food Technol 28:24–34
Stone H, Siedel JH, Bloomquist J (1980) Quantitative descriptive analysis. Cereal Foods World 25:642–644
Sunwoo Y et al (2006a) Physiological and subjective responses to low relative humidity. J Physiol Anthropol 25:7–14
Sunwoo Y et al (2006b) Physiological and subjective responses to low relative humidity in young and elderly men. J Physiol Anthropol 25:229–238

United States Department of Agriculture (USDA) (2010) United States standards for grades of olive oil and olive-pomace oil. Federal Register, 24 Oct 2010, Washington, DC
Verhagen JV, Engelen L (2006) The neurocognitive bases of human multimodal food perception: sensory integration. Neurosci Biobehav Rev 30:613–650
Voirol E, Daget N (1986) Comparative study of nasal and retronasal olfactory perception. Lebensm Wiss Technol 19:316–319
Withehead MC, Breeman CS, Kinsella BA (1985) Distribution of taste and general sensory nerve endings in fungiform papillae of the hamster. Am J Anat 173:185–201

Chapter 15
Olive Oil Consumer Research: Methods and Key Learnings

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Contents

15.1 Introduction

Consumers play a significant role in the success or failure of products, and olive oil is no exception. Sensory properties play a key role in food acceptability; aroma, taste, texture, appearance, and color are among the factors that influence final consumption by consumers (Cardello 2003; Drewnowski 1997). However, acceptability and consumption are also influenced by extrinsic factors that include contextual, cognitive, social, and cultural variables (Cardello 2003; Sijtsema et al. 2002). As a consequence, product acceptability relies on a number of complex variables that are either continuous (e.g., concentration of a particular ingredient, product age) or categorical (e.g., consumer gender and product brand). The complexity of these factors

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties*,
DOI 10.1007/978-1-4614-7777-8_15, © Springer Science+Business Media New York 2013

suggests that it is important to determine the most appropriate methods to understand how consumers perceive olive oil. This chapter is intended to introduce some of these methodologies as they relate to olive oil.

15.2 Quantitative Research

In using quantitative measures – in the form of structured questions and answers, where consumers are selected randomly from the study or target population – researchers must follow basic principles and guidelines in the preparation of questionnaires (Bradburn et al. 2004; Dillman 2007; Groves 2004). Consumer responses can then be analyzed through a diverse set of statistical procedures. The main advantage of quantitative research is the ability to test hypotheses and to obtain results from subject populations. Disadvantages include consumer bias, fatigue or boredom (for example, with long questionnaires), and variability between researcher-administered and self-administered questionnaires.

15.2.1 Survey Research

Survey research is designed to collect information concerning a topic of interest through the use of questionnaires. Answering a survey questionnaire involves four processes: comprehension, retrieval, judgment, and response (Jobe and Mingay 1989; Tourangeau et al. 2000). First, the subject needs to understand the question. Second, the subject must remember the relevant information that the question calls for. Third, the subject needs to determine which parts of the retrieved information are relevant to the question. Fourth, before responding, the subject weighs his/her answers based on several factors, including the sensitivity of the information, the social desirability of the answer, and the probable accuracy of the answers, before eventually committing to a response to the question.

The format of the questions determines the best method of contacting the consumer: survey questionnaires can be administered by telephone, in person, by mail, or over the Internet. Table 15.1 describes each method. Writing clear questions is key when designing survey questionnaires; ambiguous or complicated questions or vague terms in the questionnaire are likely to produce biased results (Bradburn and Miles 1979; Fowler 1992). Lawless and Heymann (2010) provide the following recommendations for designing a questionnaire:

- Be brief.
- Use plain language.
- Do not ask what they do not know.
- Be specific.
- Multiple-choice questions should be mutually exclusive and exhaustive.

Table 15.1 Comparison of mail, online, telephone, and in-person surveys

Survey	Kind of questionnaire	Data collection time	Advantages	Disadvantages
Mail	Self-administered	Can take months to collect data	Easy and convenient: consumers respond at their own pace	Low response rate
			Higher response for sensitive questions because no interviewer is present	Incomplete questionnaire responses
			Relatively cheap compared with other formats	
Online (Web site or by e-mail)	Self-administered	Immediate	Convenient: consumers respond at their own pace	Low response rate
			Faster distribution than mail format	Consumers may not be representative
			Software can help design questionnaire: skip questions, avoid leaving a question unanswered	Some consumers are unfamiliar with computers
Telephone	Handled by interviewer	Immediate if assisted by computer	Convenient for quick answers to simple questions	Consumers may find the calls intrusive or not trust in the person calling and how the data will be handled and refuse the survey
In person	Handled by interviewer	Longer time than the online and telephone formats but may be shorter than mail format	Presence of interviewer may be useful to clarify questions or make easier for consumers to understand the instructions of the survey	Presence of interviewer may bias the results in two ways:
			(a) Intercept them in a central location or	(a) Consumers want to impress interviewer or
			(b) Interview them at home	(b) Consumer does not respond to questions due to embarrassment. More expensive than other formats

- Do not lead the respondent.
- Avoid ambiguity.
- Beware of the effects of wording.
- Beware of halos and horns.
- Pretesting is usually necessary.

Several researchers have considered the advantages and disadvantages of various question formats, especially regarding the use of open-ended or closed-ended questions. Open-ended questions offer the advantage of being easy for the researcher to structure, and for respondents to answer, since they can use their own words. The disadvantages are that sometimes respondents fail to provide an answer, and the use of their own words makes statistical analysis and interpretation of results more difficult. With closed-ended questions, the main advantage is the ease of coding and, therefore, the relative ease of statistical analysis and interpretation; however, there is a risk that consumers will not understand the question or will choose from among answers that do not fully or accurately reflect their true opinions or attitudes (Belson and Duncan 1962; Converse 1984; Rasinski et al. 1994; Bradburn et al. 2004; Groves 2004; Smyth et al. 2006; Dilman 2007).

The format of the questionnaire can also affect the survey results, so the researcher must consider how the survey will be administered (either self-administered or handled by an interviewer). Self-administered questionnaires offer the advantage of eliminating bias from an interviewer, especially for sensitive questions; the presence of the interviewer is, however, useful in clarifying questions or instructions. Also to be considered is whether the questionnaire will be on paper or in a computer-based format (either online or via a CD). The computer-based format has the advantages of forcing the respondent to select an answer (e.g., even if one of the choices is "prefer not to answer") and of allowing the researcher access to the data almost immediately, thereby reducing the cost of transferring the data onto computer. The paper questionnaire, on the other hand, is useful when the survey needs to be conducted in a location where no computers are available – e.g., outside supermarkets – or when the respondent does not feel confident using computers (Bourque and Fielder 2002; Dillman 2007; Groves 2004). Finally, the format and style of the questionnaire also have an effect on the respondent. Recommendations for questionnaires include having a clear format, using the same font size for all questions, highlighting (in color) the different responses, and, when questionnaires are more than one page in length, indicating clearly that the questionnaire continues on the next page (Dillman 2007; Sanchez 1992).

Survey research has been applied to understand consumer behavior regarding extra virgin olive oil (EVOO) in our laboratory (Santosa 2010) and by others. In this research, we utilized different question modes to accommodate the large numbers of topics being covered. The formats included yes/no, check-all-that-apply, multiple-choice, and open-ended questions. The survey questionnaire was self-administered and consisted of three main parts: oil consumption and purchase behavior, demographics, and psychographics. The survey was administered to 178 northern California consumers from April to August 2009. Three consumer segments were

Table 15.2 Summary of hierarchical segment (cluster) analysis results of consumer segmentations based on three different criteria

	Number of consumers	*%*	*Description of types of olive oil consumed*
Cluster 1	106	59.6	Only EVOOs
Cluster 2	45	25.3	(1) EVOOs and one other type of olive oil such as non-EVOO or flavored olive oils or both types (2) Only non-EVOOs (3) Non-EVOOs and flavored olive oils (4) Only flavored olive oils (5) Not sure what types of olive oils consumed
Cluster 3	27	15.2	Both EVOOs and flavored olives oils
	Number of consumers	*%*	*Description of types of olive oil brand consumed*
Cluster 1	77	43.3	(1) Store brands (2) Don't know brand names
Cluster 2	54	30.3	(1) Imported brands (2) Imported and store brands (3) Imported and local brands (4) Imported, store, and local brands
Cluster 3	47	26.4	(1) Local brands (2) Local and store brands
	Number of consumers	*%*	*Description of origin of olive oils consumed*
Cluster 1	71	40.0	Imported
Cluster 2	71	40.0	Imported and local
Cluster 3	36	20.0	(1) Local (2) Don't know origin

identified. Using hierarchical cluster analysis (HCA), the survey study segmented consumers based on three criteria: (1) types of olive oil consumed, (2) olive oil brands consumed, and (3) origin of olive oils consumed. Based on the HCA dendrograms, three consumer clusters were identified (Table 15.2). A summary of the results from this survey follows.

15.2.1.1 Events Prompting Olive Oil Use

Most consumers were prompted to start consuming olive oil by their awareness of olive oil's health benefits. These consumers were unlikely to have consumed olive oil when they were still living at home with their family and were unlikely to have olive oil consumption as part of their cultural traditions. Few consumers who were prompted by cultural tradition or from having their parents or family use olive oil when they were still living at home continued their consumption habits into their adult life. A large majority of consumers also started consuming olive oils because of one of a number of events, such as starting to cook by themselves; their spouse or

partner using olive oil for preparing meals and cooking; a recipe calling for olive oil use; a cooking show they watched often using olive oil as an ingredient; having an olive oil tasting or sampling at a tasting room, a farmers' market, or olive-oil-related event; and having olive oil as a condiment for bread dipping at a restaurant. Very few consumers started consuming olive oil because they had lived or had a holiday overseas. Most of the consumers in the survey were unlikely to be prompted to start consuming olive oil as a result of receiving it as a gift.

15.2.1.2 Shopping Place

Consumers who purchased store brand olive oils were more likely than any other consumer group to go to discount retailers (i.e., a warehouse club such as Costco). This consumer group, together with the most diverse consumer group (cluster 2), also normally went to supermarkets. Consumers in cluster 2 were also more likely to have purchased olive oils imported from overseas, on a Web site, and from wineries. Consumers who purchased only local brands or both local and store brands were more likely to have purchased olive oils from specialty/gourmet stores, at an olive oil tasting/fair/holiday or food festival, from olive oil farmers/producers or an ethnic market. Some consumers across the three consumer clusters also went to a farmers' market to buy their olive oils. This pattern was found to be statistically significant ($p < 0.05$).

15.2.1.3 Typical Price Range and Volume

There was a tendency for store brand olive oil buyers to buy cheaper oils between \$5 and \$7.99 or \$5 or less per half-liter, for consumers who purchased either local brands or both local and store brands to buy in the higher middle price point between \$16–\$20.99 and \$21–\$25.99, and for consumers who purchased imported brands or imported brands and a combination of other types of olive oil brands to buy from the highest price range of \$26–\$30.99 or \$31 and above. However, these trends were not statistically significant ($p > 0.05$). This could be due to the fact that most of the consumers coming from the three different consumer groups bought from the middle price range between \$8–\$9.99 and \$10–\$15.99.

Consumers who could not estimate the typical olive oil volume they bought came primarily from the consumer group that bought store brand olive oil. This group tended to buy the bigger volume of 5 L, 2 gal, or bulk size. Like this consumer group, consumers who consumed only local brands or both local and store brands tended to buy bulk size as well, in addition to the smaller size of 250 mL and the bigger size of a half-gallon. Consumers who typically bought imported brands or imported brands and other types of olive oil tended to buy the medium size of 750 mL and the bigger size of 2 L. It can be seen that each consumer group had two different sizes of olive oil that they tended to buy. The trends observed were not statistically significant ($p > 0.05$). This could be because other sizes, such as

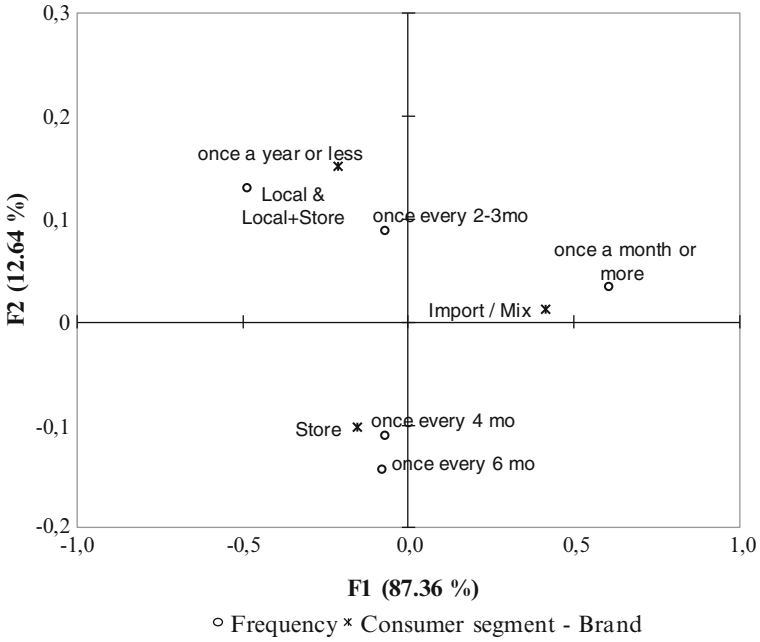

Fig. 15.1 Correspondence analysis of purchase frequency for olive oil bought for own consumption and use at home for consumer clusters based on olive oil brands purchased

350–375 mL, 500 mL, and 3 L, were shared by most consumers across the three consumer groups, or because each consumer group had the same tendency of purchasing at least two different sizes.

15.2.1.4 Purchase Frequency

Figure 15.1 shows the correspondence analysis (CA) results of how the purchase of olive oil brands affected purchasing frequency. To analyze a data set of nominal or ordinal level, CA or multiple correspondence analysis (MCA) is used to detect significant associations between two variables or several variables at once, respectively. CA may produce either a symmetrical map, which plots the principal coordinates of the rows and the columns, or an asymmetrical map, which shows the describing variable (usually the rows) in standard coordinates and the variable being described (the columns) in principal coordinates (Blasius and Greenacre 2006). The measure of variance is closely related to the chi-squared statistic. The objective of CA is to represent the maximum possible variance in a map of few dimensions. The map is interpreted in how the categories lie relative to one another and how the other categories are spread out relative to the former categories. The origin coincides with the average for the data set at hand. Therefore, the categories are judged in terms of how much they deviate from the average. Consumers who purchased only local

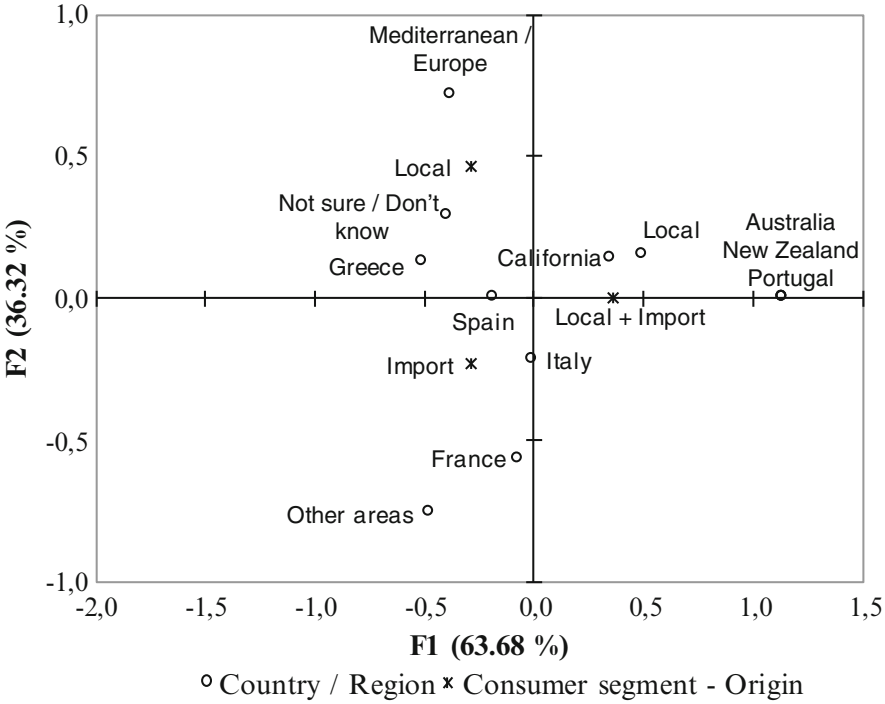

Fig. 15.2 Correspondence analysis of regions believed to be the best producers of extra virgin olive oils for consumer clusters based on origin of olive oils consumed

brands or both local and store brands tended to buy either once every 2–3 months or once a year or less. Store brand olive oil buyers tended to purchase once every 4–6 months. Consumers who purchased only imported brand oils or imported and some other brands of olive oil tended to buy once a month or more. The apparent trends were not statistically significant ($p > 0.05$). This could be because the purchase frequency of once every 2–3 months was shared by all consumer groups. Since consumers purchased different sizes of olive oils depending on the intended use, they finished the different bottles at different rates. Hence their purchasing frequency varied as well.

15.2.1.5 Beliefs About Production Region

Survey participants believed that Italy was the region producing the highest-quality EVOO. California was the second most cited. If California was combined with the local regions, then the USA was the second most cited country, with Spain coming in third. About 23 % of the consumers surveyed felt unsure or did not know what the best region was for producing EVOO (Fig. 15.2). A chi-squared test of independence showed that the consumer segments and their beliefs were significantly

associated ($p < 0.05$). It can be seen that some consumers who purchased and consumed both locally made olive oils and imported olive oils had tasted olive oils from Portugal, Australia, and New Zealand and believed these regions were producing top EVOOs comparable to those of California, while still maintaining that Italy produced the best oils. Consumers who bought imported oils cited Italy as the best producing region more often than California or other local regions. This consumer group was also more likely to be familiar with olive oils produced in France, Spain, and other areas. Some consumers who purchased only locally made olive oils, together with the few consumers who did not know where their olive oil came from, believed that the best EVOOs came from the Mediterranean region or Europe. This consumer group, together with the group that consumed only imported oils, admitted to not knowing or not being sure where the best EVOO came from, more so than the consumers who consumed both imported and local oils.

15.2.1.6 Olive Oil Consumption Motivation

Flavor, health benefits, and cuisines/cooking were shared in different proportions as the three most cited motivators behind purchasing and consuming olive oil among the three consumer segments.

15.2.1.7 Olive Oil Purchase Motivation

Some consumers purchased local olive oils out of convenience – for example, because they happened to see some at the farmers' market or at the supermarket while shopping for other household groceries. Besides finding the intended use of olive oil as an influential indicator of consumer behavior, this survey's results highlighted that the physical presence of others (i.e., farmers/producers at the table) might also impact purchasing behavior of those consumers who consumed local oils or who shopped at the farmers' market. The small size, packaging container, appearance of the olive oils, and the tasting awards the olive oil had garnered attracted this consumer segment. Awards have also been shown to affect wine choice in certain ways depending on whether the buyers are low- or high-involvement consumers (Lockshin et al. 2006). Some consumers who purchased both local and imported olive oils did so to support the local growers or farmers and because of a personal connection to the producer. The olive oils tended to be organic and of the specialty type or were perceived to be of higher quality and suitable for special purposes or occasions. Supporting the local economy was shared by this consumer segment together with consumers who purchased only local olive oils. Consumers who purchased imported olive oils tended to buy olive oils that were familiar or relatively cheaper or on sale or discounted in some other way. The oils tended to be in large-quantity containers and at a good price point. Because a liking for the taste or flavor of the olive oils was the main reason cited for buying the olive oils consumers had at home, this reason was shared rather similarly across segments.

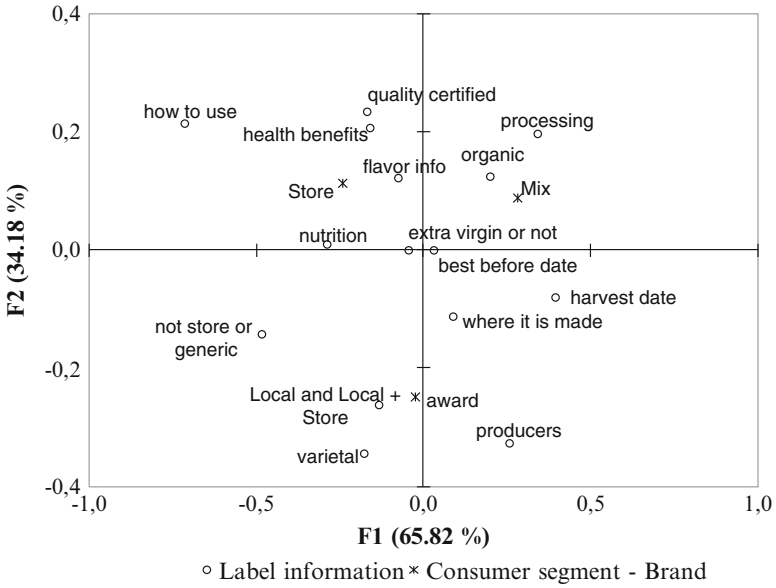

Fig. 15.3 Correspondence analysis of information read on product labels for consumer clusters based on olive oil brands purchased

15.2.1.8 Product Label Information

Consumers were asked how frequently they read the olive oil product label information and what kind of information they were interested in. About 20 % admitted to never reading the product label information prior to deciding which olive oil to buy. The majority of consumers who normally bought store brands *never* read the packaging label, although some *rarely* read it. The second highest percentage of consumers who *rarely* read the label tended to buy locally produced olive oils or both locally produced and store brands. Some of this consumer segment *sometimes* read the label. Most consumers belonging to the most diverse segment, who purchased all three types of brands, *sometimes* read the label. Some actually read it *most of the time*, with a few reading it *all of the time*.

CA was performed on consumer segments based on olive oil brands consumed and the kinds of information they would read on the product label (Fig. 15.3). The chi-squared test showed that there was a significant association between consumer segments and the kinds of information they would read on product labels ($p < 0.05$). Consumers who purchased only store brand oils were more interested in learning about recommendations on how to use the olive oil. Consumers who purchased the whole range of oils were more interested in learning whether the oil was organic

and how the olives had been processed. Flavor descriptions of the olive oils, health benefits, and quality certification were shared by these two consumer groups as information they read on the label. Consumers who purchased local brand oils or both local and store brands were more interested in the olive varietal or cultivar (single or blend) and whether the oil had won any awards. Some of these consumers wanted to learn whether the olive oil was a store or generic brand, although more consumers who purchased store brands were more interested in this than any other group. These consumers might only be buying the store private label brand and not other brands they perceived to be generic or they might be buying the brands that have been established for a while in the supermarket aisle and not the grocery store/private label brands. Whether or not the olive oil was extra virgin and still within the "best before" date were factors across all consumer segments, hence their position near the origin.

15.2.2 Use of Sensory Evaluation Methods and Survey Research to Uncover Consumer Preferences

Some authors have discussed how consumers' responses about why they preferred Product A instead of Product B tend to be ambiguous; some consumers say they like a product because of its flavor, while others say they disliked a product because of the flavor (Elmore et al. 1999; Lawless and Heymann 2010). As an alternative, it is suggested that researchers first try to obtain the sensory characteristics of the products, using any of the analytical sensory methodologies that require training of the panelists (mainly descriptive analysis methods), second ask for hedonic consumer responses, and then relate the results through statistical procedures, the most common being preference mapping (Lawless and Heymann 2010; Næs et al. 2010).

15.2.2.1 Characterization of Products

The first step in training judges is to teach them how to characterize the products, so that they can identify differences and similarities among them. A sensory methodology called descriptive analysis is typically used for this purpose. Different techniques have been used: Quantitative Descriptive Analysis (Stone and Sidel 2004), Sensory Spectrum method (Meilgaard et al. 2007), or generic descriptive analysis (Lawless and Heymann 2010). While those methods differ in theory, the principles and outputs are similar. In general, these techniques require recruiting panelists based on their availability and their previous experience, screening them for any anosmia or allergies, then training them with specific tasks to develop the vocabulary or to refamiliarize them with terms already in the literature. Training is accomplished by introducing standards, ways of quantifying the attributes, and selecting the scale to rate them. The next step is for the researcher to evaluate the panel performance after training, primarily to evaluate whether there is consistency of ratings

Table 15.3 Extra virgin olive oils used for consumer preference tasting study

ID	Country of origin	Variety
U1	USA (California)	Arbequina
U2	USA (California)	Blend
U3	USA (California)	Arbequina/Arbosana/Koroneiki
U4	USA (California)	Sevillana
U5	USA (California)	Frantoio
U6	USA (California)	Mission/Manzanilla/Sevillana/Barouni/Ascolano
U7	USA (California)	Manzanilla/Mission
U8	USA (California)	Blend
U9	USA (California)	Arbequina
U10	USA (California)	Frantoio/Leccino/Pendolino/Coratina
U11	USA (California)	Mission
S1	Spain	Picual
S2	Spain	Hojiblanca
C1	Chile	Picual
C2	Chile	Arbequina
I1	Italy	Frantoio
I2	Italy	Taggiasca (late harvest)
I3	Italy	Taggiasca
I4	Italy	Picholino
A1	Australia	Hojiblanca
GS	Spain	Generic brand (oils from several countries)
GI	Italy	Generic brand (oils from Italy)

both within the group and with each of the individuals involved (reproducibility). Once consistency is determined and additional training is given, if needed, the panelists are ready to evaluate the samples; if the panel will be used over a long term, the next step is monitoring their training and performance.

We conducted a study to characterize 22 EVOOs (50 % of them from California, 50 % imported) (Table 15.3) using a generic descriptive analysis to develop the language and methodology for the evaluation of EVOO. A panel of 18 judges (14 women, 4 men), with an average age of 29 years for the women and 30 years for the men, was assembled for this study. Each panelist completed 10 training sessions (development of the language, concept alignment, agreement). A total of 22 attributes were defined by the panel. EVOOs were evaluated in triplicate, with five samples evaluated per session. The samples were served in a randomized order according to a Latin square design provided by FIZZ software (Biosystèmes, Couternon, France). Samples were coded with random three-digit numbers. More details about the methods can be found in Delgado and Guinard (2011a). A canonical variate analysis (CVA) was conducted to understand sensory similarities and differences among the 22 EVOOs and characterize their significant sensory attributes (Fig. 15.4). CVA was selected because it restricts the classification of the sensory properties of the EVOOs to fewer groups and categories and also reveals significant differences among the products. We found that the California oils in the experimental set were defined by green-bitter, nutty, tea, ripe fruit, and pungent characteristics. Chilean

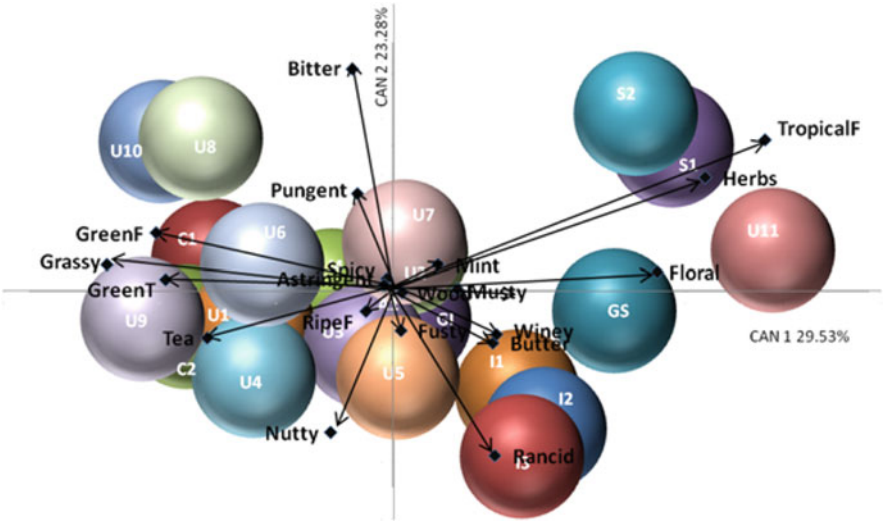

Fig. 15.4 Canonical variate analysis for 22 extra virgin olive oils (EVOOs) for first two dimensions. Note: products are represented by *spheres*; see Table 15.3 for a full description of EVOOs. Vectors represent attributes ($n=20$), short vectors show that certain attributes—namely, spicy and wood—discriminated less among the oils. Each circumference represents the 95 % confidence interval (Chatfield and Collins 1980), with two overlapping spheres indicating the products are not significantly different (*Source*: Adapated from Delgado and Guinard (2011a), with permission of John Wiley & Sons)

and Australian oils shared some attributes with the California profile. Tropical, herbal, and floral attributes were the main characteristics found in the Spanish oils in the set. Some of the main attributes for the Italian oils in the experimental set were nutty, buttery, and the defect of rancidity.

15.2.2.2 Consumer Hedonic Ratings

The next step in understanding the drivers of liking is to know consumers' opinions regarding the set of products (i.e., individual olive oils). Consumer studies require a large number of consumers in order to make the tests sensitive enough to be meaningful (the power of the test). Some recommendations regarding consumer tasting studies are as follows:

- Follow all the principles of safety
- Consider the consumer sample size and whether the sample is representative of the target population. If the sample is not random, consider whether it is a convenient sample or a quota sample.
- Show the products in blind conditions (no brand or packaging), in the same container and coded with three-digit random numbers.
- Present the samples using exactly the same volume.

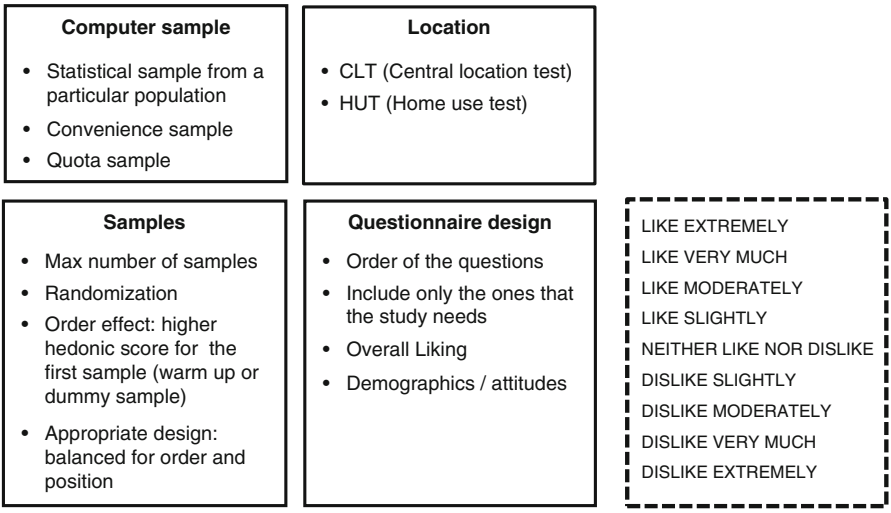

Fig. 15.5 Factors to consider when designing sensory consumer studies

- Establish an experimental design; balance the order presentation; use a warm-up sample to eliminate first-order effects.
- Provide consumers with palate cleaners; apple slices (such as Granny Smith) work well as palate cleanser.
- Determine the best environmental setting for the test.

Regarding the location, the choices are to use a central location test (CLT) or home use test (HUT). The CLT is generally used when the researcher needs more control over the products – for instance, when serving temperature is important, sensitive products will be used, or cooking must be done before the food is presented for evaluation. In addition, the CLT is recommended when the number of products to test is large, in which case it is recommended to split the products into sessions and conduct the experiment in a sensory laboratory facility. On the other hand, in cases where the objective is to intercept consumers and ask them for their preference for a few products with a short questionnaire, the location can be a supermarket or shopping center. HUTs are convenient when the researcher is interested in how consumers will use the products. The home environment is also convenient because consumers have a long period to try the product and experience it in real conditions. However, the limitations are that responses from people other than the test subject may influence the target consumer and that the tests are more expensive and take more time to conduct and to gather responses.

Figure 15.5 shows the steps that should be considered when designing sensory consumer studies. Studies today can be as complex and sophisticated as the researcher's objectives. The basic sensory consumer tests measure preference and acceptance. Preference implies that there is a comparison to be made between two or more products, with the consumer indicating which she/he prefers. Acceptance involves asking the consumers for their degree of liking of a product, using either a hedonic

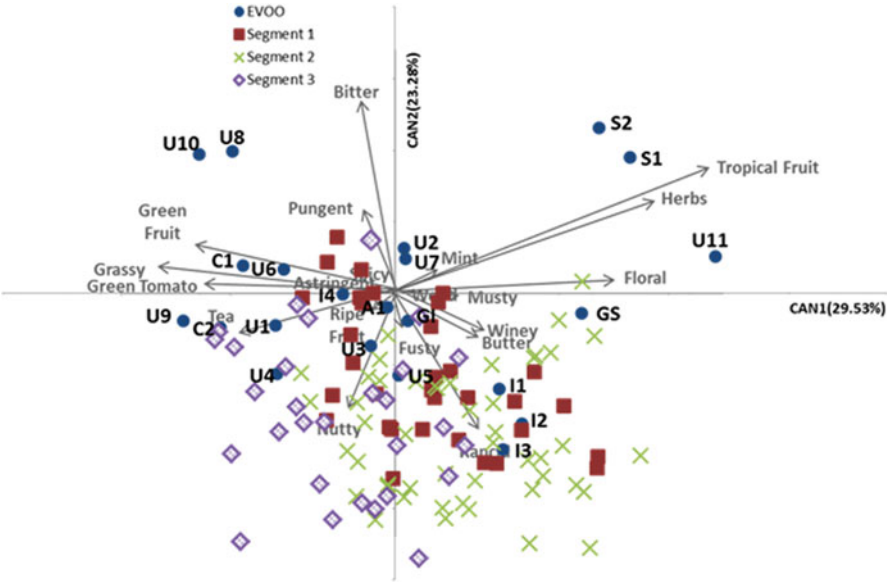

Fig. 15.6 External preference mapping for 22 extra virgin olive oils (EVOOs) (Dimension 1 versus Dimension 2). Note: EVOOs are represented by *solid circles*, attributes are represented by vectors. Consumers in segment 1 ($n=54$) are identified by a *solid red square*, segment 2 ($n=49$) by a *green cross*, and segment 3 ($n=33$) by a *diamond in purple*. See Table 15.2 for a full description of the EVOOs (*Source*: Adapated from Delgado and Guinard (2011b), with permission of Elsevier)

scale – the most common being the nine point hedonic scale by Peryam and Pilgrim (1957) – or another kind of scales such as the just about-right (JAR), where consumers indicate whether the intensity of certain attributes are just right, not enough or too much (Gacula Jr. et al. 2007; Popper 2004; Rothman and Parker, 2009). More details about these techniques can be found in Lawless and Heymann (2010).

15.2.2.3 Integrating Sensory and Consumer Data to Identify Drivers of Liking

Once the information (descriptive data and consumer hedonic scores) is collected, statistical procedures are needed to relate the sensory properties with the hedonic scores. Preference mapping has been applied for this purpose by many sensory scientists (Elmore et al. 1999; Guinard et al. 2001; Johansen et al. 2010; Suwansri et al. 2002; Yenket et al. 2011). To understand consumer preferences for EVOO, we recently used external preference mapping, in which descriptive analysis ratings were analyzed through a canonical variate analysis (CVA) (Fig. 15.4), to show the relationship to consumers' hedonic scores by regressing them into the CVA space (Delgado and Guinard 2011b). In this study, we also used cluster analysis to reveal any segments based on overall liking as well as a brief exit survey. We identified three consumer segments (Fig. 15.6). The first segment ($n=54$) was characterized by their liking of the majority of the EVOOs, by the reputation of the oil, which is

the main factor influencing EVOO purchase, and by these consumers' considering imported oils to be better than local oils. The second segment ($n=49$) considered bitter and pungent as negative drivers of liking, were less sensitive to defects, and considered price as the most important driver of EVOO purchase. These consumers thought that EVOOs were a healthier product compared with other edible oils but that the EVOOs in the study generally tasted the same. The third segment, cluster 3 ($n=33$), preferred oils with nutty, tea, ripe fruit, and buttery characteristics, while tropical fruit, floral, mint, and herbal characteristics proved to be negative drivers of liking. For these consumers the information about the product was the main driver of purchase, and they felt that EVOOs enhance the taste of salads.

15.3 Qualitative Methods

Qualitative research methods use a diverse set of techniques (e.g., in-depth interviews, focus groups, and observational studies, among others) to understand consumer issues. As a result, they produce a limited amount of quantitative data to be analyzed. Some researchers point out that they lack statistical validity, that the consumers may not be representative of the population, and that the methods can be overused or used when not appropriate (McCarthy and Perreault 2005). The advantage, however, is that qualitative data involve words and visual images of products and objects, all of which can be subjected to analysis. The main advantage lies in the depth of the researchers' analyses, which allow them to understand in more detail what consumers think of or feel about a particular topic or product.

15.3.1 Focus Group

A focus group interview is one of the most widely applied methods in qualitative research. This method involves recruiting of 3–4 groups of 8–10 consumers according to the specific requirements of the study. This technique requires that a professional moderator lead a discussion following a topic guide, and then consumers as a group interact and interchange comments (Krueger 1998; Morgan et al. 1998; Templeton 1994). Focus groups can be applied to explore how consumers perceive new ideas to help in product development, to understand whether the image of the product established by marketing matches consumers' expectations, and to obtain concepts and ideas directly from consumers.

Table 15.4 summarizes our focus group findings (Santosa 2010). The importance of sensory factors such as flavor/taste for both consumption and purchase activities cannot be understated because they were repeated across different topics as well as discussion groups. Nonsensory factors related to product variables, such as the perceived health benefits of olive oil, were important for consumption behavior, whereas cost/price were important for purchase behavior. These findings paralleled

Table 15.4 Summary of focus group findings

Consumption behavior		
Event prompting olive oil use	Key factor	Health benefits
	Other nonsensory factors	Family cooking since childhood, cultural tradition, media exposure (TV cooking show, cookbooks), restaurant dining, overseas holiday trip, or temporary education overseas
	Contexts	Life milestone: college, marriage
Form of olive oil use	Culinary method	Roasting, sautéing or frying, drizzling, marinating, and dipping
	Less common	Baking
	Less common nonculinary uses	Hair/leather conditioner, additive in lotion, face mask/body lotion, homemade infused oil, oiling decks, animal foods
	Concern	Inappropriate taste in baked sweets/desserts
		Wasted flavors as a result of baking
Habits	Other oils consumed	More common: canola oil, sesame oil
		Less common: peanut oil, mixed/blend oil, grapeseed oil
	Context	Purpose of cooking use, culinary use
	Reason	Taste and perceived healthiness of oil
	Kind/brand of oil consumed	(1) Store/private label
		(2) Supermarket bulk oil
		(3) Established imported oil
		(4) Locally produced oil
		(5) Oil brands at specialty store
	Length of consumption	Could not estimate, different rates for different bottles, 2–3 months or 4–6 months
Purchasing behavior		
Shopping location	More common	Supermarket, warehouse club, farmers' market
	Other places	Winery, specialty/gourmet store, restaurant
	Mediating effect	Convenience, proximity
	Context	Day trips/traveling vacation vs. household grocery shopping
Marketing variables	Typical volume	Unable to estimate volume, 500 mL, 1 L, 2 L
	Typical price range	Unable to estimate price, $6–$10, $12–$15, and around low $20s for bigger size or bulk oil
	Most expensive olive oil paid for	Only ballpark range cited: Lowest: $15–20 Middle: $20–25 Highest: $30–40
	Nonsensory factor	Shopping location, sampling opportunity, product appearance, purpose of use
	Contexts	Purpose of cooking use, culinary use
	Packaging material	Glass – dark or green with metal twist cap or cork closure: more common
		Glass – clear or plastic: less common

(continued)

Table 15.4 (continued)

Buying habits	Purchase frequency	Variable frequencies: average 2–5 times/year; less common: twice a month or once a year
Motivations	Reasons	(1) Sensory characteristics: flavor/taste most common, texture and smell less common
		(2) Perceived health benefits
	Other reasons	(1) Olive oil usage
		(2) Culture/tradition
		(3) Geographical area of production
	Purchasing decision factors	(1) Cost/price
		(2) Value
		(3) Quality
		(4) Place of origin
		(5) Flavor/taste
		(6) Organic
		(7) Volume or size
		(8) Purpose of use
		(9) Reliability
		(10) Familiarity
		(11) Appearance
		(12) Packaging features
	Contexts	Purpose of use
Products		
Concepts related to olive oil		(1) Olive related
		(2) Physical characteristics of olive oil packaging
		(3) Sensory characteristics
		(4) Quality
		(5) Food items or dishes
		(6) Usages
		(7) Emotion or nostalgia
		(8) Health-related concepts
		(9) Geographical location
		(10) Cartoon
Product knowledge	Little known	Meaning unknown; don't know much about it; IOC; COOC seal of quality requirements
	More common	First pressed/cold pressed, not heated, less processing, no solvents/chemicals used in extraction process, more purity
	Less common	Better for health, premium oil, better quality oil, more perishable oil, fresh oil
	Opposing views	Flavor: rich, fruity, more flavorful vs. light flavor
	Importance of "extra virgin"	Not at all – a lot
Production origin image	Common	Italy (Lucca, Tuscany)
	Runner up	California/Yolo County

(continued)

Table 15.4 (continued)

Quality image	Common criterion	Taste/flavor
	Other criteria	Reputable source, expensive oil, oil color, texture
	Color perception	The greener the oil, the more flavorful the oil
	Texture liking	Heavier vs. lighter
Consumers		
Consumer language	Most common positive attribute	Fruity
	Ambiguous positive terms	Smelled good, pleasant taste, smooth, delicious, very nice, smooth
	Most common negative attributes	Bitter aftertaste, hotness, burning sensation (irritating/stung back of throat)
	Other common negative attributes	Oily, fishy, medicinal, meat flavor, astringent, spicy, metallic, chemical taste, and off-taste or off-flavor
	Bipolar attributes preference	Butter or buttery, bitter flavor or aftertaste, pepper aftertaste/finish or peppery flavor, grass or grassy flavor
Emerging issues and concerns	Laws for certification	*Pros*: Trade fairness/local industry stability, consumer fairness
		Cons: Expensive products, products taste similar/same
	Quality	(1) Can't tell flavor differences between less and better quality
		(2) Non-extra virgin with no chemicals is acceptable
		(3) Greater confidence if olive oil is made in California (upon awareness of adulterated European olive oil)
		(4) Quality concern for oil
	Date	(1) Freshness
		(2) Length of supermarket shelf storage
	Flavor impact	(1) Not suitable for certain dishes
		(2) Impart unwanted flavor to foods
		(3) Cannot be heated at high temperature like peanut oil or grape seed oil

factors found in the food choice model of Shepherd and Sparks (1994), where food-related factors, personal factors, and economic factors were found to be important variables explaining food choice. These findings also paralleled some of the values identified in the food choice process models of Furst et al. (1996) and Connors et al. (2001), where taste, health, and cost were the three main values, and also with some of the factors in the food choice model of Köster (2009), where intrinsic product characteristics such as taste were found to be essential factors influencing eating behavior and food choice.

Sensory and nonsensory factors interacted to affect both the consumption and purchase of olive oil, depending on the context. Contextual factors clearly affecting olive oil consumption behavior were the intended use and whether consumers were

buying the olive oil for themselves or for someone else. These two contexts were considered jointly with both sensory and nonsensory factors. For example, if the purchase was intended as a gift, then consideration of where the person lived and how the person was going to use/consume the olive oil affected what kind of olive oil the consumers purchased. If the olive oil was for personal consumption, the intended use of the olive oil affected the price consumers were willing to pay. If consumers were just using the olive oil for cooking, where the flavors of other food ingredients were going to overpower the flavor of the olive oil or the flavor of the olive oil would be reduced or eliminated as a result of the cooking process, then consumers wanted to purchase a less expensive olive oil. However, if consumers were going to use the higher-quality olive oil for a special purpose, such as enjoying the taste or flavor in salad dressing or as dipping oil, then they were willing to pay more. A few consumers with Italian or Spanish roots consumed olive oil out of habit because it was a tradition of the Italian or Spanish culture to include olive oil in daily cuisine or diet. Hence, the consumption habits of this consumer group were influenced by its culture.

During the focus group discussions, some less knowledgeable consumers or consumers having less experience with EVOO had the opportunity to learn new information and modify their perceptions as a result of the acquired learning. It was discovered that after they assimilated new information about EVOO from the more experienced or knowledgeable consumers, they were willing to consider changing their current consumption and purchasing habits from using the less expensive, bigger-size/bulk-packaged olive oils toward more expensive, less bulky sizes, or from their current brand to a different brand. They did this in order to obtain better-quality and purer olive oil and thus to avoid potential unknown/unwanted chemicals or other ingredients. Chocarro et al. (2009) also found that the level of consumer knowledge of particular products could affect their perceptions of other extrinsic product characteristics.

15.3.2 Modified Sorting Task

We also carried out a modified sorting task of EVOOs with 31 consumers (Santosa et al. 2010). A sorting task asks subjects to place objects with similar characteristics into one of several groupings. In the study this pattern for sorting was selected over the typical three-group selection scheme because the researchers wanted to shorten the time involved for subjects to make a determination (Bech-Larsen and Nielsen 1999). After being presented with the oils in their original package, participants were asked to verbalize their rationale for their choices after they sorted the products to aid the researchers in understanding consumer perceptions of EVOOs. Consumers did not taste the oils. To fully investigate the product variables, the sorting task in this study was broken into two stages, allowing subjects a second opportunity to make evaluations and elaborate on their verbal descriptions for each of the test samples.

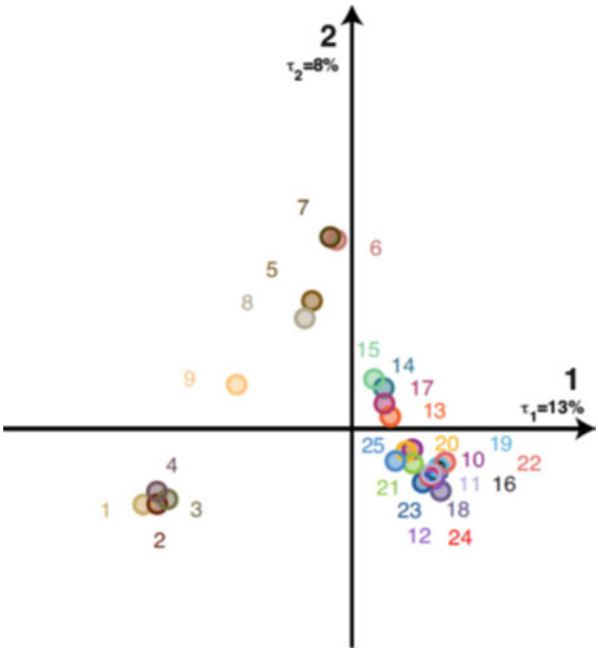

Fig. 15.7 Positioning of 25 extra virgin olive oils from second stage of sorting task. Note: For description of the 25 oils see Table 15.5. (*Source*: Santosa et al. (2010), with permission of Elsevier)

During the first stage of the sorting task, each consumer was asked to group the olive oil products based on their perceived similarities. Consumers were informed that they would be describing the characteristics that governed their choice of group after they finished the sorting task. Then, in the second stage of the sorting task, they were asked to perform the sorting task once more *within* each of the groupings they created to see whether they perceived other characteristics that might differentiate particular samples from the others within each group. In the second stage consumers were not allowed to move samples across groups, and they were asked to provide descriptions once more for any newly formed groupings. Each session lasted 35–40 min.

The modification of a simple sorting task into two stages afforded better product discriminations (Fig. 15.7). DISTATIS, a three-way extension of classical multidimensional scaling, which explores the level of consumer agreement as well as the structure of the EVOO products, was employed to analyze sorting data. A more detailed description of the general principles and operation of this statistical analysis technique can be found in Abdi et al. (2007). Table 15.5 describes the oils used in this study. Subjects created more groupings of imported oils (numbers 1–9) compared with California oils (numbers 10–25). In addition to revealing similar sensory factors of the oils (e.g., different flavor strengths and flavor profiles), the modified sorting task also allowed researchers to examine nonsensory factors that had been mentioned in focus groups (e.g., price, volume/size, origin, and intended use). We were able to associate different qualities that had determined the subjects' grouping of the oils based on place of purchase, organic vs. nonorganic, scale of production, types, and sizes.

Table 15.5 Origin, type of olive variety, and bottle color of extra virgin olive oils used in focus group, sorting, and mean-chains studies

Product ID[a]	Origin and variety	Bottle color
1	Imports	Clear
2	Imports	Clear
3	Imports	Clear
4	Italy	Clear
5	Spain	Clear
6	Greece	Dark
7	Greece	Dark
8	Italy	Clear
9	Spain	Clear
10	CA blend	Dark
11	CA blend	Dark
12	CA blend	Dark
13	CA blend	Clear
14	CA Manzanilla	Clear
15	CA Mission	Clear
16	CA blend	Dark
17	CA Frantoio	Clear
18	CA blend	Dark
19	CA blend	Dark
20	CA Arbequina	Dark
21	CA Arbequina	Dark
22	CA Taggiasca	Dark
23	CA Arbequina	Dark
24	CA Arbequina	Dark
25	CA blend	Dark

[a]Products 1–8 were bought at local supermarkets in Davis, CA, USA; *CA* California; products 9–25 were donated by producers

The modified sorting method allowed for further understanding of consumer perceptions of olive oil packaging since different kinds of bottle shape, height, and label were physically presented in front of consumers for detailed examination. Bottles that were shorter or smaller or had square or round profiles were perceived as more utilitarian due to their ability to fit easily into one's pantry. Taller, thinner bottles were viewed as cumbersome to handle or easily toppled and therefore less suitable for typical kitchen use such as cooking. However, these same bottles were seen as appropriate for gift giving due to their aesthetic appeal.

When the harvest year appeared on the label, when the area of olive oil production was known also for its wines, or when the bottle shape resembled that of a wine bottle, even if it was of smaller size, as was the case for several of the California oils, consumers sometimes described the samples using *wine references*.

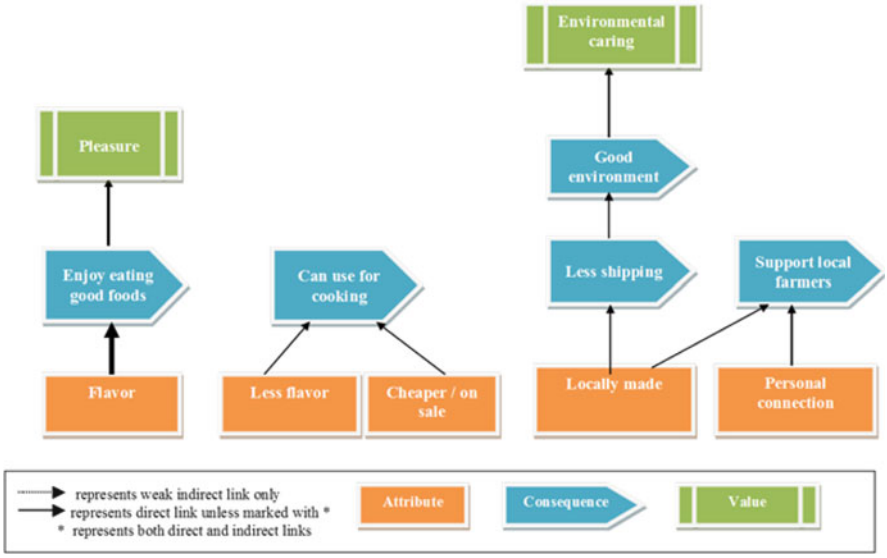

Fig. 15.8 Hierarchical value map for olive oil purchase motivations among consumers who currently consume locally made olive oil (*Source*: Santosa and Guinard (2011), with permission of Elsevier)

15.3.3 Means-End Chain Analysis

Means-end chain (MEC) theory postulates a hierarchical link among product attributes, product use benefits, and the value consumers place on the identified benefits (Gutman 1982). A soft-laddering interview was utilized to probe consumers' consumption and purchasing motivations because in this kind of in-depth interview the respondents are restricted as little as possible in their natural flow of speech. This method involved a series of *why* questions to uncover the attributes, consequences, and values associated with olive oil consumption.

An in-depth interview was conducted with 63 consumers (Santosa and Guinard 2011), in which two main questions were asked to understand purchase and consumption motivations: (1) Why do you consume olive oil? (2) Why did you buy the olive oil(s) that you currently have at home? Each consumer was given an illustration of how the interview session would proceed prior to the actual interview questions. Each interview session lasted for about 30–40 min.

Figures 15.8, 15.9, and 15.10 show the hierarchical value maps (HVMs) of consumer purchase motivations for the olive oils they currently had at home, for the three groups of consumers in the analysis – those who consumed only locally made olive oil, those who consumed only imported olive oil, and those who consumed both kinds. The HVMs show that there were some differences in consumer perceptual orientations among the three consumer groups. The least complex HVM was

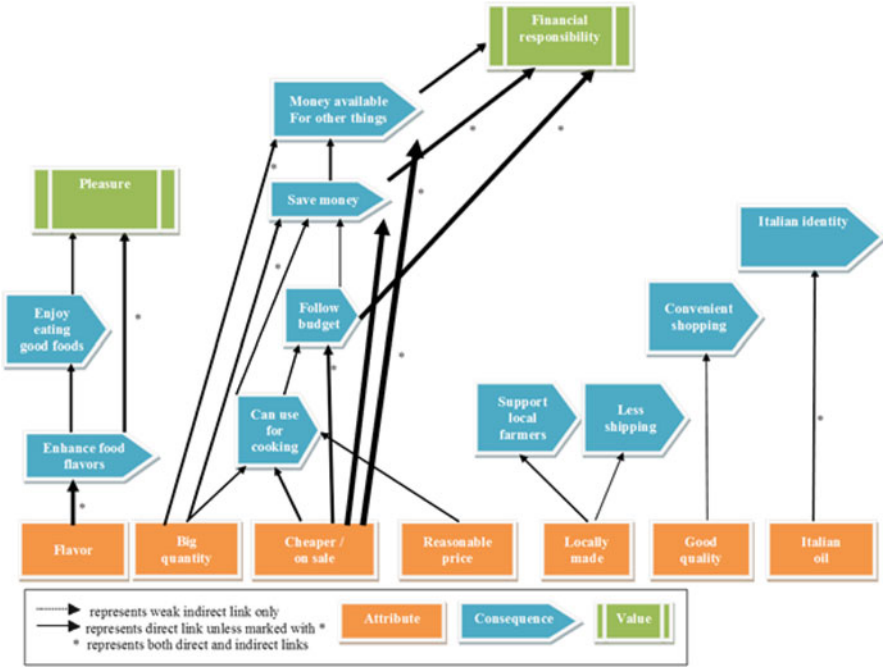

Fig. 15.9 Hierarchical value map for olive oil purchase motivations among consumers who currently consumed imported olive oil (*Source*: Santosa and Guinard (2011), with permission of Elsevier)

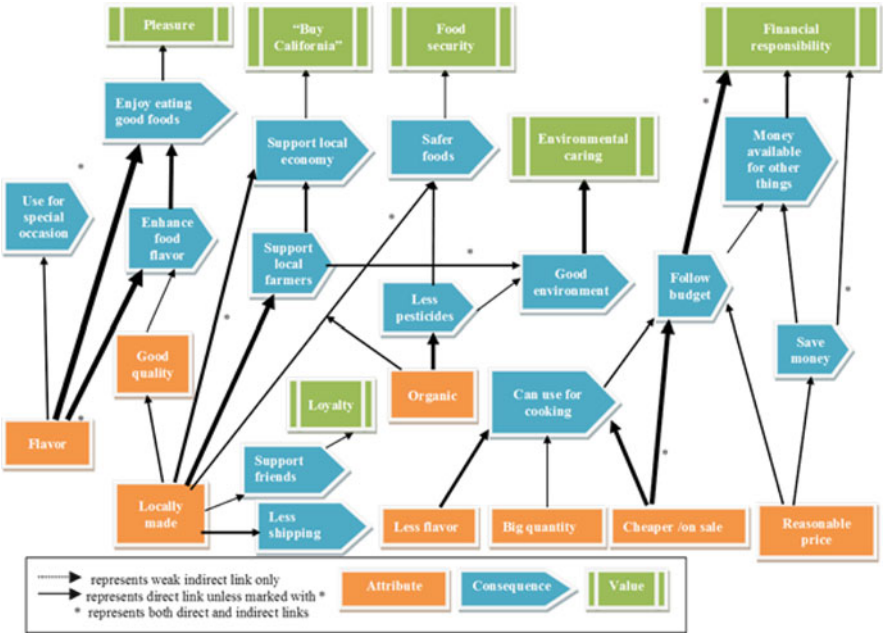

Fig. 15.10 Hierarchical value map for olive oil purchasing motivations among consumers who currently consume both imported and locally made olive oil (*Source*: Santosa and Guinard (2011), with permission of Elsevier)

that of local oil purchasers (Fig. 15.8). Culture was found to play a role in the behavior of some consumers who were of Italian descent (Fig. 15.9). These consumers used olive oil because they were used to it as part of their daily diet, just as their families of origin (e.g., parents or grandparents) had done when preparing and cooking meals or when they lived in Italy.

The MEC interview elicited a smaller variety of attributes than the focus group interviews. Still, both sensory and nonsensory factors and contexts motivating purchasing behaviors were uncovered using the MEC method. *Less flavored* olive oil was associated with the *bigger volume/sized* product and *cheaper price* or *reasonable price* and, consequently, was *for cooking*. An additional context uncovered was *social interaction* among people during meals. This *social interaction* context also interacted with another context discovered in the focus groups and the modified sorting task, i.e., *purpose of use*.

From the HVMs, it can be seen that one product attribute could be associated with multiple personal values. For example, among consumers who purchased both local and imported olive oils, *locally made* was linked to *pleasure*, *buy Californian*, *food security*, *loyalty*, and *environmental consciousness* (Fig. 15.10). Conversely, several product attributes sometimes served a single personal value. Again, among consumers who purchased both local and imported olive oils, *less flavor*, *big quantity*, *cheaper/on sale*, and *reasonable price* attributes could serve to fulfill the *financial responsibility* value (Fig. 15.10).

15.4 Conclusions and Future Trends

Asking questions and making observations are the two basic methods of obtaining information about consumers. Questions can be used in both quantitative and qualitative research, while observations are applied only in qualitative research. The method chosen must be that which best fits the research objectives and should not be interchanged with another method simply because of budget constraints or other extrinsic factors.

Using a combination of qualitative and quantitative methodologies allows the researcher to understand how discrete and nondiscrete independent variables affecting consumer consumption and purchase behaviors for olive oils interact and influence each other. As appropriate, consumer research should also combine this mixed-method approach with multivariate statistics to find patterns and trends. In some cases qualitative research is used to understand consumer perception of a product/concept and can serve as a starting point for designing a quantitative study and extend results to a target population.

All the methods illustrated in this chapter show that both sensory and nonsensory factors affect consumer choice in the purchase and consumption of EVOOs. When designing an experiment, researchers must keep in mind that each method should answer a particular question or help solve a specific problem.

Consumers differ in their preferences for the different brands of olive oil and in their attitudes. Consumer segmentation, whether preestablished by marketing concepts/demographics or determined after data collection, helps researchers address these differences.

References

Abdi H, Valentin D, Chollet S, Chrea C (2007) Analyzing assessors and products in sorting tasks: DISTATIS, theory and applications. Food Qual Prefer 18:627–640

Bech-Larsen T, Nielsen NA (1999) A comparison of five elicitation techniques for elicitation of attributes of low involvement products. J Econ Phychol 20:315–341

Belson W, Duncan JA (1962) A comparison of the check-list and the open response questioning systems. Appl Stat 11:120–132

Bourque LB, Fielder EP (2002) How to conduct self-administered and mail surveys. Sage, Thousand Oaks

Blasius J, Greenacre M (2006) Correspondence analysis and related method in practice. In: Greenacre M, Blasius J (eds) Multiple correspondence analysis and related methods. Chapman & Hall/CRC, Boca Raton, pp 3–40

Bradburn NM, Miles C (1979) Vague quantifiers. Public Opin Q 43:92–101

Bradburn NM, Sudman S, Wansink B (2004) Asking questions: the definitive guide to questionnaire design for market research, political polls, and social and health questionnaires. Jossey-Bass, San Francisco

Cardello AV (2003) Consumer concerns and expectations about novel food processing technologies: effects on product liking. Appetite 40:217–233

Chatfield C, Collins AJ (1980) Introduction to multivariate analysis. Chapman & Hall, London, p 248

Chocarro R, Cortiñas M, Elorz M (2009) The impact of product category knowledge on consumer use of extrinsic cues – a study involving agrifood products. Food Qual Prefer J 20:176–186

Connors M, Bisogni CA, Sobal J, Devine CM (2001) Managing values in personal food systems. Appetite 36:189–200

Converse JM (1984) Strong arguments and weak wvidence: the open/closed questioning controversy of the 1940s. Public Opin Q 48:267–282

Delgado C, Guinard J-X (2011a) Sensory properties of Californian and imported extra virgin olive oils. J Food Sci 76:S170–S176

Delgado C, Guinard J-X (2011b) How do consumer hedonic ratings for extra virgin olive oil relate to quality ratings by experts and descriptive analysis ratings? Food Qual Prefer J 22:213–225

Dillman DA (2007) Mail and internet surveys: the tailored design method. Wiley, Hoboken

Drewnowski A (1997) Taste preferences and food intake. Annu Rev Nutr 17:237–253

Elmore JR, Heymann H, Johnson J, Hewett JE (1999) Preference mapping: relating acceptance of "creaminess" to a descriptive sensory map of a semi-solid. Food Qual Prefer J 10:465–475

Fowler FJ (1992) How unclear terms affect survey data. Public Opin Q 56(2):218–231

Furst T, Connors M, Bisogni CA, Sobal J, Falk LW (1996) Food choice: a conceptual model of the process. Appetite 26:247–266

Gacula M Jr, Rutenbeck S, Pollack L, Resurreccion AVA, Moskowitz HR (2007) The just-about-right intensity scale: functional analyses and relation to hedonics. J Sens Stud 22:194–211

Groves RM (2004) Survey methodology. Wiley, Hoboken

Guinard J-X, Uotani B, Schlich P (2001) Internal and external mapping of preferences for commercial lager beers: comparison of hedonic ratings by consumers blind versus with knowledge of brand and price. Food Qual Prefer J 12:243–255

Gutman J (1982) A means-end chain model based on consumer categorization processes. J Mark 46(2):60–72
Jobe JB, Mingay DJ (1989) Cognitive research improves questionnaires. Am J Public Health 79:1053–1055
Johansen SB, Hersleth M, Næs T (2010) A new approach to product set selection and segmentation in preference mapping. Food Qual Prefer J 21:188–196
Köster EP (2009) Diversity in the determinants of food choice: a psychological perspective. Food Qual Prefer J 20:70–82
Krueger RA (1998) Moderating focus group. Sage, London
Lawless HT, Heymann H (2010) Sensory evaluation of food. Springer, New York
Lockshin L, Jarvis W, D'Hauteville F, Perrouty J-P (2006) Using simulations from discrete choice experiments to measure consumer sensitivity to brand, region, price, and awards in wine choice. Food Qual Prefer J 17:166–178
McCarthy EJ, Perreault WD (2005) Basic marketing: a global managerial approach. McGraw-Hill/Irwin, Homewood
Meilgaard M, Civille GV, Carr BT (2007) Sensory evaluation techniques. Taylor & Francis, Boca Raton
Morgan DL, Krueger RA, King JA (1998) Focus group kit. Sage, Thousand Oaks
Næs T, Brockhoff PB, Tomic O (2010) Statistics for sensory and consumer science. Wiley, Chichester
Peryam DR, Pilgrim FJ (1957) Hedonic scale method of measuring food preferences. Food Technol 11:9–14
Popper R (2004) Workshop summary: data analysis workshop: getting the most out of just-about-right data. Food Qual Prefer J 15:891–899
Rasinski KA, Mingay D, Bradburn NM (1994) Do respondents really "Mark all that apply" on self-administered questions? Public Opin Q 58(3):400
Rothman L, Parker MJ (2009) Just about right (JAR) scales design, usage, benefits, and risks. ASTM standards and engineering digital library. ASTM International, West Conshohocken. http://dx.doi.org/10.1520/MNL63-EB
Sanchez ME (1992) Effects of questionnaire design on the quality of survey data. Public Opin Q 56:206–217
Santosa M (2010) Analysis of sensory and non-sensory factors mitigating consumer behavior: a case study with extra virgin olive oil. Ph.D. dissertation, University of California, Davis
Santosa M, Abdi H, Guinard J-X (2010) A modified sorting task to investigate consumer perceptions of extra virgin olive oils. Food Qual Prefer J 21:881–892
Santosa M, Guinard J-X (2011) Means-end chains analysis of extra virgin olive oil purchase and consumption behavior. Food Qual Prefer J 22:304–316
Shepherd R, Sparks P (1994) Modelling food choice. In: MacFie HJH, Thomson DMH (eds) Measurement of food preferences. Blackie Academic and Professional, London, pp 202–226
Sijtsema S, Linnemann A, van Gaasbeek T, Dagevos H, Jongen W (2002) Variables influencing food perception reviewed for consumer-oriented product development. Crit Rev Food Sci Nutr 42:565–581
Smyth JD, Dillman DA, Christian LM, Stern MJ (2006) Comparing check-all and forced-choice question formats in web surveys. Public Opin Q 70:66–77
Stone H, Sidel JL (2004) Sensory evaluation practices. Academic, Oxford, UK
Suwansri S, Meullenet JF, Hankins JA, Griffin K (2002) Preference mapping of domestic/imported Jasmine rice for U.S. Asian consumers. J Food Sci 67:2420–2431
Templeton JF (1994) The focus group: a strategic guide to organizing, conducting and analyzing the focus group interview. Probus Publishing, Chicago
Tourangeau R, Rips LJ, Rasinski KA (2000) The psychology of survey response. Cambridge University Press, Cambridge, UK
Yenket R, Chambers Iv E, Adhikari K (2011) A comparison of seven preference mapping techniques using four software programs. J Sens Stud 26:135–150

Chapter 16
Olive Oil Authentication

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Contents

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties*,
DOI 10.1007/978-1-4614-7777-8_16, © Springer Science+Business Media New York 2013

16.1 Introduction

The authenticity of products labeled as olive oil has become an important issue from both commercial and health aspects. Olive oil has recently gained in popularity because of its quality, its potential health benefits derived from its consumption, and its strict purity control. But it is the high price of olive oil and its reputation as a healthy and delectable oil that makes it a preferred target for fraudsters. Adulteration may take place not only by accidental contamination during the stages of oil processing but even more often by deliberate mislabeling of less expensive olive oil categories or admixtures containing less expensive edible oils for the purpose of financial gain.

In countries that manufacture seed oils and import olive oils, the admixtures of expensive olive oils with less expensive and lower-grade oils have traditionally been more than a potential problem. Numerous adulterants have been found in virgin olive oil (VOO). These range from refined olive oil (ROO), raw olive-pomace oil, and synthetic olive oil-glycerol mixtures to seed oils, such as corn, cottonseed hazelnut, rapeseed, soybean, and sunflower, among others. This procedure is harmful for emergent VOO markets whose local consumers buy olive oil for its health benefits (Kafatos and Comas 1991; García-González et al. 2009) and are surprised to receive oil that does not fulfill this expectation. Hence, an effective regulation of olive oil purity requires tighter control by exporting countries, clear definitions for olive oil products, uniform labeling regulations, and rapid, easy, and accurate instrumental techniques and methodologies.

Several institutions (e.g., Antifraud Unit of EU-OLAF and the International Olive Council [IOC], among others) are actively involved in antifraud measures that aim to avoid any image of a hypothetical uncontrolled distribution of adulterated olive oil into the market, and their standards contain those provisions needed to ensure fair trade and prevent fraud as well as safety and consumer protection. The result is a strictly controlled olive oil market by means of trade standards that are periodically revised and upgraded.

Advances in knowledge and technology have undoubtedly led to greater success in the fight against adulteration over the years, but it is equally true that the same techniques and knowledge are also used by fraudsters to invalidate the usefulness of official methods. Such competition requires not only a considerable investment in perfecting techniques or developing new ones but also a rapid pace of research and development in methods of detection of malpractices (Aparicio et al. 2007). However, no rapid and universal method exists that is officially recognized for all the authenticity issues.

Authenticity is a multifaceted issue that covers many aspects including characterization, adulteration, mislabeling, and misleading origin, among many others. With this plethora of possible issues, the large number of olive oil categories, and the numerous edible oils that can potentially be used in spiking, it is not surprising

that numerous methods have been used or suggested to detect olive oil adulteration just during the past 10 years. Thus, many modern instrumental techniques have been used, such as high-performance liquid chromatography (HPLC), gas liquid chromatography (GLC), supercritical fluid chromatography (SFC), chiral chromatography, stable carbon isotope ratio analysis, silver ion chromatography, mass spectrometry (MS), nuclear magnetic resonance (NMR) spectrometry, near-infrared (NIR) spectroscopy, Fourier transform mid-infrared spectroscopy (FTIR), FT-Raman spectroscopy, and others (Lai et al. 1995; Aparicio 1998; Aparicio and Aparicio-Ruiz 2000; Bianchi 2002; Ogrinc et al. 2003; Aparicio et al. 2007; Frankel 2010).

The test results of those chemical analyses are used to check and classify olive oil against international specifications and legal regulations. All these decisions are, therefore, directly linked to money. Whenever such decisions are based on different methods of analysis, it is obvious that not only is the expertise of the analyst a critical item, but so are the quality of the equipment, reagents, environment (e.g., temperature, humidity, air pollution), and many other factors. Validated analytical methods are thus essential for the quality performance of analytical laboratories, and the analysts must demonstrate the quality of their results. An important first step in establishing these requirements is the quality performance of laboratories, which is directly related to the use of standardized methods of analysis. Standardized methods, such as ISO standards or other national standards, have the advantage that the performance of the methods has been checked and, as a rule, meets a required degree of precision.

16.2 Definitions of Authenticity and Official Methods

The criteria that define the genuineness of a food product depend on the commodity, although the following definition has been suggested for authenticity: “A product is authentic as long as it is firstly described accurately by the label and secondly complies with the current legislation in force in the country where it is marketed or sold” (Lees 1998). Thus, an authentic food is one that is truly derived from a specified source, where the term source (e.g., a particular category of olive oil) must be clearly defined. When the foodstuff definition is fairly broad, the legal detection of adulteration will be rather difficult due to the highly sophisticated fraudulent practices. On the other hand, a clear and strict definition requires an armory of analytical techniques that must be capable of distinguishing adulteration by picking up minor differences present, even at trace levels.

The definitions of the designations of olive oils and olive-pomace oils, according to the IOC, appear in Chap. 1, and they clearly belong to the aforementioned second type of possible definitions where clear and strict definitions are given. All of these categories are important from the adulteration viewpoint, although extra virgin olive oils (EVOOs) and VOOs are the most noteworthy due to their economic implications in either adulteration or characterization.,

Table 16.1 shows the main authenticity issues and their paradigm; two of them are still challenges for analysts – detection of the presence of deodorized VOO in

Table 16.1 Main authenticity issues and subissues and their current examples

Issue	Subissue	Paradigm
Adulteration	Addition of cheap oil to expensive oils	Detection of refined hazelnut in ROO
	Addition of refined oils to VOO	Detection of seed oils in VOO
	Addition of low to high olive oil categories	Detection of deodorized VOO in VOO
Geographical origin	Inexact label	Detection of VOO from several origins
	Traceability	Characterization/authentication of PDOs
Production systems	Organic vs. conventional	Detection of conventional in organic VOOs
Extraction system	Centrifugation and percolation	Characterization of VOO obtained by two-phase centrifugation system
	Cold-press versus solvent	
Type	Species	Characterization of edible oils
	Variety	Characterization of varietal VOOs

Note: PDO protected denomination of origin

VOOs and traceability of VOOs' geographical origin – despite the arsenal of analytical techniques at the analyst's disposal (García-González and Aparicio 2006; Aparicio et al. 2007).

Three different normative and legal sources have traditionally ruled olive oil production and international trading, although there are also other regulations in force in some producer countries. Besides IOC trade standards for its members, which are almost all the producer countries, a number of regulations exist within the European Union (EU), which, although they can only be applied within the EU, control the largest olive oil market.

As stated, olive oils are subject to worldwide trade, so that a further level of regulation is needed, and this is provided by the Codex Alimentarius Commission that is a shared rule for a large number of countries.

EU regulations are in force for EU countries, while IOC and Codex standards are agreements that signatory countries voluntarily have accepted to comply with the limits established for each quality and purity criterion including the precision values.

16.2.1 European Community Legislation

The early legislative reference dealing with olive oils in Europe dates back to 1966 within the framework of the European Economic Community (EEC) Regulation 136/66, published on 22 September, when the EEC was made up of six countries. This regulation established the rules for a common organization of the market in oils and fats, olive oil being one of the commodities.

Olive oils were described in Articles 35 and 36, and the definition for VOO already stated that free acidity should not exceed 1.0 % (w/w), while sensory characteristics should be "absolutely perfect," and definitions were established for

Table 16.2 Definition of oils extracted from olives, as established by Article 35 of Regulation EEC 136/66

Name	Definition	
Virgin olive oil[a]	Natural olive oil obtained solely by mechanical processes, enclosed pressure. Any admixtures with different oils and with oils by different tecnologies are forbidden	
	Extra	Olive oils with perfect sensory characteristics whose content of free fatty acids, expressed as oleic acid, cannot exceed 1 g/100 g
	Fino	Olive oil whose characteristics are the same of extra except for free fatty acid content, which cannot exceed 1.5 g/100 g
	Ordinary[b]	Olive oil whose taste is good except for free fatty acid content, which cannot exceed 3.3 g/100 g
	Lampante	Olive oil whose taste is not perfect or whose free fatty acid content as oleic acid exceeds 3.3 g/100 g
Refined olive oil[c]	Olive oil obtained by refining of VOOs	
Pure olive oil	Oil obtained by mixing virgin olive oil and refined olive oil	
Olive marc oil	Oil obtained by solvent extraction of byproduct listed at TARIC category ex 23.04 described by Article 1, Paragraph 2, Letter E	
Refined olive marc oil	Edible oil obtained by refining of oil described at point 4	
Refined marc and olive oil	Oil obtained by mixing refined marc oil and virgin oil	
Olive marc oil for technical purposes	Any other oil extracted from products listed in Custom Category ex 23.04 described at Article 1, Paragraph 2, Letter E, different from those listed in previous categories	

EEC European economic community
[a]The expression "pure virgin olive oil" was admitted as well
[b]The term "semifino" was also admitted
[c]The expression "pure refined olive oil" was admitted as well

other categories of oils extracted from olives as well. Table 16.2 reports the original definitions of Regulation (EEC) 136/66, now deeply modified.

The first amendment was Regulation (EEC) 2658/87 (EC 1987), which modified the definitions of VOOs, although the authentic milestone for EEC quality and purity definitions of olive oils was Regulation (EEC) 2568 published on 11 July 1991 (EC 1991). This regulation established (1) the parameters that had to be checked to assess purity and quality; (2) the limit for each parameter, depending on olive oil category; (3) and the methods that had to be applied to check whether a sample met the limits for its category. Also, Regulation (EEC) 2568/91 deeply changed the analytical approach to olive oil control. Thus, the regulation officially proposed, for example, the use of gas chromatographic open tubular columns, which were then introduced in several national official methods, and the methodology for the sensory evaluation of virgin oils by applying the so-called panel test method. VOO was the first food with legislation that included conducting a sensory evaluation.

A number of amendments have been introduced in the regulations to protect the purity and quality of the oils as knowledge and analytical instrumentation have improved. Table 16.3 shows the evolution of the main amendments in the successive regulations from 1991 until today.

Table 16.3 Evolution of European Union regulations on olive oils. Information at http://eur-lex.europa.eu/JOIndex.do

Regulation	Subject	EEC official journal
3682/91	Gives details on realization of panel test: Annex XII of Reg. (EEC) 2568/91	L349; 18/12/1991; p. 36
1429/92	Amends method for analyzing fatty acid composition, *trans* isomer evaluation, and their related limits	L150; 2/06/1992; p. 6
1683/92	Amends information of sensory descriptors: Positive, depending on quality More or less positive, but not to be considered as defects Negative, to be considered as defects	L176; 30/06/1992; p. 27
1996/92	Amends trilinolen method for raw olive-pomace oils	L199; 18/07/1992; p. 18
3288/9	Informs about following panel test aspects: Application of panel test method is postponed to 1. Sensory assessors must be formed and trained. Tolerance must be added to score established by panel as follows: +1.5 in 1992/1993; +1.0 in 1993/1994; and +0.5 in 1994/1995.	L327; 13/11/1992; p. 28
183/93	Substitutes method based on aliphatic alcohols for waxes.	L22l; 30/01/1993; p. 58
620/93	Amends limits of trilinolein for olive-pomace oil categories	L66; 18/03/1993; p. 29
177/94	Amends some details within regulation text	L24; 29/01/1994; p. 33
2632/94	Indicates suggestions for panel test method	L280; 29/10/1994; p. 43
826/93	Establishes that method for wax evaluation will be applied on 1 May	L87; 7/04/1993; p. 6
656/95	Adopts method based on stigmastadienes; amends some limits for other chemical parameters	L69; 29/03/1995; p. 1
2527/95	Waiting for revision of panel test method; a tolerance of + 1.0 point is stated for oils obtained in 1993/1994	L258; 28/10/1995; p. 49
2472/97	ΔECN42 method replaces trilinolein one	L341; 12/12/1997; p. 25
282/98	Amends some figures of ΔECN42 method	L28; 4/02/1998; p. 5
2248/98	Amends limit of linolenic acid for oils produced in Morocco in 1998/1999, 1999/2000, and 2000/2001	L282; 20/10/1998; p. 55
379/99	Establishes that sampling must be carried out according to ISO-EN 661 and ISO-EN 5555 norms (ISO 2003, 2001)	L46 l; 20/02/1999; p. 15
455/01	Amends Regulation 378/99 by adding regulation to sampling As carried out according to ISO-EN 661 and ISO-EN 5555 norms Rules for sampling storage, handling, and time limit to send sample to laboratories and to carry out analysis are introduced as well	L65; 7/03/2001; p. 9
2042/01	Amends limit of linolenic acid for olive oils produced in Morocco	L276; 19/10/2001; p. 8
796/02	Amends panel test method with new adopted method. Furthermore: A procedure of arbitration for panel test results is adopted A method for analysis of aliphatic alcohols (need for olive-pomace oils) is adopted Limits and their relationship for waxes, aliphatic alcohols, erythrodiol in order to identify oils obtained by second centrifugation of pomace are established A standardized method for fatty acids methyl esters is adopted	L128; 15/05/2002; p. 8 (to be applied on 1/9/2002)
1989/03	Amends Annex 1 according to (CEE) Regulation 1513/01: lower free acidity for EVOO, deletion of ordinary category, deletion of word "virgin" in definition of lampante olive oils	L292; 13/11/2003; p. 57
702/07	Adopts a new method for determination of saturated fatty acids at second position of triacylglycerol. The method is substituted by determination of 2-monopalmitine; a lower limit is fixed. The stigmastadiene limit is lowered to 0.10 mg/kg	L161; 22/06/2007; p.11
61/11	Adopts method for alkyl esters and related limit	L23; 27/01/2011; p. 1
61/11	Corrigendum of Italian version of Reg. (EU) 61/2011	L48; 23/02/2011; p. 19

Table 16.4 Quality criteria established for EVOOs; limits of parameters are shown in Tables 16.6 and 16.7

Parameter	EU limit	Explanation of quality criteria
Free acidity	≤0.8 g % w/w[a]	The higher the value, the worse the olive quality
		The processing of unhealthy olives increases free acidity
Peroxide value	≤20 meq O_2/kg oil	After extraction from olives, oil undergoes oxidation depending on several external variables
		Management of olive oil storage should be checked
K_{232}	≤2.50	Oxidation products absorb at this wavelength
		The higher the oxidative status, the higher the value
K_{270}	≤0.22	Oxidation products absorb at this wavelength
		The higher the oxidative status, the higher the value
ΔK	≤0.01	The higher the oxidative status, the higher the value
Panel test	Me (sensory defects) = 0	Sensory defects induce rejection of VOO by consumers
	Me (fruity attribute) > 0	EVOOs must have a fragrant fruity odor
		A low value does not fit with extra-virgin designation
Alkyl esters	([b])	EVOOs should have no ethyl esters or at trace level
		High values characterize lampante olive oils

Me median value, *FAME* fatty acid methyl esters, *FAEE* fatty acid ethyl esters
[a]As oleic acid
[b]$\sum$(FAME + FAEE) ≤ 75 mg/kg or [75 mg/kg < $\sum$(FAME + FAEE) ≤ 150 mg/kg and (FAEE/FAME) ≤ 1.5]

Tables 16.4 and 16.5 show the limits established for each quality and purity criterion of EVOO as well as the objective of its implementation, while Tables 16.6 and 16.7 display the same information but for all the olive oil categories, according to Regulation (UE) 61/2011 (EC 2011). It is important to note that VOO is the only oil whose sensory characteristics are used as quality criteria. The sensory assessment (so-called panel test) contributes to distinguish between VOO categories only. Thus, it is important to bear in mind that according to the EU regulation, sensory assessment is used only to classify the sample as extra virgin or virgin or lampante, but not to establish a quality score inside the same category of the European regulation. A sample, for instance, is classified as extra virgin if its median of defect is zero and at the same time its median of the fruity attribute is higher than zero.

Chemical parameters, on the other hand, which are qualified by their maximum or minimum limits detectable in the oil by chromatographic means, are used to classify all the olive oil designations as well as to check purity by measuring the maximum admitted concentration of some selected compounds that are typical of oils of different botanical origins. Thus, the analytical methods have fixed limits, maxima, or minima, for the percentages of certain FAs and sterols, total of *trans-oleic* isomers, total of *trans*-linoleic and *trans*-linolenic isomers, trilinolein, triterpene dialcohols (erythrodiol and uvaol), and saturated FAs of triglycerides in position 2. The total content of waxes, sterols, halogenated solvents, and stigmastadienes are also limited by maximum or minimum values. The chromatographic results are supplemented with the measurement of quality parameters, such as peroxide value, free acidity, and spectrophotometric values of K^{232}, K^{270}, and ΔK.

Table 16.5 Purity criteria established for EVOOs; limits of parameters are shown in Tables 16.6 and 16.7

Parameter	Explanation of purity criteria
Waxes (mg/kg)	A higher value is related to presence of olive-pomace oil
2-glyceryl monopalmitate (%)	A higher value is related to presence of esterified oils whose TAGs are obtained by chemical synthesis
Stigmastadienes (mg/kg)	A higher value is related to presence of refined oil, even if UV absorption or fatty acid *trans* isomers are within the limits, as well as of desterolized oils
Δ ECN42	A higher value is related to presence of seed oils even if fatty acid composition fits with olive oils, and to presence of olive-pomace oil
K_{232}	A higher value is related to presence of refined oil
K_{270}	
ΔK	
Myristic acid (%)	A higher value is related to presence of seed oil, mainly fractionated palm oil
Linolenic acid (%)	A higher value is related to presence of seed oils such as soybean oil and low erucic rapeseed oil
Eicosacoic acid (%)	
Eicosecoic acid (%)	
Behenic acid (%)	
Lignoceric acid (%)	A higher value is related to presence of seed oils such as peanuts oil
∑C18:1 *trans* isomers (%)	A higher value is related to presence of refined oils, even if UV absorption is inside the limits
∑(C18:2 + C18:3) *trans* isomers	
Cholesterol (%)	A higher value is related to presence of animal fats or of fractionated palm oil
Brassicasterol (%)	A higher value is related to presence of *Brassicaceae* oils (e.g., rapeseed, canola) even if with "zero" erucic acid content
Campesterol (%)	A higher value is related to presence of seed oils
Stigmasterol (%)	
Apparent β-sitosterol (%)*	A lower value depends on presence of seed oils
Δ^7-stigmastenol (%)	A higher value is related to presence of compositae oils (e.g., sunflower, safflower) even if with high oleic acid content
Total sterols (mg/kg)	A lower value is related to presence of seed oils or of desterolized oils
∑(Erythrodiol + Uvaol) (%)	A higher value is related to presence of olive-pomace oil as well as grape seed oil

*Apparent β-sitosterol is ∑ (β-sitosterol + $\Delta^{5,23}$-stigmastadienol + sitostanol + Δ^5-avenasterol + $\Delta^{5,24}$-stigmastadienol + clerosterol)

16.2.2 International Olive Oil Council: Trade Standards

EU regulations are useful tools within the EU, but there are producing countries that do not belong to the EU, and in addition, there are a number of issues not covered by the EU regulations, for example, the promotion of worldwide consumption of olive oil and table olives, international cooperation on research and development, the creation of permanent technical forums for all the actors, the analysis of the

Table 16.6 Olive oil purity and quality characteristics according to Commission Regulation (EU) 61/2011 (EC 2011)

Designation	(1)	(2)	(3)	(4)	(5)	(6[a])	(7)	(8)	(9[b])
EVOO	≤0.05	≤0.05	≥1,000	≤4.5	≤250	≤0.10	≤ 0.2	*B*	≤2.50[c]
VOO	≤0.05	≤0.05	≥1,000	≤4.5	≤250	≤0.10	≤ 0.2	*B*	≤2.60[c]
Lampante VOO	≤0.10	≤0.10	≥1,000	≤4.5[c]	≤300[c]	≤0.50	≤ 0.3	*C*	–
Refined olive oil	≤0.20	≤0.20	≥1,000	≤4.5	≤350	–	≤ 0.3	*C*	–
Olive oil	≤0.20	≤0.20	≥1,000	≤4.5	≤350	–	≤ 0.3	*B*	–
Crude olive-residue oil	≤0.20	≤0.10	≥2,500	≤4.5[d]	>350[d]	–	≤ 0.6	≤1.4%	–
Refined olive-residue oil	≤0.40	≤0.40	≥1,800	≤4.5	>350	–	≤ 0.5	≤1.4%	–
Olive-residue oil	≤0.40	≤0.40	≥1,600	≤4.5	>350	–	≤ 0.5	≤1.2%	–

Designation	(10[b])	(11[b])	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)
EVOO	≤0.22	≤0.01	≤0.8	≤20	≤0.5	≤0.5	≤0.1	≤4.0	<Camp	≥93.0
VOO	≤0.25	≤0.01	≤2.0	≤20	≤0.5	≤0.5	≤0.1	≤4.0	<Camp	≥93.0
Lampante VOO	–	–	>2.0	no limit	≤0.5	≤0.5	≤0.1	≤4.0	–	≥93.0
Refined olive oil	≤1.10	≤0.16	≤0.3	≤5	≤0.5	≤0.5	≤0.1	≤4.0	<Camp	≥93.0
Olive oil	≤0.90	≤0.15	≤1.0	≤15	≤0.5	≤0.5	≤0.1	≤4.0	<Camp	≥93.0
Crude olive-residue oil	–	–	No limit	No limit	≤0.5	≤0.5	≤0.2	≤4.0	–	≥93.0
Refined olive-residue oil	≤2.00	≤0.20	≤0.3	≤5	≤0.5	≤0.5	≤0.2	≤4.0	<Camp	≥93.0
Olive-residue oil	≤1.70	≤0.18	≤1.0	≤15	≤0.5	≤0.5	≤0.2	≤4.0	<Camp	≥93.0

Notes:
(1): *Trans*-oleic fatty acid (%)
(2): Sum of *trans*-linoleic and linolenic fatty acids (%)
(3): Total sterol content (mg/kg)
(4): Erythrodiol and uvaol content (% total sterols)
(5): Wax content: C40 + C42 + C44 + C46 (mg/kg)
(6): Stigmastadiene content (mg/kg)
(7): Difference between actual and theoretical ECN42 TAG content
(8): Content of 2-glyceryl monopalmitate; *B*, ≤0.9 if total C16:0 ≤ 14.0% or 1.0 if C16:0 > 14:0%, *C*, ≤0.9 if total C16:0 ≤ 14.0% or 1.1 if C16:0 > 14:0%
(9): Absorbency in UV at K_{232}
(10): Absorbency in UV at K_{270}[e]
(11): Absorbency in UV (ΔK)
(12): Free acidity (%m/m expressed in oleic acid)
(13): Peroxide value (in milleq. peroxide oxygen per kg/oil)
(14): Δ^7-stigmastenol (%)
(15): Cholesterol (%)
(16): Brassicasterol (%)
(17): Campesterol (%)
(18): Stigmasterol (%)
(19): The value of β-Sitosterol is calculated as
$\Delta^{5,23}$-Stigmastadienol + Clerosterol + β-Sitosterol + Sitostanol + Δ^5-Avenasterol + $\Delta^{5,24}$-Stigmastadienol
[a]Total isomers that could (or could not) be separated by capillary column
[b]Quality characteristics
[c]When the oil has a wax content of between 300 and 350 mg/kg, it is considered a lampante olive oil if the total aliphatic alcohol content is ≤ 350 mg/kg or if the erythrodiol + uvaol content is ≤ 3.5%
[d]When the oil has a wax content of between 300 and 350 mg/kg, it is considered a crude olive-residue oil if the total aliphatic alcohol content is > 350 mg/kg and if the erythrodiol + uvaol content is > 3.5%
[e]Maximum wavelengths of 268 if iso-octane is used and of 270 nm if cyclohexane is used

Table 16.7 Olive oil purity and quality characteristics according to Commission Regulation (EU) 61/2011 (EC 2011)

Designations	(20[a])	(21[a])	(22[a])	(23)	(24)	(25)	(26)	(27)	(28)	(29)
EVOO	Mf > 0	Md = 0	([b])	≤0.05	≤1.0	≤0.6	≤0.4	≤0.2	≤0.2	([c])
VOO	Mf > 0	0 < Md ≤ 3.5	–	≤0.05	≤1.0	≤0.6	≤0.4	≤0.2	≤0.2	([c])
Lampante VOO	–	Md > 3.5([d])	–	≤0.05	≤1.0	≤0.6	≤0.4	≤0.2	≤0.2	([c])
Refined olive oil	–	–	–	≤0.05	≤1.0	≤0.6	≤0.4	≤0.2	≤0.2	([c])
Olive oil	–	–	–	≤0.05	≤1.0	≤0.6	≤0.4	≤0.2	≤0.2	([c])
Crude olive-residue oil	–	–	–	≤0.05	≤1.0	≤0.6	≤0.4	≤0.3	≤0.2	([c])
Refined olive-residue oil	–	–	–	≤0.05	≤1.0	≤0.6	≤0.4	≤0.3	≤0.2	([c])
Olive-residue oil	–	–	–	≤0.05	≤1.0	≤0.6	≤0.4	≤0.3	≤0.2	([c])

Notes:
(20): Organoleptic assessment: median of fruity attribute (Mf)
(21): Organoleptic assessment: median of defect (Md)
(22): Fatty acid methylesters (FAMEs) and fatty acid ethylesters (FAEEs)
(23): Myristic acid (% m/m methylesters)
(24): Linolenic acid (% m/m methylesters)
(25): Arachidic acid (% m/m methylesters)
(26): Eicosenoic acid (% m/m methylesters)
(27): Behenic acid (% m/m methylesters)
(28): Lignoceric acid (% m/m methylesters)
(29): Other fatty acids (% m/m methylesters)
[a]Quality characteristics
([b])$\sum$(FAME + FAEE) ≤ 75 mg/kg or [75 mg/kg < $\sum$(FAME + FAEE) ≤ 150 mg/kg and (FAEE/FAME) ≤ 1.5]
([c])Palmitic: 7.5–20.0; Palmitoleic: 0.3–3.5; Heptadecanoic: ≤ 0.3; Heptadecenoic: ≤ 0.3; Stearic: 0.5–5.0; Oleic: 55.0–83.0; Linoleic: 3.5–21.0
([d])Of where the median of defects is less than or equal to 3.5 but the fruity median is equal to 0

agronomical aspects of olive tree orchards and their environmental impact, the implementation of procedures for improving olive oil quality, the perfection of extraction technology, and technical assistance to new producing countries.

All these topics are covered by the IOC (formerly IOOC), which is the largest association of producer countries and was established in 1956 as a consequence of a first agreement on olive oil trade that had been signed in Geneva 1 year earlier. The IOC counted 43 countries – 27 from the EU – as members in December 2010, in addition to the observers involved in IOC activities.

In the quite simple structure of the IOC, there is a technical committee that objectively reviews and discusses the areas of olive oil chemistry and standardization, research and development, technology transfer, training, and customized technical assistance for IOC member countries. Within the technical committee is the Chemists' Group (IOC-CG), whose delegates are chemists from IOC member countries, as well as from nonmember countries, who meet at least twice a year to study and elaborate analytical methods to check the purity and quality of olive oils.

These studies have been the principal basis for EU regulations. However, the European regulations are focused on olive oils and olive-pomace oils produced by EU member states, whereas the IOC issues trade standards for its association members. As olive tree varieties and climate have a significant effect on the

Table 16.8 Particular characteristics of VOO designations according to IOC trade standard (IOC 2010); ordinary VOO has the same values as VOO for remaining criteria in Tables 16.6 and 16.7

Designations	(1)	(2)	(3)	(4[a])	(5)
EVOO	≤0.8	Md = 0	Mf > 0	≤2.50[b]	≤0.22
VOO	≤2.0	0 < Md ≤ 3.5	Mf > 0	≤2.60[b]	≤0.25
Ordinary VOO	≤3.3	3.5 < Md ≤ 6.0[c]	–	–	≤0.30[d]
Lampante VOO	>3.3	Md > 6.0	–	–	–

Notes:
(1): Free acidity (%m/m expressed in oleic acid)
(2): Organoleptic assessment: median of defect (Md)
(3): Organoleptic assessment: median of fruity attribute (Mf)
(4): Absorbency in UV at K_{232}
(5): Absorbency in UV at K_{270}
[a]This determination is solely for application by commercial partners on an optional basis
[b]Commercial partners in country of retail sale may require compliance with these limits when oil is made available to end consumer
[c]Or when median of defect is ≤3.5 and median of fruity attribute is equal to 0
[d]After passage of sample through activated alumina, absorbency at 270 nm/268 nm shall be equal to or less than 0.11

chemical and sensory composition of olive oil designations, the limits adopted for each criterion can sometimes vary from the EU regulations to IOC trade standards, and harmonization is always in progress to minimize hurdles to international trade. For example, a nonharmonized issue between EU and IOC is the category "ordinary" VOO that at present has been deleted from the latest EU regulation but is still in IOC trade standards. Table 16.8 shows the characteristics that distinguish the ordinary VOO from the VOO designation described in Tables 16.6 and 16.7. The main differences concern quality criteria.

In 1987 the IOC published the international trade standards to be applied to olive oil and olive-pomace oil. The designation for the mixture of ROO and VOO was published in 1990. In May 1991, the IOC improved the international trade standard in order to cover the definition of VOOs, and in November 1991 this standard incorporated the analytical methods and limits adopted for each criterion. The minimum organoleptic overall grading for EVOO and fine VOO was included in May 1992. The initial limits for FA, waxes and contaminants were amended in June 1993, while the designations and definitions for olive oils and olive-pomace oils were amended in June 1994. On 17 November 1994, the IOC adopted a new release of the general methodology for the organoleptic assessment of VOO, and the guide for the selection, training, and monitoring of sensory assessors that was later adopted by the EU as well. The need to fight new frauds led to an amendment of the trade standard in 1995, which added a method for the determination of steroidal hydrocarbons in vegetable oils. The proof of polycyclic aromatic hydrocarbon (PAH) contaminations resulted in a method for the detection of PAHs in edible olive oils and olive-pomace oils, which was added to the trade standard in November 2005. A duly validated revision of the method for the determination of the differences between the actual and theoretical contents of triacylglycerols (TAGs) with ECN42 was added to the trade standard in November 2010.

The IOC-CG carries out experimental studies and validation of analytical methodology. The usual procedure involves a member country presenting to IOC-CG results of a new method at some stage of the experimentation/research. IOC-CG can decide, after analyzing the results, to proceed with a collaborative trial for its validation and to propose it as an official IOC method if the results are suitable. IOC-CG is also involved in issuing opinions about topics that are not always related to analytical methods, such as the establishment of limits for selected parameters or the variation of the olive oil chemical composition depending on the environment, climate, variety, and other external factors. Consequently, IOC-CG is organized into temporary and permanent working groups in accordance with the tasks (e.g., validation of a particular analytical method versus studies on pesticides).

The proficiency of chemical laboratories previously accredited by their relevant national accreditation body is verified in two or three collaborative trials organized by the IOC every year. The award of recognition by the IOC lasts for one olive oil campaign only.

Another important issue to be kept in mind is that IOC trade standards are based on the agreement signed by each member country once a year, while EU regulation is a law in force for each country of the EU. This allows the IOC to be quicker in including methods, even if they are labeled as provisional, a term that is not allowed within the framework of a law. Consequently, the EU needs much more time, according to its protocols, to publish regulations. Moreover, and for the same reason, the IOC can standardize and validate methods to determine parameters that have no associated detection limits (e.g., phenols and tocopherols) with the sole aim of providing standardized methods for determining chemical parameters used in the trade. Thus, the IOC validated and adopted an HPLC method for determining phenols in 2009, whereas the colorimetric method, proposed for the same purpose, still remains provisional through lack of reliable precision data (repeatability and reproducibility).

For many years, IOC methods were used worldwide for the analytical control of olive oils. Today, however, most of the methods refer to ISO methods, although some still refer to American Oil Chemists Society (AOCS) methods. This choice has led the IOC to compare already adopted methods, which had been developed for olive oils, with ISO methods that had been developed for a wide number of fats and oils. Sometimes, however, the peculiar characteristics of olive oils have allowed certain methods to be included as ISO norms. The IOC is an official liaison organization of ISO/TC 34/SC 11 – Animal and vegetable fats and oils, which is a subcommittee of ISO/TC-34 – Food products.

16.2.3 Codex Alimentarius

The Codex Alimentarius Commission was created in 1963 by the Food and Agriculture Organization (FAO) and World Health Organisation (WHO) of the United Nations to develop food standards and to produce guidelines and related texts, such as codes of good practice, under the Joint FAO/WHO Food Standards

Programme. The main purposes of this program are protecting the health of consumers, ensuring fair trade practices in the food trade, and promoting coordination of all food standards work undertaken by international governmental and nongovernmental organizations. The food standards contain provisions that are intended for voluntary application by commercial partners and not for application by governments.

Codex STAN 33–1981 (formerly CAC/RS 33–1970), which applies to olive oils (VOO, ROO, and olive oil) and olive-pomace oils (refined olive-pomace oil and olive-pomace oil) was revised in 1989 and 2003 and amended in 2009. The essential composition and quality factors of each designation are identical to those described by IOC trade standards. Codex is also concerned with possible food additives and contaminants (limits for heavy metals, pesticide residues, and halogenated solvents). Codex recommends that olive oils and olive-pomace oils be prepared and handled in accordance with General Principles of Food Hygiene (CAC/RCP 1–1969) and other relevant Codex texts such as Codes of Hygienic Practice and Codes of Practice, that the oils intended for human consumption comply with the Principles for the Establishment and Application of Microbiological Criteria for Foods (CAC/GL 21–1997), and that the oils intended for international trade be packed in containers labeled in accordance with the Codex General Standard for Labelling of Prepackaged Foods (CODEX STAN 1 – 1985). Today, interest is focused on the limits of certain compounds (e.g., linolenic acid) because genuine olive oils of some producer countries do not comply with current trade standards and official regulations (CAC 2009a).

16.2.4 Further Legislation Regulating Production and Quality

The IOC trade standard contains not only the designations, definitions, and purity and quality criteria for olive oils and olive-pomace oils as described previously but also other quality criteria, as displayed in Table 16.9. Furthermore, the IOC also describes aspects of hygiene and packaging, with recommendations that coincide with Codex Alimentarius, although the IOC trade standard is much more explicit in the characteristics of the containers, recommending four classes (IOC 2010). The IOC standard is also more specific than the Codex Alimentarius standard in the aspects of container filling tolerance and labeling (IOC 2010). Concerning the former, under no circumstance shall the volume be less than 90 %, or 80 % in the case of containers with a capacity equal to or less than 1 L. With respect to labeling, the IOC distinguishes between three different uses: (a) containers intended for direct sale to consumers, (b) packs of oils intended for human consumption, and (c) containers allowing the transportation in bulk of olive oils. With respect to the first use, specific provisions providing the following information shall be applied: product name, designation of olive oil and olive-pomace oil, free acidity of oil, net contents, name, address, country of origin, indications of source and appellations of origin, lot identification, date marking and storage conditions, date of packaging, date of minimum durability, storage instructions, and so forth.

Table 16.9 Quality and purity criteria of IOC trade standard that are not described in Commission Regulation (EU) 61/2011 (*Source*: EC 2011)

Designation	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
EVOO	≤0.2	≤3.0	<0.1	<0.1	≤0.1	≤0.1	–	–	–	–
VOO	≤0.2	≤3.0	<0.1	<0.1	≤0.1	≤0.1	–	–	–	–
Ordinary VOO	≤0.2	≤3.0	<0.1	<0.1	≤0.1	≤0.1	–	–	–	–
Lampante VOO	≤0.3	≤3.0	<0.1	<0.1	≤0.1	≤0.2	–	–	–	–
Refined olive oil	≤0.1	≤3.0	<0.1	<0.1	≤0.1	≤0.05	Acceptable	–	*D*	200
Olive oil	≤0.1	≤3.0	<0.1	<0.1	≤0.1	≤0.05	Good	–	*E*	200
Crude olive-pomace oil	≤1.5	–	<0.1	<0.1	–	–	–	≥120 °C	–	–
Refined olive-pomace oil	≤0.1	≤3.0	<0.1	<0.1	≤0.1	≤0.05	Acceptable	–	*F*	200
Olive-pomace oil	≤0.1	≤3.0	<0.1	<0.1	≤0.1	≤0.05	Good	–	*E*	200

Notes:
(1): Moisture and volatile matter (% m/m)
(2): Trace of iron (mg/kg)
(3): Trace of copper (mg/kg)
(4): Content of lead (mg/kg)
(5): Content of arsenic (mg/kg)
(6): Insoluble impurities in light
(7): Organoleptic characteristics: odor and taste
(8): Flash point
(9): Organoleptic assessment: color; *D* light and yellow, *E* light and yellow to green, *F* light, yellow to brownish yellow
(10): Food additives: maximum content of alpha-tocopherol (mg/kg)

16.3 Standard Methods for Determining Authenticity

The methods of analysis and sampling are internationally refereed methodologies, although some differences do exist between the official methods and those for trade standards or in-house methodologies. The development of standard or official methods normally results from an industry or commercial need and through their validation by collaborative studies. The associations that have developed, adopted, or recommended standard methods for olive oil include the Association of Official Analytical Chemistry (AOAC), the AOCS, the IOC, the International Union of Pure and Applied Chemistry (IUPAC), and the Federation of Oil Seeds and Fats Association (FOSFA). The following methods of analysis basically correspond to IOC trade standards for olive oil and olive-pomace oil and ISO methods, although alternative methods fostered by other associations are cited as well. Table 16.10 displays the methods proposed for the determination of the physicochemical parameters and their principles, which also require the sampling procedure (ISO 2001) and the preparation of the test sample to be added (ISO 2005a).

16.4 Current Analytical Solutions

In the days when adulterations and misleading labeling practices were fairly simple, their detection by straightforward analytical techniques was relatively easy. As advanced technology has become more widely used, fraudulent practices have become ever

Table 16.10 Analytical methods proposed for determining physicochemical parameters for authentication criteria of olive oils and olive-pomace oils and their principles

Parameter	Principle	Method
Fatty acid composition	GC[a]	COI/T.20/Doc. No. 24 or ISO 5508 or AOCS Ch 2-91
Unsaponifiable matter	Titrimetry	ISO 18609 (hexane) or AOCS Ca 6b-53 or ISO 3596 (diethyl ether)
Free acidity	Titrimetry	ISO 660 or AOCS Cd 3d-63
Peroxide value	Titrimetry	ISO 3960 and AOCS Cd 8b-90
Trans fatty acids	GC	COI/T.20/Doc. No. 17/Rev.1 or ISO 15304 or AOCS Ce 1f–96
Sterol composition and content	GC	COI/T.20/Doc. No. 10/Rev. 1 or AOCS Ch 6–91 or ISO 12228
Absorbency in ultraviolet	Absorption in ultraviolet	COI/T.20/Doc. No. 19/Rev. 2 or ISO 3656 or AOCS Ch 5-91
α-tocopherol	HPLC	ISO 9936
Moisture and volatile matter	Gravimetry	ISO 662
Insoluble impurities	Gravimetry	ISO 663
Copper, nickel, and iron	AAS[b]	ISO 8294 or AOAC 990.05
Lead	AAS	ISO 12193 or AOCS Ca 18c-91 or AOAC 994.02
Arsenic	Colorimetry	AOAC 952.13[c] or AOAC 942.17[d] or AOAC 985.16
Trace of halogenated solvents	GLC	COI/T.20/Doc. No. 8/Corr.1
Waxes and alkyl esters	GC	COI/T.20/Doc. No. 28
Waxes	Titrimetry	COI/T.20/Doc. No. 18/Rev.2; or AOCS Ch 8-02
Erythrodiol and uvaol	GC	IUPAC No. 2431
Aliphatic alcohols	GC	COI/T.20/Doc. No. 26
Stigmastadienes	GC	COI/T.20/Doc. No. 11/Rev. 2 or COI/T.20/Doc. No. 16/Rev. 1 or ISO 15788–1 or AOCS Cd 26-96
2-glyceryl monopalmitate	GC	COI/T.20/Doc. No 23
Difference between theoretical and actual ECN 42 TAG contents	Calculation	COI/T.20/Doc. No. 20/Rev.3 or AOCS Ce 5b-89
Refractive index	Refractometry	ISO 6320 or AOCS Cc 7-25
Iodine value	Wijs-Titrimetry	ISO 3961 or AOAC 993.20 or AOCS Cd 1d-92
Flash point		FOSFA
Biophenols	HPLC	COI/T.20/Doc. No. 29
Organoleptic characteristics	Sensory assessment	COI/T.20/Doc. No. 15/Rev. 3

Notes

Information at www.internationaloliveoil.org/; www.iso.org; www.aocs.org/methods/; www.aoac.org/

AAS atomic absorption spectrometry, *GC* gas chromatography, *GLC* gas–liquid chromatography, *HPLC* high-performance liquid chromatography

[a]Gas chromatography of methyl esters

[b]Atomic absorption spectrometry with direct graphite furnace

[c]Colorimetry using diethyldithiocarbamate

[d]Colorimetry using molybdenum blue

Table 16.11 Main characteristics of techniques used in authentication of olive oils

Characteristic	Technique
Separation	LGC, HRGC, HPLC, RP-HPLC, SFC
Structural and pattern recognition	NMR, MS, MOS and SAW sensors, FT-Raman, NIR, FTIR, IR, DNA
Stable isotope analysis	IRMS
Trace elemental analysis	ICP-AES, AAS, FAAS, ETA-AAS
In-tandem instruments	δ^2H-EA-Py-IRMS, HPLC-MS, ICP-MS, HS-MS, GC-MS, δ^2H-GC-Py-IRMS, HS-GC, GC×GC, LC×LC

GC gas chromatography, *HPLC* high-performance liquid chromatography, *SFC* supercritical fluid chromatography, *NMR* nuclear magnetic resonance spectrometry, *MS* mass spectrometry, *NIR* near-infrared reflectance spectroscopy, *FTIR* Fourier transform mid-infrared, *MOS* metal oxide semiconductor, *SAW* surface acoustic wave, *IRMS* isotopic ratio mass spectrometry, *ICP-AES* inductively coupled plasma atomic emission spectrometry, *FAAS* flame atomic absorption spectrometry, *ETA-AAS* electrothermal atomization atomic absorption spectrometry, *HS-GC* headspace gas chromatography, *H-EA-Py-IRMS*, elemental analysis-pyrolysis-isotopic ratio mass spectrometry, *H-GC-Py-IRMS* gas chromatography-pyrolysis-isotopic ratio mass spectrometry, *RP* reverse-phase

more complex and ingenious. Thus, the traditional standard methods based on the identification or quantification of a parameter are evolving to become an arsenal of analytical techniques (Table 16.11) in which a considerable amount of data are at the disposal of the analyst who must habitually apply pattern-recognition algorithms to identify the samples (Aparicio 2002; Aparicio and Aparicio-Ruiz 2000; Vandeginste et al. 1998).

In the past, the quality of vegetable oils was usually evaluated using index values of control tests, such as, for example, acidity (free fatty acid [FFA] content), peroxide number, color, flavor, and specific extinction in ultraviolet light, while purity was evaluated by other methods that measure the chemical composition of an analysis matrix, such as, for example, refractive index, iodine value, saponification value, flash point, slipping point, and melting point (Rossell 1986). The superior performance of new techniques, however, has meant a decline in the use of the classic tests. Today, only a few of these physical and chemical determinations, for example, the Fitelson test (AOAC 1995), are considered reliable in establishing the quality of olive oil, whereas a sensory assessment (panel test) is believed to be the most decisive for rating the categories of VOO.

The genuineness of olive oils is monitored using the methodologies and instrumental techniques described in the aforementioned tables. The official methods (EC 2011; IOC 2010) have undoubtedly enabled the control of olive oil adulteration, although some authors (Mailer et al. 2010; Salvador et al. 1998) have pointed out that some genuine EVOOs are classified outside their natural categories, while, on the other hand, certain kinds of sophisticated adulterations are still undetected with the current official methods (Gertz 2008). These problems require a considerable investment to improve the current official methods or develop new ones that will offer not only solutions to these problems but also reduce the numerous official

methods that require high expenditures in manpower, time, and money (for qualified personnel and laboratory equipment).

To improve current official methods, other research has focused on the detection of adulteration or contamination of olive oils. Some of this research has given rise to new official methods, whereas other research, based on emergent techniques, represents powerful alternatives to the current regulations or trade standards. Currently, however, some new methods are able to detect adulterations in a range of more than 10 %, which is no advantage compared with the official methods, or they claim that they can detect up to 1 % that seems to be beyond the current state of the art and, consequently, its reliability needs to be proven.

Current instrumental techniques can be clustered into two groups: (1) those based on contributions from almost all possible analytes in an oil sample, the so-called physical methods, and (2) those that rely on measurement of more definite information attained from fractionation of olive oil components, the so-called chemical methods. The latter group of techniques, which quantify and identify series of chemical compounds, analyte by analyte, have as their objective to seek compounds that do not appear, or appear only at trace levels, in genuine olive oil but that do appear in adulterated oils. Because these techniques provide information about why these compounds are present in the adulterated food, this information can also be used to remove or diminish the amount of these analytes in the adulteration process, e.g., by removing sterols or by bond double bonds of unsaturated FAs to maleic anhydride.

The physical group of techniques is based on the analysis of the chemical composition of oil using, for instance, a spectroscopic technique. In this area, fraudsters are complete ignorant, but analysts can have problems in interpreting the information with plausible chemical explanations. This group of techniques can be helpful with the use of multivariate statistical techniques. Even then, the conclusions should be supported by chemical or biochemical explanations that therefore rule out noise or random effects in the samples.

This section does not consider all the investigations on olive oil authenticity published in recent years, mainly because many papers have appeared in journals that the author was not able to find in the main databases or they were described in earlier chapters of this book. Moreover, the attention given to the various chemical methodologies has been made roughly proportional to the innovation made, the importance of the contribution in the near future, or simply because they are currently applied in some countries to detect adulteration or contamination.

16.4.1 Spectrometric Techniques

The existence of a standard method usually requires 5–7 years from development to designation as a validated official method. Today, new instruments and new methodologies are developing so rapidly that an official method is often out of date by the time it is released. Spectroscopic techniques offer plausible solutions to current

problems of chromatography, a time-consuming technique that requires several steps for carrying out quantification, uses polluting solvents, and is impracticable for online control – a common demand by farmers and governmental institutions in the fight against adulteration. In contrast, the chromatographic techniques rarely need univariate statistical algorithms for the interpretation of their results versus spectrometry, whose results must be analyzed with the assistance of multivariate statistical procedures (Martens and Naes 1989; Aparicio 2002).

Many methods based on spectroscopic techniques that have been published in the past decade for the detection of the type and amount of adulteration in olive oils. Frequently used spectroscopic approaches are fluorescence, Raman, mid- and near-infrared, and NMR. However, some of the published results have been criticized because of, for example, the manner in which the authors applied the statistical procedures, the number and kind of samples selected for the study, the usefulness of the marker, and the kind of studied adulteration. Many studies are hyperoptimistic in that the statistical procedures are used in a lax way or because they require large numbers of samples that the authors have not analyzed or results are often expressed in ranges that are not always precise enough. Furthermore, the selection of the markers by authors is often carried out in an arbitrary way; for example, it makes no sense to try to detect "deodorized" hazelnut oil in VOO or ROO using volatiles since the volatiles are removed in the deodorization process; likewise, refined hazelnut will not be detected using phenols that disappear in the refining process, as demonstrated in the literature.

Little or no sample work-up is required for pyrolysis-mass spectrometry (Py-MS), which has the advantage of speed of analysis. Py–MS is an analytical fingerprinting technique in which the sample is quickly decomposed at several hundred degrees Celsius, and the resulting fragments are analyzed by MS, and the MS spectra are subsequently submitted to multivariate data analysis. A combination of mathematical procedures and Curie-point Py-MS has enabled the design of an artificial neural network (ANN) for rapid assessment of the adulteration of EVOOs with various seed oils (soybean, sunflower, peanut, corn) or ROO (Goodacre et al. 1993), although the main applications have focused on verifying the geographical origin (Guillou et al. 1999) by means of diverse isotopic ratios ($^{13}C/^{12}C$, D/H, $^{18}O/^{16}O$), for example, in EVOOs of Italian protected designations of origin (PDOs) and protected geographical indications (PGIs) (Camin et al. 2010).

Spectroscopic techniques have been considered promising tools for rapid sample screening (Baeten et al. 1998). Indeed, the intensities of Raman spectroscopy bands near 1,656 cm^{-1} and 1,670 cm^{-1} were related to the *cis* and *trans* isomer contents in the early 1970s (Bailey and Horvat 1972), whereas infrared (IR) spectroscopy had already been applied in the 1950s (Barlett and Mahon 1958). Technical developments have allowed the application of these techniques in olive oil authentication (Baeten and Aparicio 1997; Hourant et al. 2000; Vlachos et al. 2006; Gurdeniz et al. 2007) beyond the determination of *trans* unsaturated FAs by IR spectroscopy (van de Voort et al. 1995), the determination of the iodine value and saponification number by an automated attenuated total reflectance (ATR)/FTIR method (van de Voort et al. 1992), and the monitoring of the frying process (Tena et al. 2009). Thus,

Wesley et al. (1995, 1996) reported a methodology based on an IR technique for predicting the level of adulteration (in a range of 5–30 %) in a set of olive oils adulterated with corn oil, sunflower oil, and pomace oil. More recently, other authors have analyzed NIR and ATR-FTIR spectra with different multivariate statistical procedures to detect the adulteration of olive oil with sunflower at 2 % and with hazelnut oil at percentages higher than 25 % (Ozen and Mauer 2002) and 8 % (Baeten et al. 2005), with cottonseed, rapeseed, and mixtures of corn and sunflower oils at 5 % (Gurdeniz and Ozen 2009), with peanut, corn germ, pumpkin, and sunflower oils (Alexa et al. 2009), with sunflower and corn oils around 4 % (Özdemir and Öztürk 2007; Öztürk et al. 2010), and with palm oil at 1 % (Rohman and Che Man 2010). The first application of FTIR-photoacoustic spectroscopy (PAS) for the detection of adulteration in olive oil was carried out with sunflower oil by Yang and Irudayaraj (2001). The FTIR-PAS sample cell can be easily cleaned after analysis compared to the ATR crystal, which requires an elaborate cleaning procedure to avoid the memory effect of oil samples on the surface of the ATR crystal.

Fourier transform-Raman spectroscopy (FT-Raman), however, has been used only to a limited extent in olive oil authenticity until now. FT-Raman can be regarded as a complementary method to the IR technique for analysis and, like FTIR spectroscopy, can also provide information on the functional/chemical groups for qualitative and quantitative characterization (Zou et al. 2009). Baeten et al. (1996) predicted the level of adulteration in a set of VOO samples that were adulterated with soybean, maize, and olive-pomace oils at 1 %, 5 %, or 10 % (w/w). The authors used multivariate statistical procedures to select only those wavenumbers (3,007, 2,980, 2,954, 1,758, 1,745, 1,630, 1,440, 1,414, and 1,297 cm^{-1}) that, having a chemical explanation, are significant for detecting adulteration; 91.3 % of samples were correctly classified. The relative intensities of Raman bands near 3,010 and 1,630 cm^{-1}, assigned to = C–H symmetric stretching vibration and C = C stretching vibration, were useful indicators of different levels of unsaturation. Figure 16.1 shows the Raman bands at 1,752–1,764 cm^{-1} and 1,200–1,250 cm^{-1} of the ring test. It is noteworthy that the model was able to detect the adulteration of VOO with 10 % of the same VOO but refined under mild conditions (approx. 200°C) and using charcoal instead of bleaching earth. The intensity ratio of the *cis* (=C–H) and *cis* (C = C) bonds normalized by a band at 1,441 cm^{-1} (CH_2) has also been used to determine the presence of soybean, rapeseed, sunflower seed, and corn oils in olive oils at 5 % (Zou et al. 2009), whereas Raman spectroscopy with a visible spectral range has been used by El-Abassy et al. (2009) to detect the presence of sunflower oil in EVOO at a concentration of 1 % by applying a partial least-squares regression algorithm.

A comparison of the prediction models from the different spectroscopic methods has been carried out (Marigheto et al. 1998; Yang and Irudayaraj 2001). The latest results indicate that FT-Raman spectroscopy give the best results in terms of correlation and prediction error, although NIR and MIR techniques also provide good results. The nondestructive evaluation, ease of operation, and fast determination merit the use of a Raman technique for studies about the adulteration of olive oil with lower-quality oils.

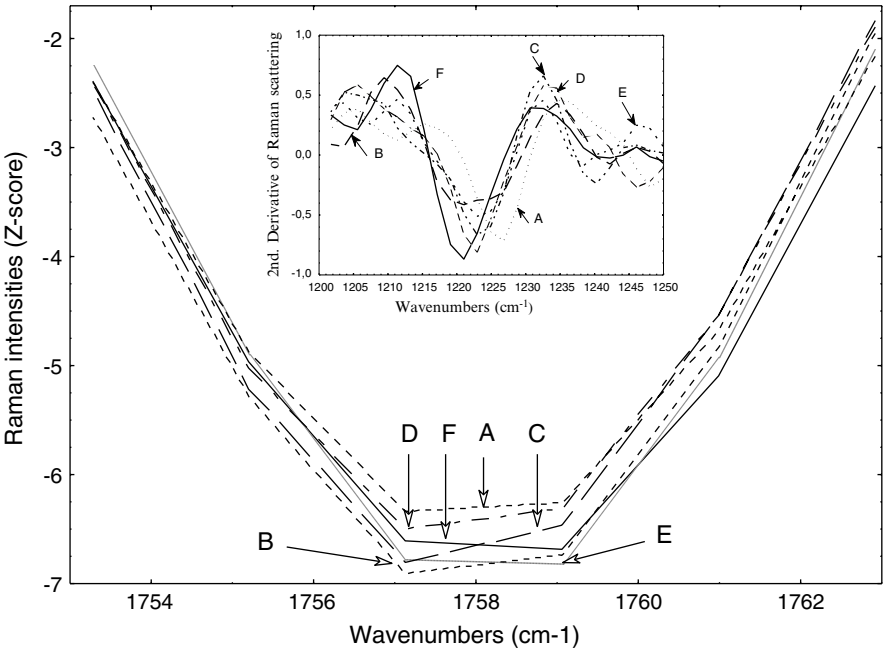

Fig. 16.1 Raman shifts 1,753–1,763 cm^{-1} of a genuine virgin olive oil (sample A) and five samples adulterated with its refined virgin olive oil at 10 % (sample C), maize oil at 2–10 % (samples E and B), and high oleic sunflower oil at 2–10 % (samples D and F). The shift at 1,758 cm^{-1} corresponds to C=O stretching vibration (molecule –CO–O-). Small figure shows Raman shifts 1,200–1,250 cm^{-1} that basically correspond to C–H bending vibration (molecule *cis* H–C=C–H)

Fluorescence spectroscopy, combined with supervised and unsupervised multivariate statistical procedures, has been applied to assess the adulteration of VOO with other edible oils. Excitation-emission fluorescence spectroscopy (EEFS) (range λ_{ex}=300–500 nm at λ_{em}=655 nm and range λ_{em}=650–900 nm at λ_{ex}=350 nm) was used, together with principal component analysis and ANNs, to detect the presence of refined hazelnut oils in ROOs at percentages higher than 9 % (Sayago et al. 2007), while other authors used ranges of λex=300–390 nm and λem=415–600 nm to detect the presence of olive-pomace oil in EVOO at 5 % (Guimet et al. 2005a). Total synchronous fluorescence (TSyF), with excitation wavelengths between 250 and 720 nm, with the help of regression models, has also made it possible to determine the presence of corn, olive-pomace, rapeseed, soybean, and sunflower oils in VOOs at levels of 2.6–13.8 % (Poulli et al. 2007). For example, TSyF contour plots for sunflower, in contrast to VOO, show a fluorescence region in an excitation wavelength range of 325–385 nm (Poulli et al. 2006)

The detection of adulteration of VOO with low proportions of ROO or olive-pomace oil (olive-residue oil) may be detected based on the presence of conjugated polyene systems. In fact, a specific extinction coefficient of 270 nm, corresponding to the absorption of conjugated trienes induced by certain types of bleaching

earths during the bleaching process (Mariani et al. 1993), is used as an index of genuineness of the different categories of olive oil (IOC 2010; EC 2011). EVOO, for example, must have specific extinction maxima of 2.50 at 232 nm and 0.22 at 270 nm (Table 16.6).

However, a vast number of oxidation products formed during VOO oxidation absorb in the same region (Guimet et al. 2005b), which results in a decrease in the sensitivity of the method even after the oil has been poured over activated alumina to eliminate most of the oxidized products. A region that overcomes such problems is 310–320 nm, where the conjugated tetraene systems have their absorption maxima. In fact, Kapoulas and Andrikopoulos (1987) had already reported that the ratio between the absorptions of two consecutive extremes of a second-derivative reflection at 315 nm showed differences between VOO, ROO, and olive-pomace oil. In fact, the ranges of $\Delta K^{1\%}{}_{315}$ allowed for the unequivocal detection of 5 % adulteration of virgin with the other, cheaper, olive oils. Later, Passaloglou-Emmanouilidou (1990) found similar possibilities for the same region calculating $\Delta K^{4\%}{}_{315}$, from direct measurements of the UV spectra, rather than from the second derivative. Aued-Pimentel et al. (1993), distinguished VOO from ROO, rice bran oil, soybean oil, and sunflower oil using ΔK for second-derivative spectra at 310–313 nm. The second-derivative reflections were also used by Calapaj et al. (1993) to distinguish between VOO and refined vegetable oils. However, the modern technology of deodorizing process made possible to sell deodorized oils with characteristics of UV absortion very similar to those of VOO.

The literature also includes methods based on NMR. Table 11.4 (Chap. 11) displays the nuclei (^{13}C, ^{1}H, and ^{31}P) and frequencies of the instruments used to detect the presence of different edible oils in olive oil categories by the quantification of specific biomarkers.

The admixure of olive oils with esterified oils was first examined with ^{13}C-NMR by Sacchi et al. (1992), who reported that the method could be applied to detect a level of 2–3 % saturated components in the *sn*-2 position. Later, Mavromoustakos et al. (2000) studied 12 peaks of the olefinic region (127.5–130.0 ppm) of ^{13}C-NMR spectra, as well as the R/β ratios of oleic acid (1.1) and linoleic acid (1.5), to detect the presence of seed oils (cottonseed, sunflower seed, soybean, and corn oils) in EVOO. ^{13}C-NMR can precisely and easily distinguish olive oils from mixtures of olive oils containing 5 % by volume of other edible oils, such as soybean oil, rapeseed oil, sunflower seed oil, or corn oil (Zamora et al. 2001).

^{1}H-NMR spectroscopy has also been a useful tool to simultaneously determine the chemical shifts of the saturated and unsaturated (C18:1, C18:2, C18:3) FAs that allow determination of edible oils in olive oils (Guillen and Ruiz 2003). This spectroscopic technique has also been used to detect the presence of hazelnut oil (either crude or refined) in olive oil categories at 10 % (Mannina et al. 2009) and in lower percentages (8 %) in combination with ^{13}C-NMR spectra (García-González et al. 2004). Furthermore, high-power gradient NMR diffusion coefficients (D) were determined for the rapid screening of EVOO for adulteration with cheaper vegetable oils (Šmejkalová and Piccolo 2010). Changes in D values could be detected with adulteration of 10 % for sunflower and soybean oils and 30 % for hazelnut and peanut oils.

16.4.2 Chromatographic Techniques

Chromatographic techniques, as described in Chap. 6, are probably the most effective analytical approach when separation of each component of oil is required. In fact, it has extensively been applied to lipids – their chemical and physical characteristics make them one of the more suitable classes of organic compounds to undergo such analyses – and most of the current work on olive oil adulteration is based on chromatographic analysis. Thus, HPLC and HRGC analysis have been applied to the quantification of FAs, TAGs, sterols, tocopherols, and hydrocarbons (IOC 2010; EC 2011). Although HPLC presents important advantages compared with GLC, and it has increasingly been used in the past decade, the number of analytical methods for olive oils is still lower than those applying GLC.

16.4.2.1 Fatty Acid Methyl Esters

FAs were some of the very early compounds to be analyzed by GC that made possible an in-depth characterization of edible oils since FA composition depends on the botanical family to which the oil-producing plant belongs. FA composition was extensively applied to purity control of olive oils with very good results until the late 1960s when seed oils with a modified FA composition similar to olive oil appeared. The first was safflower *(Chartamus tinctorius*) cultivar with 70–80 % of oleic acid, while its original composition was characterized by 70 % linoleic acid. Later, high oleic sunflower and "zero" erucic rapeseed (called canola) were also developed. More recently, rapeseed with high oleic content and high oleic soybean oil became available to the trade as well.

In light of such issues, the method for the determination of FA composition seemed to lose its relevance. Its analytical performances, however, were improved and made the method suitable to determine *trans* isomers of FAs, which were then evaluated by IR spectroscopy of oil in a carbon sulphide solution.

The presence of the *trans* isomers of oleic (octadecenoic), linoleic (octadecadienoic), and linolenic (octadecatrienoic) above their maximum levels (Table 16.6) can indicate adulteration with hydrogenated seed oils (Gurr 1986), esterified olive oils (Gegiou and Georgouli 1983), illegally treated VOOs (Mariani et al. 1991), or mutant (or genetically altered) seed oils desterolized at high temperatures (Paganuzzi 1997). Today, GC analysis of FAs is carried out as a tool for the calculation of the theoretical composition of TAGs with the final objective of determining the value of the theoretical ECN42 (equivalent carbon number), which may be compared to the experimental ECN42 obtained by HPLC.

Special attention must be paid to the methylation step that can be carried out by acid or alkaline catalysis. Several reagents can be used in the acid catalysis – e.g., hydrochloric acid in methanol (5 % w/v solution), several concentrations of boron trifluoride in methanol (most usual 14 % w/v), sulfuric acid in methanol – while alkaline catalysis is usually performed by means of sodium methylate in methanol.

The analysts should also take into account that even if the strong acidity of some reagents can catalyze the isomerization of unsaturated FAs, it is mandatory to use such a reagent if FFAs are present because alkaline catalysis is not effective in methylation, only in *trans* methylation.

In summary, FA composition is no longer the ultimate determination to check the olive oil purity due to new edible oils from genetically modified seeds. In addition, the analysis of FAs is really carried out on their methyl esters (FAMEs), and this means that FAs are analyzed without any relation to the TAG to which they are bound. This leads to a loss of information that theoretically should be obtained from analytical data, while if FAs are considered within the framework of TAG composition, more restricted modification can occur.

16.4.2.2 Triacylglycerols

TAGs (trihydric alcohols esterified with three FAs) make up the major part of olive oil, and they largely determine its main characteristics. The molecular structure of each individual TAG species has three basic characteristics: (1) the total carbon number (CN), which is the sum of the alkyl chain lengths of each of the three FAs; (2) the degree of unsaturation in each FA; and (3) the position and configuration of the double bonds in each FA (Buchgraber et al. 2004). Thus, the large number of possible FA combinations on the glycerol backbone of TAGs makes the analysis of the TAG composition a very challenging task either with GLC or high-performance chromatography in normal and reverse-phase modes (HPLC).

The main advantage of GLC over the other competing techniques is the availability of a flame ionization detector (FID), a simple but universal linear response detector. Selectivity in GLC, however, depends on the length and chemical nature of the column stationary phase. For a number of years, even after the spread of capillary columns, the very high temperature suitable to elute TAGs from gas chromatographic columns required the use of very short columns with the consequence of a rather poor resolution. Polar stationary phases could not be used at high temperatures so that only some apolar phase columns of dimethyl polysiloxane were used, and separation was obtained on the basis of molecular weight only. The kind of injection port also affects the analysis results, as discrimination phenomena occur when a vaporizing injection port is used (e.g., split/splitless). Programmed temperature vaporizer (PTV) and on-column (OC) are the most suitable injectors.

It was not until 1985 that a polar stationary phase, so-called RSL-300, was designed for a suitable separation of compounds on the basis of molecular weight and degree of saturation of molecules. Later, an improved phase (called the tryglyceride analysis phase [TAP]) became available and was used extensively by several researchers (Frega et al. 1990) and was followed by other stationary phases with the same column dimensions (25 m × 0.25 mm × 0.1 μm) (Table 16.12).

With these new approaches, the presence of trilinolein (LLL) in olive oils was highlighted and a limit was established at 0.5 % by the IOC. LLL is a polar

Table 16.12 GLC and RP-HPLC conditions used for determination of olive oil TAG profile

GLC

Column stationary phase	Oven temperature program	Injection mode	Carrier gas	Pressure	Ref
CP-TAP PhMeSi	340°C; 1°C/min; 360°C	Split	He	130 kPa	Frega et al. 1990
OV-17 PhMeSi 50%	330°C; 1 min; 1°C/min; 355°C; 4 min	Split	H_2	100 kPa	Antoniosi Filho et al. 1993
CB-TAP PhMeSi	343°C; 1 min; 1°C/min; 344°C	–	H2	–	García-Pulido and Aparicio 1993
OV-17-OH PhMeSi 50%	330°C; 1 min; 1°C/min; 340°C; 9 min	–	H2	–	Antoniosi Filho et al. 1995

RP-HPLC

Column dimension	Stationary phase	Mobile phase	Detector	Flow rate[a]	Ref
60×6.2 mm; 3 µm	ODS Zorbax	ACN-DCM	FID		Phillips et al.1984
250×4.0 mm; 5 µm	Hibar RP18	PPN	–	0.6	Frede 1986[b]
(250×4.9 mm)×2; 5 µm	Zorbax C-18	ACN-DCM	–	0.8	Zeitoun et al. 1991[c]
250×4.6 mm; 5 µm	Supercosil LC-18	AC-ACN	RI	1.5	Flor et al. 1993
250×4.6 mm; 5 µm	Supercosil LC18	ACN-AC	RI	1	Salivaras and McCurdy 1992
244×4 mm; 5 µm	Supersphere 100 RP-18	ACN-iProp-Hex; EtOH-Hex	DA-ELSD	0.8	Damiani et al. 2000[d]
244×4 mm; 5 µm	Supersphere 100	Propionitrile	RI	0.5/0.8	Ollivier et al. 2003
250×4.6 mm; 5 µm	Kromasil 100 C-18	AC/ACN	ELSD	1	Cunha and Oliveira 2005
55×2 mm; 3 µm	Purospher Star RP-18e	Met/Hex	APCI-MS	0.2/0.7	Nagy et al. 2005
300×3.9 mm; 4 µm + 150×3.9 mm; 4 µm	Nova Pak C-18	ACN/iProp	APCI/MS	3	Lisa et al. 2009

AC acetone, *ACN* acetonitrile, *DCM* dichloromethane, *EtOH* ethanol, *Hex* hexane, *iProp* iso-Propanol, *Met* methanol, *PPN* propionitrile. Sample solvent: [a]mL/min; [b]PPN-ether; [c]AC; [dCHCl3-iProp]. Detector: *FID* flame injector detector, *RI* refractive index, *DA* diode array detector, *ELSD* evaporative light-scattering detector, *APCI* atmospheric-pressure chemical ionization, *MS* mass spectrometry

molecule with a long retention time in GC analysis on polar phases, which results in a poor peak shape and a not very reliable measurement. In contrast, in reverse-phase high performance liquid chromatography (RP-HPLC), LLL is eluted within several minutes and the peak area measurement is more reliable. For this reason HPLC was chosen as the official method.

By then, interesting research had already been carried out to detect the presence of vegetable oils in olive oil. Thus, Kapoulas and Passaloglou-Emmanouilidou (1981) used column chromatography on silicic acid impregnated with ammoniacal silver nitrate to isolate a fraction enriched in polyunsaturated triacylglycerides. They studied the linoleic acid content and the ratio of oleic to linoleic acid to detect mixtures of VOO with refined seed oils at 5–10 %, although the hypothetical ranges of linoleic acid for either VOOs or seed oils were narrower than those described in the literature by then. Later, Gegiou and Georgouli (1983) proposed different ratios of TAG to detect the presence of reesterified oils in olive oils: (1) 1-oleo-2,3-dipalmitin to 1,3-dipalmito-2-olein is 8:92 for olive oil and 55:45 for reesterified oils; (2) 1,3-dioleo-2-palmitin to 1-palmito-2,3-diolein is 7:93 for olive oil and 55:45 for reesterified oils; and (3) 1,3-dioleo-2-stearin to 1-stearo-2,3-diolein is 16:84 for olive oil and 46:54 for reesterified oils.

Gallina-Toschi et al. (1993) studied the TAG composition of olive oil samples by stereospecific analysis after partial hydrolysis with ethyl magnesium bromide, derivatization, preparative chiral HPLC, transesterification, and GLC quantification of FA methyl esters. The stereospecific analysis determined the substituents at all positions of the glycerol backbone. The stereospecific analysis of olive oil by enzymatic and nonenzymatic methods showed qualitative and quantitative results similar to those shown by Damiani et al. (1994).

As HPLC methodology was applied to every sample that must be checked for purity, an increasing number of olive oils that did not fit the limit (0.5 %) was highlighted. Research showed that not only LLL but also a cluster of incompletely resolved peaks were eluted at closed retention times.

As opposed to GLC, where CN components do not overlap even on medium-polarity columns, in RP-HPLC the components are separated according to the combined effect of the chain lengths of the FA moieties contained in a given TAG species plus their degree of unsaturation. Thus, a number of parameters have been suggested for describing the chromatographic properties of TAG molecules. Equivalent carbon number (ECN) (El-Hamdy and Perkins 1981) was one of them, and it had the greatest success detecting certain admixtures. ECN is based on the empirical results that the CN is linearly related to the logarithm of retention volume of a TAG in isocratic elution. This observation showed that in RP-HPLC each double bond of a TAG decreased the retention volume to approximately that of a saturated TAG with two fewer carbon atoms. As a consequence, ECN can be defined by the equation $ECN = CN - 2n$, where CN is the sum of the carbon atoms and n is the sum of the double bonds per TAG.

Salivaras and McCurdy (1992) explored the use of RP-HPLC in the detection of adulterations of VOO with canola oil. Genuine VOOs from various geographical origins were mixed with canola oil from 2.5 to 30 %, but samples spiked at less than

7.5 % could not be detected. Later, El-Hamdy and El-Fizga (1995) detected adulterations with approximately 1 % of linoleic-rich vegetable oils (soybean, sunflower, and corn oils) in less than 15 min by RP-HPLC. However, detection of olive oil admixed with seed oils nonrich in linoleic acid is more difficult.

ECN was also an incentive for using computer programs to calculate the theoretical composition of TAGs from FAs according to their 1,3-random 2-random distribution or full random distribution (Cortesi et al. 1990). Then, the experimental composition of TAGs, determined by HPLC, and their theoretical values calculated from FAs were compared. The difference between both values should be zero for an authentic olive oil sample, but the analytical error – for example, only some theoretical TAGs show good mathematical significance (García-Pulido and Aparicio 1993) – directed attention to the difference between the real and theoretical ECN42 triacylglyceride content (ΔECN42). The IOC trade standard and European regulation (EC 1997) included a limit of $\leq |0.2|$ for ΔECN42. The official method is an invaluable tool for the discovery of the fraudulent addition of seed oils to olive oil, even in low percentages, although significant deviations were found when the addition was to olive-pomace oil (Sinouri et al. 1995).

Concerning RP-HPLC technical aspects, the success of the selected analytical procedure depends upon the elution conditions (isocratic adversus gradient) and the type of detector. A UV detector with gradient elution, for example, cannot be used because some baseline drift can occur in the absorption region used for lipids, and in addition, many suitable solvents, such as acetone, cannot be used since they absorb strongly in the detection region of TAGs (between 200 and 220 nm). The alternative is the use of a refractive index (RI) detector with isocratic elution. The mobile phase also has a major effect on the separation of TAGs. Mobile phases are generally composed of an organic solvent, usually acetonitrile and an organic modifier, mostly acetone or, alternatively, benzene or tetrahydrofurane, which is added to improve the solubility of TAGs in the mobile phase. In fact, critical pairs are effectively separated with acetone/acetonitrile as the mobile phase in isocratic elution, although with this phase, saturated long-chain TAGs are not sufficiently dissolved. As a consequence, some researchers (Moreda et al. 2003; Ollivier et al. 2003) carried out experiments on isocratic elution with propionitrile as previously investigated (Podlaha and Toregard 1982; Fiebig 1985). The result is an improved separation of the groups of peaks clustered as ECN42 (Fig. 16.2). The method is at present under evaluation by IOC to assess whether the change in chromatographic conditions could somewhat influence the results of analysis. The improved separation permitted Cert and Colbs (IOC 2006a) to submit a method called a “global method for the detection of extraneous oils in olive oils” to the IOC. This has been suggested as a provisional application for the detection of the admixture of hazelnut oil to olive oil and thus is also in the evaluation stage.

A different approach to TAG analysis was focused on the discrimination of natural (biosynthesized) TAGs from chemically synthetized ones that were obtained from FFAs esterified with glycerol. Synthetized TAGs were sold as olive oil in the early 1970s.

The biosynthesis of TAG in vegetable oils, however, does not allow high concentrations of saturated FAs, as well as FAs with a carbon chain longer than 18 carbon atoms, to be present at 2-position of the molecule; 1 % of such acids esterified at

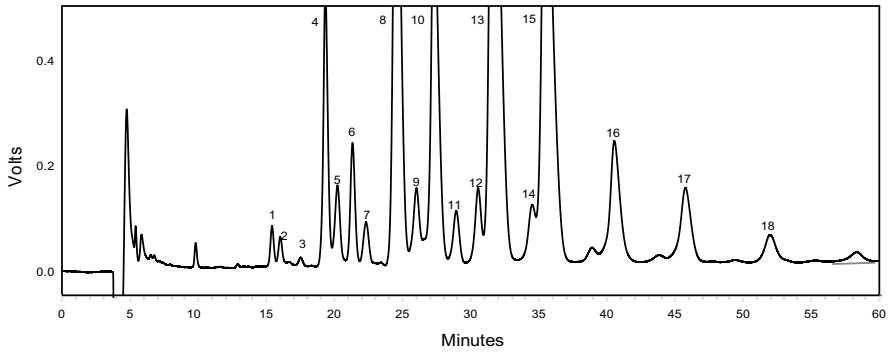

Fig. 16.2 HPLC trace obtained by isocratic elution with propionitrile mobile phase. Main components of chromatographic peaks: *1* LLL, *2* OLLn + PoLL, *3* PLLn, *4* OLL, *5* OOLn + PoOL, *6* PLL + PoPoO, *7* POLn + PPoPo + PPoL, *8* OOL + LnPP, *9* PoOO, *10* SLL + PLO, *11* PoOP + SPoL + SOLn + SPoPo, *12* PLP, *13* OOO + PoPP, 14 SOL, 15 POO, 16 POP, 17 SOO, 18 POS + SLS (*Source*: IOC (2006a), with permission of the International Olive Council)

the 2-position of glycerol is enough to conclude that the oil is not genuine. Thus, the analytical approach was based on the use of pancreatic lipase, an enzyme that has the ability to attack the 1- and 3-position of natural TAGs, thereby producing 2-monoacylglycerols. In the initial analytical method, TAGs, DAGs, and monoacylglycerols (MAGs) were fractionated by TLC and the band of MAGs was scraped off; FAs were then esterified with methanol, and the resulting methyl esters were analyzed by GC. However, Motta et al. (1983) demonstrated that the direct analysis of a lipolysis reaction was possible by capillary GC that, with some amendments (Lercker et al. 1985), avoided the TLC separation step, thus making the analytical process easier and faster (Lipp and Anklam 1998). The method was validated and included in national regulations (NGD 2005).

The initial limit for the sum of palmitic and stearic acids at the 2-position was substituted by the content of 2-glyceryl monopalmitate (IOC 2006b). The method was submitted to IOC chemists, and after collaborative trials, mainly aimedat checking for possible authentic samples not fitting the new limit (Table 16.6) due to a high content of palmitic acid, it was included in the IOC trade standard in 2006 and 1 year later brought into EU Regulation 702/2007 (EC 2007).

16.4.2.3 Diacylglycerols

As was already stated, diacylglycerols (DAGs) are major compounds of the polar fraction in VOOs (1–3 %) and are found as 1,2- and 1,3-isomers. 1,2-DAGs are attributable to TAGs' incomplete biosynthesis (Kennedy pathway), whereas 1,3-DAGs are attributable to TAGs' enzymatic or chemical hydrolysis; in fact, the amount of 1,3-TAGs is higher in cloudy VOOs. Several researchers have analyzed the amount of DAGs to assess the quality of oils in trade based on the fact that the amount of 1,2-DAGs decreases while the amount of 1,3-DAGs increases during

VOO storage (Frega et al. 1993; Pérez-Camino et al. 1996; Conte et al. 1997; Cossignani et al. 2007). The observation that 1,2-DAGs are more represented than 1,3-DAGs in fresh oils, whereas the latter increases in old oils, leads to the adoption of the ratio between 1,2-DAGs and 1,3-DAGs isomers to indicate VOO freshness. Even though the rate of isomerization of 1,2-DAG to 1,3-DAG is influenced by the free acidity of the oil, and thus it depends on the quality of the concerned oil, the ratio of 1,2-DAG to 1,3-DAG remains a good quality parameter, and it is widely applied in trade. Currently, two official methods cover them: DGF Standard Method C-VI 16(06) (DGF 2006) and NGD C 87–05 (NGD 2005). In addition, the ratio has been proposed as a hypothetical marker for the presence of deodorized olive oil in VOO (Gertz and Febig 2006).

Some other approaches have tried to relate DAG isomerization to the illegal treatment of lampante olive oils (e.g., deacidification) that could be applied together with the deodorization of the oil (Serani et al. 2001). The TMP-value (difference between real diacylglycerols and their theoretical content), for example, shall characterize the isomerization time of the 1,2-DAG but it does not give reliable results in practice (Gertz and Fiebig 2005).

16.4.2.4 Fatty Acid Alkyl Esters

Fatty acid alkyl esters (FAAEs) are a family of natural neutral lipids present in olive oils and formed by the esterification of FFAs with low molecular weight alcohols (Pérez-Camino et al. 2002). Inappropriate practices during the olive oil extraction process or the bad quality of the olive fruits increases the formation of FAAEs. Certain conditions are required to allow the synthesis of alkyl esters: the presence of a suitable concentration of FFAs due to the lipolytic activity of endogenous lipase of parasitic origin, the presence of methanol originating from fruit pectin demethylation, and the presence of ethyl alcohol due to fruit sugar fermentation. These conditions usually happen in poor-quality or unhealthy olives that produce lampante (virgin) olive oil.

A mild deodorization that removes volatile compounds, responsible for odor, but does not modify any other oil compounds (e.g., no *trans* isomerization of FAs, no dehydration of sterols) induced fraudsters to add the resulting deodorized olive oil (called deodorato) to VOO. A kind of fraud whose detection is still a challenge for analysts, although it has made much progress in its detection with the combined action of some analytical methods, one of them is based on the determination of alkyl esters (Pérez-Camino et al. 2008).

Collaborative trials led by the IOC Chemists' Working Group allowed the standardization and validation of the method, which was officially adopted as an IOC trade standard (IOC 2010) and later by the EU (EC 2011). Limits (Table 16.7) were established on a twin-track approach: concentrations less than 75 mg/kg or between 75 and 150 mg/kg. In this latter case, the sample must also have a ratio of ethyl esters to methyl esters below 1.5. The latter limit was established on the basis of the fact that good-quality VOOs contain a certain amount of methyl esters, but the content of ethyl esters is at a trace level.

Quantification can be determined by isolation with a silica LC chromatography (as is used for wax analysis) or by a silica gel solid-phase extraction cartridge followed by analysis on a gas chromatograph equipped with an on-column injector or with a programmed temperature vaporizer injector using a short nonpolar capillary column or a polar capillary column.

16.4.2.5 Sterols

If major compounds are useful for detecting some kinds of adulteration, the composition of the minor compounds provides information about the identity of oils, mostly after the genetic improvement of seeds for producing oils. Sterols, which comprise a major portion of the unsaponifiable matter, are the most important of the minor compounds. The ranges of concentration of some of them are characteristic of the genuineness of vegetable oils; for example, rapeseed oils contain significant levels of brassicasterol, while safflower and sunflower seed oils contain high levels of Δ^7-stigmastenol. In the case of olive oil, sterols have a profile of high levels of β-sitosterol and Δ^5-avenasterol and low levels of campesterol and stigmasterol (Table 16.13). These differences in the profiles of the edible oils can be described as the fingerprint of the oil.

These apparent differences in concentration attracted the attention of researchers, who used them not only for determining the botanical origin of an oil, but also for detecting olive oil adulteration with other vegetable oils, mostly thanks to the wide availability of GC-MS instruments that allowed for a reliable identification of the sterolic compounds. GLC analysis of sterols, for example, was dramatically influenced when some polar stationary phases for packed columns (e.g., 100 ft OV-17; OV225) became available as it allowed the separation of the peak called β-Sitosterol into three peaks: β-sitosterol, sitostanol, and Δ^5-avenasterol. This improvement increased as capillary columns were widely adopted. Their power of separation highlighted the presence of many more compounds, namely, campestanol, Δ^7-campesterol, $\Delta^{5,23}$-stigmastadienol, 24-methylencholesterol, clerosterol, and Δ^{24}- stigmastadienol; some of them had already been determined using a TLC/$AgNO_3$ fractionation of sterols obtained by preparative TLC (e.g., 24-methylencholesterol).

Concerning the application of sterols in olive oil authentication, the first results date back to the mid-1980s when Brumley et al. (1985) detected the presence of brassicasterol in olive oil by GC-electron ionization mass spectrometry (GC-EIMS). Grob et al. (1994a) were able to detect adulteration of VOO with seed oils (rapeseed, soybean, sunflower, grape seed) via direct analysis of the sterols by online coupled LC-GC-FID. The content of free brassicasterol would allow detection of the addition of 2 % rapeseed oil to VOO. By increasing the percentage to 10 %, the concentration of free campesterol increases by a factor of 5 and there are also increases in γ-tocopherol and the campesteryl-C_{18}-ester that are almost negligible in olive oil. The addition of 10 % soybean oil to olive oil is easily detected by the concentration of free campesterol and stigmasterol that are increased to at least double,

Table 16.13 Basic composition of sterols determined in several oils, estimated maximum and minimum percentages

Sterolic compounds	Olive	High linoleic safflower	High oleic safflower	Low erucic rapeseed	Sunflower high linoleic	High oleic sunflower	Hazelnut	Soybean
Cholesterol	≤ 0.5	nd–0.7	nd–0.5	nd–1.3	nd–0.7	nd–0.5	0.8	0.2–1.4
Brassicasterol	≤ 0.1	nd–0.4	nd–2.2	nd–13.0	nd.0.2	nd–0.3	<0.15	nd–0.3
Campesterol	≤ 4.0	9.2–13.3	8.9–19.9	24.7–38.6	6.5–13.0	5.0–13.0	3.8–5.6	15.8–24.2
Stigmasterol	≤ Camp	4.5–9.6	2.9–8.9	0.2–1.0	6.0–13.0	4.5–13.0	0.8–1.4	14.9–19.1
β-sitosterol	≥ 93.0	40.2–50.6	40.1–66.9	45.1–57.9	50–70	42.0–70.0	87.0–94.0	47.0–60.0
Δ^5-avenasterol		0.8–4.8	0.2–8.9	2.5–6.6	nd–6.9	1.5–6.9	1.1–5.2	1.5–3.7
Δ^7-stigmastenol	≤ 0.5	13.7–16.4	3.4–16.4	nd–1.3	6.5–24.0	6.5–24.0	0.9–3.7	1.4–5.2
Δ^7-avenasterol		2.2–6.3	nd–8.3	nd–0.8	3.0–7.5	nd–9.0	0.3–1.6	1.0–4.6
Others		0.5–6.4	4.4–11.9	nd–4.2	nd–5.3	3.5–9.5		nd–1.8
Total sterols (ppm)	≥ 1,000	2,100–4,600	2,000–4,100	4,500–11,300	2,400–5,000	1,700–5,200	1,000–2,000	1,800–4,500

Camp campesterol, *nd* not detected

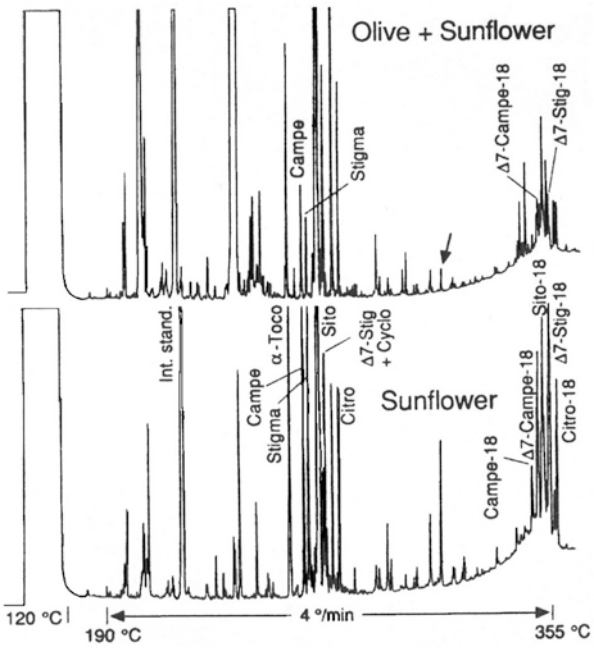

Fig. 16.3 Minor components of sunflower oil as such and as a 10 % admixture to olive oil. Note: Δ^7 stig, Δ^7 stigmastenol, *arrow* presence of a straight-chain C_{41}-ester

along with a substantial content of γ-tocopherol. The addition of 5 % sunflower oil increases the stigmasterol, campesterol, and Δ^7-stigmastenol concentrations significantly above those found in nonadulterated olive oils [also detected by Alonso et al. (1997)] (Fig. 16.3). A 10 % addition of grape seed oil would significantly increase the concentrations of free campesterol and stigmasterol, but an admixture of 5 % would be critical with the methodology suggested because tocotrienols, specific for grape seed oils, are too small to be detected from such a small contamination. Dionisi et al. (1995), however, set up RP-HPLC with amperometric detection that was able to detect the addition of palm and grape seed oils to any tocotrienol-free vegetable oil (e.g., olive oil) at 1–2 %.

The methodologies of the 1990s, however, would not detect admixtures with adulterants whose minor compounds had been removed by a strong refining process, another kind of fraudulent practice that arose then. Mariani et al. (1992) described the removal of free sterols by bleaching at forced conditions; parts of the sterols were absorbed by the earth, whereas others produced olefinic degradation products. Grob et al. (1994b) revealed that numerous edible oils were adulterated with cheaper oils whose sterols had been removed in order to make these admixtures undetectable, although this fraudulent practice was detected by the amount of *trans* unsaturated C_{18}-fatty acids that are produced from normal *cis* acids during the deodorizing process. The investigation then turned toward the analysis of the olefinic sterol degradation products, an aspect that is described in Sect. 16.4.2.6.

Results of tests on sterols were expressed as a percentage of the total area of sterols, but the illegal process of removing sterols without forming FA *trans* isomers suggested giving the information in absolute concentrations. Thus, authentic VOOs usually cannot have less than 1,000 mg/kg sterols. Below this value, it is reasonable to think that olive oil might have been spiked with desterolized seed oils.

More recently, the detection of adulteration with refined hazelnut oil was the target of researchers. One of the most successful proposals, from among the numerous ones with enough of a scientific basis, was based on the quantification of sterols. A double mathematical model based on three free and esterified sterols (campesterol, Δ^7-stigmastenol, and Δ^7-avenasterol) was able to detect the presence of hazelnut oil in olive oil at percentages in a range of 6–8 % after analyzing 116 samples (Mariani et al. 2006; García-González et al. 2007). Another model system, although now based on the sum of campesterol and stigmasterol, was recently proposed for determining the presence of some vegetable oils (corn, soybean, sunflower, and cottonseed) in olive oil (Al-Ismail et al. 2010).

The nonexistence of a database with information from all producer countries when trade standards were implemented as well as the increase in olive-tree-growing areas as far as the latitude and hemisphere of the Mediterranean basin as New Zealand and Argentina have resulted in exceptions to the limits described in Table 16.6 when analyzing the sterolic composition of olive oils from diverse geographical origins and cultivars. Thus, some olive oils from Syria, Palestine, and eastern Greek islands show levels of Δ^7-stigmastenol slightly exceeding the legal limit (0.5 %), while a part of Australian (Mailer et al. 2010) and Argentina (Ceci and Carelli 2007) olive oil productions present higher values for campesterol. This latter situation was already noted in the Cornicabra (Rivera del Alamo et al. 2004) and Corniche (Sánchez-Casas et al. 2004) olive oil varieties, and there are many more known exceptions.

16.4.2.6 Hydrocarbons

The availability of genetically modified seeds with FA composition similar to that of olive oil with the aim of improving their oxidative stability and nutritional characteristics was also used to create frauds. Sterol composition, however, remains an important obstacle to this purpose. Such a complicated structure as the sterol molecule cannot be converted to water and carbon dioxide without destroying oil, so it was simply modified by losing water because of the high temperatures used in the refining process.

Lanzón et al. (1989) were the pioneers suggesting stigmasta-3,5-diene (derived from β-sitosterol) as a marker for detecting admixtures of VOO with refined vegetable oils (Fig. 16.4), whose quantification was later improved (Grob and Bronz 1994). Lanzón et al. (1994) also found that the most notable feature of refined oils was the presence of hydrocarbons such as n-alkanes, n-hexacosadiene, and stigmasta-3,5-diene, isomerization products of squalene, isoprenoidal polyolefins produced from hydroxy derivatives of squalene, and steroidal hydrocarbons derived

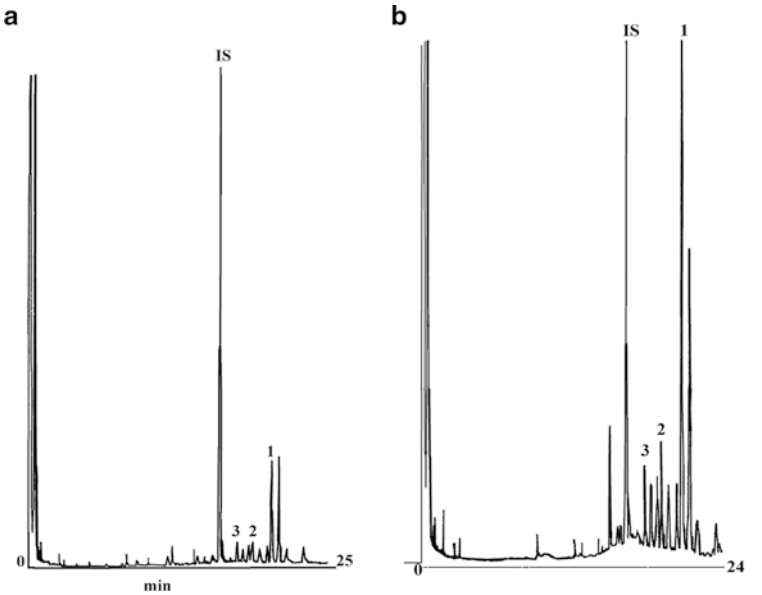

Fig. 16.4 High-resolution gas chromatography of major steradiene compounds in VOO (cv. Picual) spiked with high-oleic sunflower at (**a**) 2 % and (**b**) 10 %. Note: *1* stigmasta-3,5-diene, *2* stigmasta-3,5,22-triene, *3* campesta-3,5-diene

from 24-methylene cycloarthanol. Sterol dehydration products have been used to detect the presence of desterolized rapeseed oils by quantifying campestatriene. The ratio of the degradation products of sitosterol (stigmastadienes) and campesterol (campestadienes) is a good marker for desterolized sunflower, soybean, palm, or grape seed oil (Grob et al. 1994b), whereas the determination of 3,5-cholestadiene, coming from cholesterol dehydroxylation, allows the evaluation of lards deodorized (>230°C) or bleached by activated earths (Mariani et al. 1993). Later, it was also determined that the desterolizing process involves isomerization, thus converting Δ^7-sterol into $\Delta^{8(14)}$-and Δ^{14}-sterols (Bierdermann et al. 1995), which were coeluted with Δ^5-sterols when apolar GC capillary columns were used. Mariani et al. (1995) set up a technique for the separation of $\Delta^{8(14)}$-and Δ^{14}-sterols from Δ^5-sterols making it possible to detect slight additions of desterolized high oleic sunflower oil to olive oil. Later Biedermann et al. (1996) detected desterolized sunflower oil in olive oil through isomerized Δ^7-sterols, and Mariani (1998) showed the presence of ergosterol in some samples of olive oils as a consequence of the presence of molds and yeasts.

The method was almost immediately included in IOC trade standards, and it became an EU official method in 1995. A limit of 0.15 mg/kg was established initially for EVOOs and 0.50 mg/kg for lampante olive oils in 2007. Later, the limit was lowered to 0.10 mg/kg for all VOOs.

The EU regulation includes a footnote pointing out that the limit is for the sum of all the isomers that can (or cannot) be separated by capillary GC. Although 3,5-stigmastadiene is the major peak, the dehydration reaction is not selective for β-sitosterol (its precursor molecule), so it was proposed initially to use the ratio between different sterene isomers to check for the presence of desterolized seed oils in refined olive oil. However, the ratio of dehydration depends on the availability of free hydroxyl groups, and the distribution of single sterols between free and esterified fractions depends on the edible oil (Grob et al. 1992).

As a consequence, the yield of dehydration reaction will not be the same for every sterol, and because of this, the proposal was rejected. In addition, the presence of sterenes with three double bonds depends on the presence of hydroxysterols originating from sterol oxidation (Bortolomeazzi et al. 2000).

The official procedure involves a saponification step followed by LC on a silica gel column to remove interfering compounds such as squalene isomers and n-alkanes. Despite the high separative power of capillary columns, some overlaps can still occur, which may produce false results. Different approaches using offline or online preseparation by HPLC have also been validated (Gallina-Toschi et al. 1996; Amelio et al. 1998; Verleyen et al. 2002).

16.4.2.7 Triterpene Dialcohols and Alcohols

Erythrodiol and uvaol are the triterpene dialcohols found in olive oils. These compounds are mainly present in the fruit skin, so that they are coextracted with oil during mechanical extraction and are much more concentrated in pomace that undergoes solvent extraction. Because of this, erythrodiol and uvaol had been considered as suitable markers to detect the presence of olive-pomace oil in olive oil.

Their analytical determination is carried out together with sterols by scraping their TLC band with the band of sterols. Results are calculated, in percentage, as the ratio between the sum of the areas of erythrodiol + uvaol and the sum of sterols + erythrodiol + uvaol. VOOs must not have a value exceeding 4.5 %; higher values would indicate blending with residue olive oil, although this figure has been discussed by various authors; Albi et al. (1990) showed that certain genuine Spanish VOOs (*cv*. Verdial de Huevar) would be classified as adulterated. According to some researchers, a better result would be obtained by calculating their concentrations in milligrams per kilogram, while others assert that the monoester and diester must be calculated only after the free and esterified sterols have been determined.

Concerning the series of triterpene alcohols, Morchio et al. (1989) found that the ratio between triterpene alcohols and 24-methylene cycloarthanol to cyclobranol allowed for the detection of admixtures of VOOs with refined oil by UV values. Values greater than 160 corresponded to VOOs, while a contamination of only 15 % produced values lower than 100.

16.4.2.8 Aliphatic Alcohols

Aliphatic alcohols, also called alkanols, were adopted as a tool to detect admixtures with olive-pomace oils when it became clear that an illegal technology had been developed. The technology was able to remove erythrodiol and uvaol, perhaps by oxidizing them to the corresponding acids that were then removed by alkaline washing.

Aliphatic alcohols result from the hydrolysis of waxes that takes place in the saponification step, so that the alcohol evaluation is an indirect measure of the amount of waxes. Analyses showed that the concentration of waxes was higher in olive-pomace oil. Therefore, a high concentration of aliphatic alcohols in VOOs is a very good indication of the presence (admixture) of olive-pomace oil. Initially this new method was known as the alcohol index, but later this term was replaced by the term alkanol. In 1991, the EU (EC 1991) proposed a limit of 250 mg/kg as the maximum content of aliphatic alcohols in EVOOs. This could be understood as meaning that a high content of aliphatic alcohols in olive oils indicates the presence of oil extracted with solvents (e.g., olive-pomace oil). This is not always true because there are VOOs with high contents of aliphatic alcohols due to natural causes such as pedoclimatic conditions or infestations (e.g., *dacus oleae*, *prays oleae*). The wide application of the method also highlighted the existence of numerous genuine olive oils (mainly from Greece and southern Italy) that exceeded the proposed limit. Grob et al. (1990) demonstrated that such oils had high concentrations of free alcohols but low concentrations of waxes, which meant that aliphatic alcohols did not originate from wax hydrolysis exclusively. Thus, new EU regulations (EC 1995; 1997) proposed the determination of wax content as an alternative to the quantification of aliphatic alcohols.

16.4.2.9 Waxes

When the reliability of alkanols became weak, because of the considerations given in the previous paragraph, the possibility of detecting cold-pressed virgin oils spiked with solvent extracted oils dropped. The initial method was based on oil fractionation – after the addition of an internal standard on a hydrated silica gel column – and then the determination by capillary GC with an on-column injection port of the peaks corresponding to C40–C46. Because the silica column separation of waxes from TAGs is rather critical, Pérez-Camino et al. (2003) improved the original method by adopting Sudan I as an indicator of the separation, since its chromatographic behavior is intermediate between waxes and TAGs. As the sample preparation is time consuming, HPLC has been suggested as a preparative technique to purify the wax fraction prior to injection (Amelio et al. 1993). Figure 16.5 shows a gas chromatogram of waxes of a VOO sample; it can be noted that some separations are rather critical and some peaks (e.g., phytil behenate) interfere in the peak area measurement.

As in the case of aliphatic alcohols, data have been published about genuine olive oils with the content of waxes exceeding the IOC limit (Ceci and Carelli 2007).

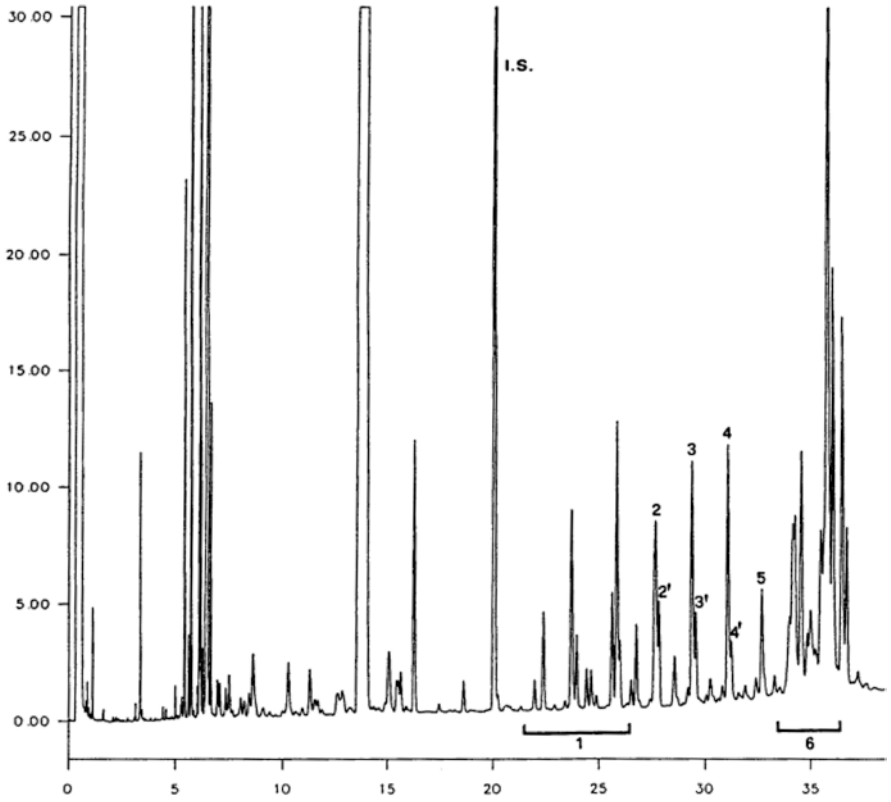

Fig. 16.5 GLC trace of waxes of an EVOO according to Commission Regulation (EU) 61/2011 (EC 2011) and Method COI/T20/Doc No. 18. Note of peak identification: I.S., internal standard (lauryl arachidate); *1* diterpenic esters, *2 + 2'* C40 esters, *3 + 3'* C42 esters, *4 + 4'* C44 esters, *5* C46 esters, *6* sterol esters and triterpene alcohols (*Source*: IOC (2006c), with permission of the International Olive Council)

16.4.2.10 Phenolic Compounds

The typical taste of VOOs, characterized by bitter and pungent (sensory) perceptions, is related to the presence of phenolic compounds. Despite their importance in describing olive oil quality, phenolic substances are not listed among official parameters, although the protocols of many PDO oils determine the minimum concentration of total phenols for characterizing their VOOs.

No harmonized analytical method to measure the content of phenolic substances was available until a few years ago. Today, there are two main approaches, colorimetric evaluation and HPLC analysis (Cortesi and Fedeli 1983; Montedoro et al. 1992; Hrncirik and Fritsche 2004; Carrasco-Pancorbo et al. 2005). Their results, however, do not seem comparable. The colorimetric method, though quicker and cheaper than the HPLC method, has a severe drawback: the lignans cannot be quantified even if a prior fractionation is carried out. Furthermore, the standard used for

calibration in the colorimetric method (caffeic acid or syringic acid or tyrosol) affects the response factor, which can vary up to 1.5 times. Thus, a method based on the quantification of phenols by HPLC was adopted as the official method for the IOC trade standard (IOC 2009).

16.4.3 Other Techniques

Although most of the analytical proposals for the detection of the adulteration of olive oil designations are based on the use of chromatographic techniques and only a small percentage on the application of spectroscopic techniques, researchers have investigated the application of other techniques with the aim of implementing rapid methodologies or of identifying and quantifying hitherto undetectable compounds with the help of sophisticated and recently developed hyphenated techniques. Calorimetry, pyrolysis, and bidimensional chromatography (GC×GC, LC×LC) represent, among others, new frontiers of research in the field of olive oil authenticity.

There are little data in the literature on how to authenticate olive oils through calorimetry techniques. However, differences in the solid–liquid phase transitions of olive oil and seed oils have been known to researchers for many years. Perhaps the problem was in obtaining good reproducible thermograms that take into account the specific characteristics of particular oils. The technique seems to be useful in preliminary stages for quality testing, although it also allows for the detection of the adulterant (approximately 10 % of seed oils or ROOs in VOOs) before more complex and expensive procedures and analyses are applied (Angiuli et al. 2009). Simultaneous thermogravimetry and differential scanning calorimetry analyses are being applied also, though the research is currently focused on identifying deconvoluted peaks related to olive oil chemical composition (TAGs, FAs, and phenols) (Vecchio et al. 2009)

The technique of pyrolysis–isotope ratio mass spectrometry (Py-IRMS) is an analytical fingerprinting technique that, coupled with suitable multivariate data analysis procedures, has been applied in fields where stable isotopic ratios enable the detection of frauds in food like olive oil (Guillou et al. 1999). Little or no sample cleanup is required with this technique, which has the advantage of being a rapid analysis. An example of the usefulness of this technique was already reported by Goodacre et al. (1993), who combined Curie-point PyMS and artificial neural network (ANN) for a rapid assessment of the adulteration of VOOs with refined olive oil and various seed oils (e.g., soybean, sunflower, peanut, and corn).

Hydrogen isotope ratios may be useful for detecting low-level adulterations. Information collected by ^{2}H-EA-Py-IRMS (the precision of ^{2}H measurements is around 3.3‰) has made it possible to detect olive oil samples adulterated with refined hazelnut oils at percentages higher than 10 %. Its main drawback is the lack of D/H certified reference material and geographical origin dependence; more information about analytical techniques for the detection of hazelnut oil in olive oil is available in MEDEO, an EU funded project (Bowadt and Aparicio 2003).

Application of isotopic techniques based on $^{18}O/^{16}O$ ratios has found that the pyrolysis of compounds with low oxygen content creates more problems than expected with conventional instruments. Thus, studies based on $\delta^{18}O$ isotopic abundance in olive oils have also been focused on olive variety and geographical origin; authors have found a correlation between enrichment in heavy isotopes and latitude, whereas no clear-cut effect of altitude has been observed (Aramendia et al. 2007)

$\delta^{13}C$ isotope ratio measurements have been widely used for traceability purposes in some foods (e.g., wine, honey, and juices) but rarely in olive oil authentication. Kelly et al. (1997), although examining other edible oils, found that the values of this ratio by themselves did not allow fraud to be ascertained but provided valuable complementary information. Angerosa et al. (1997) used $\delta^{13}C$ measurements of the aliphatic alcohol fraction to detect adulteration of olive oil with olive-pomace oil at 5 %, although results have not been validated with other samples yet.

Blending of olive oil with edible oils with slightly different FA composition (olive-pomace, sunflower, and hazelnut) can be detected using $\delta^{13}C16:0$ *versus* $\delta^{13}C18:1$ covariations combined with molecular information and carbon isotopic composition of the bulk oil (Ogrinc et al. 2003). However, variability in $\delta^{13}C$ values of FAs has already been related to a combination of environmental conditions (e.g., climate and plant growing condition, including atmospheric carbon dioxide and cultivation practices), geographical origin of the oil, and genetic factors (Woodbury et al. 1998). Therefore, it seems important to establish a database that provides isotopic information for olive oils as a prior step to tackling olive oil authenticity with these kinds of techniques.

Chromatography is, however, the most widely utilized analytical technique because it offers sufficient resolution for a large number of samples and analytical problems, and in addition it has a good capability to separate and yield quantitative information for chemical components in oily mixtures. But the probability of separation of every component is not very high; it has been estimated as ranging from 19 % to 37 % depending on sample complexity. The new sophisticated adulterations seem to require techniques with increased resolution power that is not provided by single-dimension chromatography.

Multidimensional chromatography is a separation technology that utilizes two orthogonal separation mechanisms to increase the resolution power and peak capacity of an experiment by increasing the selectivity of the experiment (Vlaeminck et al. 2007; Cortes et al. 2009). Thus, a versatile system for multistep thermal desorption coupled to GC×GC-TOFMS (column set: VF-5MS×BPX50) has been used for the characterization of fresh *against* aged olive oil at a qualitative level (de Koning et al. 2008), whereas a GC×GC-TOF-MS (column set: BPX5×BPX50) was utilized to determine polycyclic aromatic hydrocarbons (PAHs) from olive oil, diluted with hexane, by means of a Carbopack Z/PDMS fiber (LOQ: 1–5 ppb) (Purcaro et al. 2007). In addition, the analysis of FAs of olive and hazelnut oils (column set: SP-2560×Equity-1) by GC×GG has been of tremendous help in identifying species where mass spectra may be ambiguous (Tranchida et al. 2009a, 2009b). Thus, saturated FAs were eluted along a straight diagonal line, monounsaturates along a separate diagonal line, diunsaturates along another, and so on. GC×GC was

also especially helpful in identifying low-level odd-numbered FAs, according to the authors. Furthermore, esters have been analyzed by comprehensive GC×GC by FID. GC×GC-FID has allowed the clustering of various classes of wax esters, in particular the phytol esters, geranylgeraniol esters, and straight-chain esters of palmitic acids and the unsaturated C18 acids (column set: PS-255×SOP-50) (Biedermann et al. 2008a).

The development of new systems of geographical identification could benefit from the recent advances in so-called ohmic technologies (Montealegre et al. 2010). DNA extracted from olive oil is being used in traceability (Consolandi et al. 2008; Muzzalupo and Perri 2002), although intensive research is required to avoid DNA contamination from other sources and to overcome the difficulties in extracting good-quality DNA (Woolfe and Primrose 2004).

16.5 Current Problems with the Official Methods

With the abundant information on the chemical composition of edible oils produced all over the world that is accessible to everybody over the Internet, fraudsters can easily prepare, with the aid of computer software, fraudulent recipes on the basis of certain olive oils with the values of their chemical composition upper far enough above the minimum or far enough below the maximum limits of the official regulations (Tables 16.6 and 16.7). For example, a sophisticated adulteration composed of 65 % refined olive oil, 15 % refined hazelnut, 15 % desterolized sunflower, and 5 % palm olein without free sterols would have been undetectable at the beginning of this millennium, although the economic benefits of this fraudulent practice did not really yield a profit.

Although an arsenal of analytical techniques is available at the moment, some adulterations are still difficult to detect. Today, for example, there is an available industrial technology that allows the deodorization of VOOs with defects at moderate temperatures (Biedermann et al. 2008b), and the question is whether deodorized oils with mild defects have enough ethyl esters to be detected in a blend with authentic VOOs.

Future problems will be, however, the consequence of near-sighted planning for the elaboration of trade standards and international regulations that have not been mainly founded on a large sample base with olive oils of almost all varieties and producing regions. The result is an large group of varieties and olive tree orchards that produce genuine (virgin) olive oils with chemical compositions that are, in one or more parameters, outside the limits of the current trade standards or European regulations. The exceptions are not only of olive tree orchards placed in latitudes that do not correspond to those of the Mediterranean area but also include varietal olive oils of Mediterranean countries, e.g., *cv.* Verdial in Spain. In the current context, the purpose of olive oil authenticity is not only to detect possible adulterations but to determine that an olive oil is genuine with regard to other authenticity issues, such as geographical origin or botanical variety.

Instrumental advances, as was already stated, have led to greater success in the fight against adulteration, but there is no rapid and universal method that can be used for all authentication purposes. Instead, each authenticity issue can be solved by determining selected chemical parameters by different techniques; Table 16.5 displays the numerous methods currently utilized for the detection of olive oil adulteration.

The growing global market, together with the increasingly stricter international regulations, has increased the number of samples that should be characterized by several analytical parameters. Therefore, there is a growing demand for rapid methods – widely demanded by farmers, dealers, and consumers – since the current alternative seems to be leading to the collapse of official analytical laboratories. However, there is not uniform agreement on a valid definition of the term rapid method due to a lack of conceptual clarity of the adjective rapid. Even though rapid methods are characterized by time, the concept of rapid does not include the condition of a fixed time limit that, incidentally, should include the entire analysis time, from sample pretreatment or sample preparation to data analysis and evaluation of results.

The alternative is the design of a global procedure based on chemical analyses that could be reduced to one or two of all the current methods. This alternative, however, would be confronted with the wide variety of edible oils and their different chemical compositions, which would inevitably come up against the so-called jackknife paradigm. Since this kind of proposal is based on mathematical algorithms designed to detect low concentrations of extraneous oils in olive oil, the result is an unstable system that oscillates between the percentages of false positives and false negatives, which would be the knife side. Thus, each new datum added to the procedure implies rebuilding the mathematical decision rules in an endless process that in most cases makes things worse.

The solution may come from the research on the DNA of edible oils (Bracci et al. 2011), which could achieve unquestionable results in the authenticity of olive oil, but currently the results are on the same order of magnitude as the chromatographic techniques (Kumar et al. 2011). The recent sequencing of the chloroplast genome has provided new information on the olive nucleotide sequence, heralding the olive genomic era. Whereas this technique and its methodologies look for solutions to current problems (Pafundo et al. 2010), the solution may come from databases with chemical information, obtained using chromatographic techniques, which could be used to perfect certain limits of the official parameters or facilitate the calibration of spectroscopic techniques that are neither destructive nor time consuming.

16.6 Selection and Validation of Analytical Methods

Today, many important decisions are based on the test results of chemical analyses. The test results are needed, for example, to assess the oil content of olives or to check and classify olive oil against international specifications and legal regulations. Thus, validation of the official methods is crucial for the quality of results,

and as a consequence, the validation process, though initially perhaps done by the laboratory itself, should be conducted under the auspices of national or international organizations for standardization. Such standards have been evaluated with a properly organized collaborative study.

ISO 5725 (ISO 1994a, b, c, d) uses the terms trueness and precision to describe the accuracy of a measurement method and gives the following definitions. Trueness refers to the closeness of agreement between the arithmetic mean of a large number of test results and the true or accepted reference value, which means that it can be measured only if a reference value is available, e.g., certified reference materials. Precision refers to the closeness of agreement between test results and is usually expressed in terms of repeatability and reproducibility. Precision is not related to the true value. It is necessary to consider precision because tests performed on presumably identical materials in presumably identical circumstances do not give identical results because unavoidable random errors occur in every measurement procedure. There are too many factors that cannot be completely controlled.

16.6.1 Method Validation

In 1987 an IUPAC Workshop was held in Geneva, Switzerland, to discuss and set minimum requirements for a collaborative study. The consensus of this meeting, in which 27 organizations participated, was published as the *Protocol for the Design, Conduct and Interpretation of Collaborative Studies* (Horwitz 1988). This was the starting point of a series of documents dealing with method validation by international collaborative trials. The original guidelines were revised in 1993 and 1994 (Horwitz 1995). These revised and harmonized guidelines have been adopted by AOAC International. Today two documents have been accepted and are used worldwide as validation guidelines. These documents are the *Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis* (AOAC 2002), based on the aforementioned IUPAC guidelines, and ISO 5725:1994, *Accuracy (Trueness and Precision) of Measurement Methods and Results* (ISO 1994a, b, c, d).

An international collaborative trial (CT) is an international interlaboratory study (sometimes called a ring test) in which several laboratories participate and measure a quantity in several identical samples. The aim is the validation of an analytical method, which means that the repeatability (precision under repeatability conditions) and reproducibility (precision under reproducibility conditions) are determined. The term repeatability means the variation s_r^2 between replicate determinations by the same analyst in the same laboratory, with the same apparatus at the same time. It defines how well an analyst can check himself using the same method on (blind) replicates of the same material. The term reproducibility describes the variation s_R^2, which includes the between-laboratory and within-laboratory variations. It measures how well an analyst in a given laboratory can check the results of another analyst in another laboratory using the same method to analyze the same

test material under different conditions, that is, using different apparatus at a different time. The repeatability limit (r) and the reproducibility limit (R) are the values less than or equal to which the absolute difference between two test results obtained under repeatability or reproducibility conditions may be expected with a probability of 95 %.

The CT is organized by a member of the working group that is responsible for the development of the method. This person, the executive officer (EO), should take full responsibility for the trial, supported by the working group.

It is very important that the test samples be homogeneous and stable. Both homogeneity and stability should have been tested before and during the CT. For the selection of the test materials refer to point 6.4 of ISO 5725–1 (ISO 1994a). The minimum number of samples should be five, if possible with different concentrations of the analyte. The minimum recommended number of valid data for each material is eight. Considering this and the fact that not more than two sets of data shall be eliminated, the minimum number of participating laboratories should be ten, but more is better. The larger the number of participating laboratories, the greater is the confidence in the resulting estimates of the statistical parameters. The required number of analyses per test sample is two replicates. Blind replicates are recommended, which means that ten test samples randomly coded are sent to the participants. The laboratories analyze each test sample only once, not knowing which ones of the test samples are the same. Nevertheless, it should also be possible to send five test samples asking the participants to carry out a duplicate determination on each test sample. This procedure will reduce the cost of a CT. More replicates provide additional information only on individual within-laboratory variability, which is usually the less important component of error. It is more effective to increase the number of levels or materials for the analysis rather than to increase the number of replicates for individual materials.

The participating laboratories shall be neither specialized laboratories nor inexperienced laboratories (6.3 of ISO 5725–1) (ISO 1994a). In the first case, the precision data for the tested method will be too good and in the second case too poor. Therefore, it is recommended that, depending on the degree of difficulty of the method, the collaborative be preceded by a pretrial in which participating laboratories must demonstrate that they are sufficiently familiar with the methodology. For such a pretrial, the EO sends one sample with known and two samples with unknown content. Sometimes the pretrial shows that the protocol of the method or the method itself is not yet good enough and must be improved before the real CT.

The EO sends the test samples, pretreated if necessary, to the participating laboratories together with the test method, a questionnaire according to Fig. 16.6, a report sheet, and all necessary instructions, e.g., the storage and handling of the test samples, and a deadline for sending in the results (see 5.2 of ISO 5725–2) (ISO 1994b).

The report sheet – usually an Excel spreadsheet – shall indicate the number of significant figures to be reported depending on the methodology and instrumentation. In many cases, it is easier to ask for a number of decimal places, as the number of significant figures is not unambiguous in all cases. A good way to achieve this is to use a preset Excel datasheet. The participants are also asked to report any difficulties and, in particular, any deviation from the protocol of the method.

Questionnaire for interlaboratory study

Title of the interlaboratory study: _____

1. *Our laboratory is willing to participate in this interlaboratory study to determine the precision data for this standard measurement method.*
2. *As a participant, we confirm that all the apparatuses, chemicals and other equipments necessary for the method are available and checked for their function.*
3. *The testing method will be strictly followed.*
4. *The samples will be treated in accordance with the given instructions.*
5. *A skilled and qualified operator will perform the measurements.*
6. *Comments.*

*Signed:*_____ *Laboratory:* _____

Fig. 16.6 Questionnaire for interlaboratory study following ISO 5725–2:1994

In any set of results, there will be one or even more results that seem to fall outside a normal distribution. These results are statistically referred to as outliers. The analysis of the data is a statistical problem and is best carried out by a statistical expert. After having obtained all the results, the EO should make a critical examination of the data in order to identify and treat outliers or other irregularities. This should be done by a first plot of the collaborative study results (results versus laboratory number). Usually major discrepancies will be apparent for different reasons, e.g., displaced means, unduly spread replicates, outlying values, and even differences in the methods used. Furthermore, ISO 5725–2 (ISO 1994b) recommends in point 7.3.1 Mandel's plot of h and k test statistics. The first is the ratio of the difference between the mean for a particular set of data and the mean of all sets of data to the standard deviation of the means from all the sets of data. The latter is the quotient of the standard deviation of results and the mean or pooled standard deviation. The calculated quotients are then plotted and compared with tabulated ratio values obtained at 95 % and 99 % confidence levels. From these plots, results that differ from the expected distribution can be identified.

Sometimes a laboratory may have carried out and reported more than the n test results officially specified. If the laboratory has stated that all results are equally valid, then a random selection should be made to choose the required number of test results for analysis. In other cases, some of the test results may be missing or the laboratory has reported only one result per sample in nonreplicate analyses. If so, the results from this laboratory will not be taken into consideration.

Only valid data are included in the statistical analysis. Valid data are values that for various reasons are considered not to be wrong by the EO. As a rule, collaborative studies show an inherent level of outliers, the number depending on the definition of outliers and the basis for calculation (analytes, materials, laboratories, or determinations). As was already mentioned, a rejection of more than two out of nine of the

data from each material in a study is not allowed, and the study must include valid data from a minimum of eight laboratories. For larger studies, a smaller acceptable percentage of rejections may be more appropriate.

According to part 2 of ISO 5725 (ISO 1994b), the repeatability and reproducibility standard deviations are to be estimated. First, it is necessary to check the presence of individual laboratories or values that appear to be inconsistent with all other laboratories or values. For this task ISO 5725 recommends a graphical consistency technique (see above) and numerical outlier tests (7.3.2 of ISO 5725–2) (ISO 1994b). For the numerical technique the following practice is recommended: Cochran's test (7.3.3 of ISO 5725–2) (ISO 1994b) and Grubbs' test (7.3.4 of ISO 5725–2) (ISO 1994b) are applied to identify stragglers and outliers, which are defined as follows:

- If the test statistic is less than or equal to its 5 % critical value, then the item tested is accepted as correct.
- If the test statistic is greater than its 5 % critical value and less than or equal to its 1 % critical value, then the item tested is called a straggler and is indicated by a single asterisk.
- If the test statistic is greater than its 1 % critical value, then the item is called a statistical outlier and is indicated by a double asterisk.

16.6.1.1 Cochran Test

It is assumed that between laboratories, only small differences exist in the within-laboratory variances. For the removal of laboratories with extreme individual values from a set of laboratory values Cochran's test is applied by computing the within-laboratory variance for each laboratory and dividing the largest of these by the sum of all of these variances. The resulting quotient is the Cochran statistic, which indicates the presence of a removable straggler or outlier if this quotient exceeds the critical value listed in the Cochran tables for 5 % or 1 % (straggler or outlier) and p (number of laboratories):

$$c = \frac{s^2_{\max}}{\sum_{i=1}^{p} s^2_i}.$$

Cochran's criterion applies strictly only when all the standard deviations are derived from the same number (n) of test results obtained under repeatability conditions. This number may vary due to missing or discarded data. However, ISO 5725–2 (ISO 1994b) assumes that in a properly organized experiment such variations in the number of test results per cell will be limited and can be ignored. Cochran's criterion tests only the highest (not lowest!) value in a set of standard deviations and is therefore a one-sided (one-tail) outlier test. However, small values of standard deviation may be very strongly influenced by the degree of rounding of the original

data and are for that reason not very reliable. In addition, it seems unreasonable to reject the data from a laboratory because it shows a better precision in its test results than the other laboratories. If the highest standard deviation is classed as an outlier, then the value should be omitted and Cochran's test repeated on the remaining values. This process is repeated as long as outliers are detected. But it may lead to excessive rejections when, as is sometimes the case, the underlying assumption of normality is not sufficiently well reached. The repeated application of Cochran's test is only a helpful tool in view of the lack of a statistical test designed for testing several outliers together.

16.6.1.2 Grubbs' Test

To compute whether the mean values of the single test results differ, Grubbs' I and II tests of the arithmetic means are applied. Both Grubbs tests mark those laboratories that are suspected as stragglers (significance level of 5 %) or outliers (significance level of 1 %). For the Grubbs I test the means of all laboratories are arranged in ascending order, then it is determined whether the largest observation

$$G_p = \frac{(x_p - \overline{x})}{s}$$

or the smallest observation is a straggler/outlier:

$$G_p = \frac{(\overline{x} - x_1)}{s}.$$

The Grubbs II test determines whether the two largest observations

$$G = \frac{s^2_{p-1}}{s^2_0}$$

or the two smallest observations are stragglers/outliers:

$$G = \frac{s^2_{1,2}}{s^2_0}.$$

The Grubbs I test is applied for the lowest and highest values only. If the highest value is an outlier, then the test is repeated for the lowest value. Only if the Grubbs I test does not compute an outlier is the Grubbs II test applied.

If the single-value Grubbs test signals an outlier, then the value is removed. After this elimination one recycles back to the Cochran test, as shown in the flow chart in Fig. 16.7.

If the single-value Grubbs test is negative, then the pair-value Grubbs test is performed. If this second test is positive, then both values responsible for activating the test are eliminated and the loop is restarted with the Cochran test (Fig. 16.7),

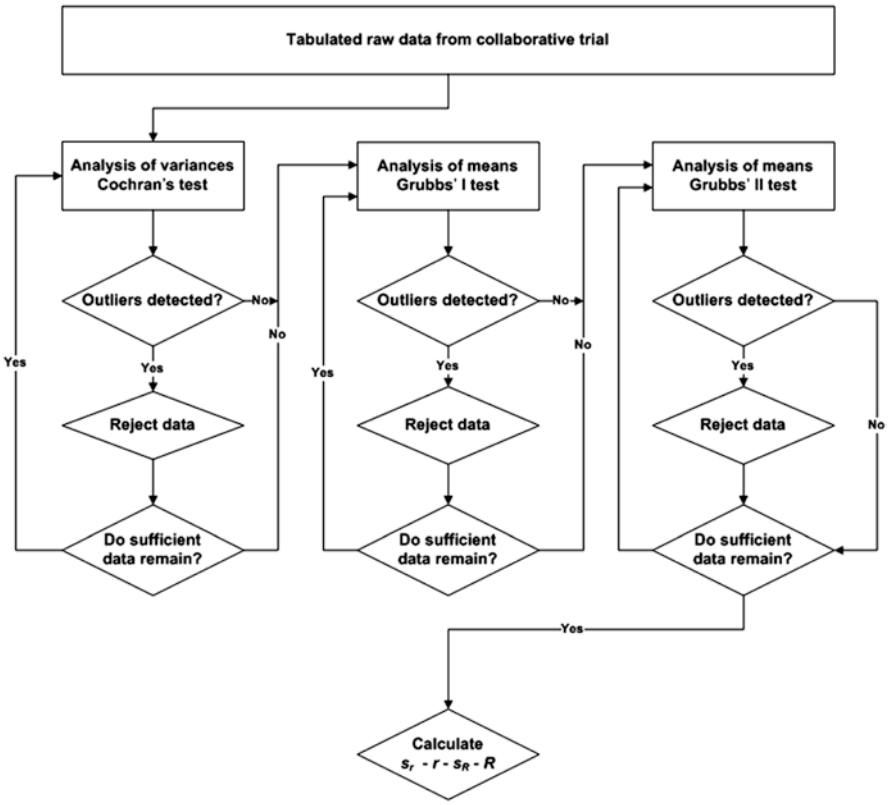

Fig. 16.7 Schematic flow chart for statistical treatment of outliers

single-value Grubbs, and pair-value Grubbs test. However, the removal of outliers shall stop before more than two of nine laboratories are removed.

If no outliers are removed for a given cycle (Cochran, single Grubbs, pair Grubbs), the outlier removal is complete. With a removal rate of greater than two out of nine, either the precision parameters must be without removal of all outliers or the method must be considered suspect. Nevertheless, the decision to remove outliers must be made by the EO on the basis of the indicated probability given by the outlier test and any other pertinent information.

16.6.1.3 Precision

Precision is defined as the closeness of agreement between independent test results obtained under stipulated conditions. Precision depends only on the distribution of random errors and does not relate to the true value or to a specified value. The precision of analytical methods is usually characterized for the within-laboratory replication or repeatability and between-laboratory replication or reproducibility. The measure of precision is usually expressed in terms of imprecision and computed

as a standard deviation of the test results. Less precision is reflected by a larger standard deviation. It must be considered that repeatability and reproducibility conditions are particular sets of extreme conditions. Repeatability is a measure of how well an analyst in a given laboratory can check her performance using the same analytical method to analyze the same test sample at the same time. Repeatability conditions means repeat execution of the complete method from the point at which the test portion is taken from the laboratory sample, and not just repeat instrumental determinations on prepared extracts.

Reproducibility is a measure of how well an analyst in one laboratory can check the results of another analyst in another laboratory using the same analytical method to analyze the same test sample at the same or a different time.

However, intermediate conditions between these two extreme conditions are also imaginable. This means that one or more factors within a laboratory (intralaboratory, e.g., the operator, the equipment used, the calibration of the equipment used, the environment, the batch of reagent, and the elapsed time between measurements) are allowed to vary. ISO 3534–2 (ISO 2006b) defines intermediate precision conditions as conditions where test results or measurement results are obtained with the same method, on identical test/measurement items in the same test or measurement facility, under different operating conditions. According to part 3 of ISO 5725 (ISO 1994c), there are four intermediate precision measures due to the change in observation conditions, which are time, calibration, operator, and equipment.

Finally the general mean and the variances are calculated from the outlier-free data following point 7.4 of ISO 5725–2 (ISO 1994b):

For level j, the general mean is

$$\overline{\overline{y}}_j = \frac{\sum_{i=1}^{p} n_{ij}\,\overline{y}_{ij}}{\sum_{i=1}^{p} n_{ij}}.$$

The repeatability variance is

$$s_{rj}^2 = \frac{\sum_{i=1}^{p} (n_{ij}-1)s_{ij}^2}{\sum_{i=1}^{p} (n_{ij}-1)}.$$

The between-laboratory variance is

$$s_{Lj}^2 = \frac{s_{dj}^2 - s_{rj}^2}{\overline{\overline{n}}_j}.$$

The reproducibility variance is

$$s_{Rj}^2 = s_{rj}^2 + s_{Lj}^2.$$

Results of interlaboratory tests

The precision of the method is the result of an interlaboratory study on an international basis. The interlaboratory test gave the statistical results, evaluated in accordance with ISO 5725:1994, given in Tables 1 to ……..

Table 1.- Summary of statistical results for free fatty acid content in Extra Virgin Olive Oil (A) and Lampante Olive Oil (expressed as a percentage mass fraction)

Sample	**A**	**B**
Number of participating laboratories, *N*	39	28
Number of laboratories retained after eliminating outliers, *n*	37	26
Number of individual test results in all laboratories	74	52
Mean, *m*	**0.343**	**3.80**
Repeatability standard deviation, s_r	0.007	0.03
Repeatability relative standard deviation, *RSD(r)*	1.9	0.8
Repeatability limit, *r* (s_r * 2,8)	**0.018**	**0.07**
Reproducibility standard deviation, s_R	0.019	0.12
Reproducibility relative standard deviation, *RSD(R)*	5.5	3.2
Reproducibility limit, *R* (s_R *2,8)	**0.053**	**0.33**
HorRat Value	1.2	1.0

Fig. 16.8 Presentation of precision data of a collaborative trial

16.6.1.4 Repeatability Limit and Reproducibility Limit

Using the measuring series with statistically homogeneous mean values and taking into account the validity of the remaining laboratories the repeatability standard deviation (s_r) and the reproducibility standard deviation (s_R) are calculated. From s_r and s_R the repeatability and the reproducibility limit are calculated as follows (4.1.4 of ISO 5725–6) (ISO 1994d):

Repeatability limit:	$r = 2.8\ s_r$,
Reproducibility limit:	$R = 2.8\ s_R$.

These relationships are valid for a probability level of 95 %. If different probability levels are required, the factor 2.8 must be replaced by 2.3 for 90 % or by 3.65 for 99 %. The result of a CT shall be summarized as shown in Fig. 16.8 (Table 1).

The calculated statistical quantities (mean, repeatability and reproducibility standard deviation, repeatability and reproducibility limit) shall be indicated using one decimal place more as the test results and in the same units (Fig. 16.8). It is also recommended to quote the HorRat value.

16.6.1.5 HorRat Value

The results of a CT – repeatability and reproducibility standard deviations – are taken as measures of the performance of the tested analytical method. Horwitz et al. (1980) analyzed the results of thousands of CTs and noticed a pattern in the relative standard deviations. At 100 % concentration of analyte the RSDR was approximately 2 %, at 1 % the RSDR was approximately 4 %, and at 0.01 % (100 ppm) the RSDR was approximately 8 %. This pattern persisted at least down to sub-ppm levels (μg/kg).

Table 16.14 Acceptable predicted and target values in accordance with concentration of analyte (%)

Analyte (%)	Predicted RSD(R) (%)	Target RSD(r) (%)
100	2	1
1	4	2
0.01	8	4
1×10^{-6}	16	8
10×10^{-9}	32	16
1×10^{-9}	45	22

These findings gave rise to the famous Horwitz trumpet, which shows this relationship. And surprisingly enough the results of CTs obey this law. The reproducibility relative standard deviation calculated from the Horwitz formula is

$$PRSD(R) = 2 * c^{-0.15},$$

where c is the concentration found or added, expressed as a mass fraction. For calculating HorRat values (HorRat) the data must be reported as a mass fraction where the units of the numerator and denominator are the same. For 100 % the mass fraction is 1.00, for 1 μg/g it is 0.000001. Today, the HorRat value is an important acceptability criterion for CTs and has been adopted by many organizations. According to AOAC, the following guidelines shall be used for the evaluation:

- HorRat ≤ 0.5: method reproducibility may be in question due to lack of study independence, unreported averaging, or consultations.
- HorRat 0.5–1.5: method reproducibility as would normally be expected.
- HorRat > 1.5: method reproducibility higher than normally expected; reasons will be examined (e.g., sample homogeneity).
- HorRat > 2.0: method reproducibility is problematic. A high HorRat may result in the rejection of a method because it may indicate unacceptable weaknesses in the method or the study.

The corresponding repeatability standard deviation *PRSD(r)* typically is one-half to two-thirds of the *PRSD(R)* (Table 16.14).

16.7 Accuracy of Measurement Methods and Results

16.7.1 Use in Practice of Accuracy Values

Part 6 of ISO 5725 (ISO 1994d) gives some indications of the way in which accuracy data can be used in various practical situations and provides a way of checking the acceptability of analytical results obtained under repeatability or reproducibility conditions. Analysis of certified reference materials (CRMs) should be used for this purpose. A CRM is a reference material accompanied by documentation issued by an authoritative body and providing one or more specified property values with associated uncertainties and traceability using valid procedures. Documentation is given in the form of a certificate. Procedures for the production and certification of

certified reference materials are given, for example, in ISO Guide 34 (ISO 2009) and ISO Guide 35 (ISO 2006a). Laboratories should use CRMs to check whether the laboratory standard deviation s_i fulfills the repeatability standard deviation s_r of the standard measurement method.

As mentioned previously, the precision (r and R) of a standard measurement method can be used to obtain a measurement uncertainty through the estimation of the so-called critical differences. If the difference between two analytical results or a limit value is greater than the critical differences as calculated below, then it may be assumed that the sample in question does not fulfill any statutory or contractual requirements. ISO 5725 uses CD_P as the abbreviation for the critical difference at probability P, but very often CrD_P is used. The calculation of critical differences can also be understood as a definition of the measurement uncertainty. Examples of different situations follow.

16.7.1.1 Comparison of Test Results in One Laboratory

Two test results (x_1 and x_2) are performed for each sample under repeatability conditions (4.2.1 of ISO 5725–6) (ISO 1994d). If the difference $|x_1–x_2|$ is less than the repeatability limit r of the method, the mean of these two results is quoted as the final result. If the absolute difference does exceed r, then the result is considered suspicious and two further results are required. The range ($x_{max}–x_{min}$) of all four test results should be equal to or less than the critical range at the 95 % probability level for $n = 4$ [$CR_{0.95}(4)$]. In this case, the arithmetic mean of the four test results is reported as the final quoted result. Critical range factors $CD_{0.95}(n) = f(n)s_r$ are taken from the corresponding table (ISO 5725–6, 5.2.2.2) (ISO 1994d) and are used to calculate the critical range according to the following equation:

$$CD_{0.95}(n) = f(n) \cdot s_r.$$

Example of four test results:

x_1	x_2	x_3	x_4	n	s_r	$x_{max}–x_{min}$	
10.0	9.2	9.0	8.6	4	0.5	10.0–8.6	**1.4**

$$CD_{0.95}(4) = 3.6 \cdot s_r \qquad CD_{0.95}(4) = 3.6 \cdot 0.5 \qquad CD_{0.95}(4) = 1.8$$

Because **1.4** is smaller than 1.8 the final quoted result is the mean $\frac{10.2 + 9.2 + 9.0 + 8.6}{4} = 9.2$ of the four test results, otherwise the median is reported.

The results may also be the calculated arithmetic mean of $\overline{y_1}$ and $\overline{y_2}$ from two groups of measurements n_1 and n_2 in one laboratory. The standard deviation s of $\left(\overline{y_1} - \overline{y_2}\right)$ is

$$s = \sqrt{s_r^2 \left(\frac{1}{n_1} + \frac{1}{n_2} \right)}$$

and the critical difference CD at the 95 % probability level for $\left(\overline{y_1} - \overline{y_2}\right)$ is

$$CD_{0.95} = 2.8 s_r \sqrt{\left(\frac{1}{2n_1} + \frac{1}{2n_2}\right)},$$

$$CD_{0.95} = r \sqrt{\left(\frac{1}{2n_1} + \frac{1}{2n_2}\right)}.$$

If n_1 and n_2 are both 1, the result is reduced to $CD_{0.95} = 2.8\ s_r = r$.

16.7.1.2 Comparison of Test Results from Two Laboratories

The first laboratory obtains n_1 test results with an arithmetic mean of $\overline{y_1}$ and the second laboratory n_2 test results with an arithmetic mean of $\overline{y_2}$; both laboratories are working under repeatability conditions. The critical difference $CD_{0.95}$ at the 95 % probability level for $\left(\overline{y_1} - y_2\right)$ is then (4.2.2 of ISO 5725–6:1994):

$$CD_{0.95} = \sqrt{R^2 - r^2 \left(1 - \frac{1}{2n_1} + \frac{1}{2n_2}\right)}.$$

If n_1 and n_2 are both 1, then the result is reduced to $CD_{0.95} = 2.8\ s_R = R$.

If the critical difference is greater than R, then it must be proved whether this is caused by poor precision during the analyses or by an inhomogeneity of the test sample.

16.7.1.3 Comparison of Mean of One Laboratory with Reference Value

Within one laboratory, n test results are obtained under repeatability conditions that give an arithmetic mean of $\overline{y}$. This mean shall be compared with a given reference value μ_0. For a two-sided problem, which means exceeding or falling below a minimum value (4.2.3 of ISO 5725–6) (ISO 1994d), the critical difference ($\overline{y} - \mu_0$) between the mean and the reference value is

$$CD_{0.95} = \frac{100}{\sqrt{2}} \cdot \sqrt{R^2 - r^2 \left(\frac{n-1}{n}\right)}.$$

16.7.1.4 Comparison of Mean of Two Laboratories with Reference Value

In p laboratories, n_i test results with arithmetic means of $\overline{y_i}$ are obtained under repeatability conditions (4.2.4 of ISO 5725–6) (ISO 1994d). The grand mean $\overline{\overline{y}}$ is calculated by

$$\overline{\overline{y}} = \frac{1}{p} \sum \overline{y_i}.$$

The grand mean is compared with a reference value μ_0. For a two-sided problem (exceeding or falling below a reference value), the critical difference ($\bar{\bar{y}} - \mu_0$) between the grand mean $\bar{\bar{y}}$ and the reference value μ_0 is

$$CD = \frac{100}{\sqrt{2}} \sqrt{R^2 - r^2 \left(1 - \frac{1}{2n_1} - \frac{1}{2n_2}\right)}.$$

16.7.2 Other Validation Characteristics

The following definitions follow the Codex Committee on Methods of Analysis and Sampling (CCMAS) recommendations of 2009 (CAC 2009b).

16.7.2.1 Selectivity

Selectivity is the degree to which a method can detect a particular analyte in a mixture or matrix without interference from other components. Selectivity can be graded. CCMAS recommends not using the term *specificity* for the same concept as this often leads to confusion.

16.7.2.2 Sensitivity

Sensitivity is the quotient of the change in the indication of a measuring system and the corresponding change in the value of the quantity being measured. Sensitivity can depend on the value of the quantity being measured. The change considered in the value of the quantity being measured must be large compared with the resolution of the measurement system.

16.7.2.3 Robustness (Ruggedness)

Robustness is a measure of the ability of an analytical procedure to remain unaffected by small but intentional variations in the parameters of a method. The term provides an indication of its reliability during normal usage.

16.7.2.4 Linearity

Linearity is the ability of a method of analysis, within a certain working range, to provide an instrumental response or results proportional to the quantity of analyte to be detected in a laboratory sample. This proportionality is expressed by an a priori

defined mathematical expression. The linearity limits are the experimental limits of concentrations between which a linear calibration model can be applied with an acceptable uncertainty.

16.7.2.5 Detection Limit/Limit of Detection

The limit of detection (LOD) is the concentration or amount of analyte in a sample for which the probability of falsely claiming the absence of a component in a material is β and which will lead to the conclusion that the concentration or amount of the analyte in the analyzed material is larger than that in the blank. IUPAC recommends default values for α and β equal to 0.05. An α error, or a false positive, means the error of rejecting a null hypothesis when it is actually true. A β error, or a false negative, is the error of failing to reject a null hypothesis when in fact it should have been rejected.

The LOD, expressed as the concentration (c_L) or the quantity (q_L), is derived from the smallest measure (x_L) that can be detected with reasonable certainty for a given analytical procedure (IUPAC 1997, 2006). The value of x_L is given by the equation

$$x_L = \overline{x}_B + k \cdot s_B,$$

where x_B is the mean of the blank measures, s_B is the standard deviation of the blank measures, and k is a numerical factor chosen according to the desired confidence level. The LOD is estimated as the mean of 20 control sample (from at least 6 separate sources) assay results plus 3 times the standard deviation of the mean. The abbreviation LOD can be used; the term sensitivity shall not be used for detection limit.

16.7.2.6 Limit of Quantification

The limit of quantification (LOQ) is the smallest measured content from which a quantification of an analyte is possible with a specified degree of accuracy within one laboratory. The LOQ is calculated by adding the tenfold amount of the residue standard deviation to the blank mean value. The LOQ then becomes the mean of the same results plus 6 or 10 times the standard deviation of the mean.

16.8 Measurement of Uncertainty

16.8.1 Uncertainty

Knowledge of the uncertainty of measurement results is essential for the interpretation of the results. Without quantitative assessments of uncertainty, it is impossible to decide whether observed differences between results reflect more than experimental

variability, whether test items comply with specifications, or whether laws based on limits have been broken. Without information on uncertainty, there is a risk of misinterpretation of results. Measurement uncertainty relates to individual results not to a method. The CAC (2007) recommended in 2007 that the best way to define the uncertainty estimate is to base it on interlaboratory studies (CT) utilizing the Harmonized IUPAC/AOAC or ISO 5725 protocol. This approach is considered acceptable by both the Eurachem guide (Ellison et al. 2000) and ISO TS 21748 (ISO 2010). Furthermore, CCMAS explains: "By utilising a sample of presumably typical laboratories operating in different environments on at least five materials covering the range of interest, it is very likely that most of the potential error factors that are likely to be encountered in practice will have been introduced. Therefore, if we equate this s_R to measurement uncertainty and call it standard measurement uncertainty (standard uncertainty for short), we are at least about 70 % certain that our result plus and minus s_R will encompass the true value. If we multiply s_R by a coverage factor of 2, we obtain the expanded measurement uncertainty (expanded uncertainty for short); we are now at least 95 % certain that our result plus and minus $2s_R$ will encompass the true value."

Repeatability, reproducibility, and bias relate to the performance of a measurement or testing process. For studies under all parts of ISO 5725, the measurement or testing process will be a single measurement method used by all laboratories taking part in the study.

Bias means the difference between the expectation of the test results and an accepted reference value.

Combined standard uncertainty *u(y)* is the standard uncertainty of the result of a measurement when that result is obtained from the values of a number of other quantities, equal to the positive square root of a sum of terms, the terms being the variances or covariances of these other quantities weighted according to how the measurement result varies with changes in these quantities.

Coverage factor *k* is a numerical factor used as a multiplier of the combined standard uncertainty to obtain an expanded uncertainty; typical values for *k* are in the range of 2–3.

Expanded uncertainty *U* is the quantity defining an interval about a result of a measurement expected to encompass a large fraction of the distribution of values that could reasonably be attributed to the measurand.

Precision is the closeness of agreement between independent test results obtained under stipulated conditions. Precision depends upon the distribution of random errors and does not relate to the true value or the specified value. The measure of precision is usually expressed in terms of imprecision and computed as a standard deviation of the test results. Less precision is reflected by a higher standard deviation. Repeatability and reproducibility conditions are particular examples of extreme stipulated conditions.

Standard uncertainty $u(x_i)$ is the uncertainty of the result of a measurement expressed as a standard deviation.

16.9 Accreditation of Laboratories

The general requirements for the competence of laboratories to carry out analytical tests, calibrations, and sampling are specified in ISO 17025 (ISO 2005a). This international standard should be used by laboratories to develop their quality, administrative, and technical systems, but it is not the basis for certification of laboratories. This standard should also be used by accreditation bodies responsible for the authorization of the competence of test and calibration laboratories.

ISO 17025 (ISO 2005) covers testing and calibration on the basis of standard methods, nonstandard methods, and laboratory-developed methods. This standard is applicable to all organizations performing tests and calibrations. This includes, for example, first- (supplier), second- (customer), and third-party (independent) laboratories where testing and calibration are part of inspection and product certification. The application of ISO 17025 does not depend on the number of personnel or the extent of the testing and calibrating activities. By using this standard, laboratories will be able to develop their administrative and technical systems to ensure quality.

ISO 9001 (ISO 2008) was the first basic management standard and can be applied to any kind of management, e.g., business enterprises, public administrations, or government departments. The growing need for such management systems has likewise increased the need to ensure that laboratories can operate according to a quality management system that complies with ISO. With ISO 17025, all the ISO 9001 requirements that are relevant to the scope of testing and calibration laboratories have been laid down in a separate standard. Therefore, testing and calibration laboratories that comply with ISO 17025 operate in accordance with ISO 9001. Certification only under ISO 9001 (ISO 9000 family) does not demonstrate the competence of a laboratory to produce technically valid data and results. Laboratories are therefore accredited under ISO 17025, rather than certified under the ISO 9000 series (ISO 2005b).

The two main sections of ISO 17025 (ISO 2005a) are Management Requirements and Technical Requirements. Management requirements are primarily related to the operation and effectiveness of the quality management system within the laboratory. Technical requirements include factors that determine the correctness and reliability of the tests and calibrations performed in the laboratory. ISO 17025 addresses the need for internal audits in a 1-year cycle. The reports on these audits are also useful in the audits of laboratories by their clients.

In summary, today analysts must be aware of the important role of quality assurance in their laboratories. Due to the worldwide accreditation system comprising internal and external audits, it is important to know and understand most of the described statistical parameters. A customer who has to pay for analytical work expects that the results he receives are the most accurate achievable. In the past, we were often faced with new analytical methods – because of a sudden need, as happened with methods for Acrylamid, 3-MCPD, and furan determinations, where no standardized methods were available initially. Customers cannot rely on the results of such determinations due to a lack of standardized methods. The results of different (in-house) methods obtained in various laboratories are not comparable if

standardized methods are not used. Confidence in the laboratory results are directly related to the use of validated methods and other procedures laid down in various ISO standards and CAC reports. Progress in all these areas continues.

16.10 Future Trends and Perspectives

Food authentication is continuously evolving to include situations that are basically governed by a global market trend. Analytical techniques should be developed or modified to give plausible solutions to future demands, and this explains why, from time to time, EU regulations and IOC trade standards are revised and upgraded in light of new advances in analytical methods or challenges created by fraudsters. Sometimes, however, the potential problems lie in the semantic definition of authenticity rather than in devious adulterations.

Mutants or genetically modified seed oils, for example, represent a challenge in oil authenticity in general, and with olive oil in particular. It is well known that FA composition determines the physical and chemical properties of the oil and their potential applications, and the food industry is offering tailored oils for specific purposes that represent a problem, not only for detecting contamination or fraud, but also for the global meaning of authenticity.

Upcoming problems do not seem, however, to be focused on the authenticity of olive oils added to canned fish or inside bottles labeled as spiced VOOs, or even in the presence of deodorized oil in VOO, but in the geographical origin of olive oil and the resulting overlap between quality and authenticity.

Today the production and consumption of olive oil are moving slowly but inexorably beyond the Mediterranean countries, and olive trees are being planted in countries as far from the Mediterranean basin as New Zealand and Argentina. Research on agricultural practices has allowed the development of high-density plantations of alien cultivars in geographical locations where the cultivation of olive trees was once inconceivable. This revolution in agricultural techniques is not, however, exempt from challenges and even problems from the chemical viewpoint.

While orchards were planted with autochthonous varieties within the ranges of latitude of the Mediterranean basin, there were few varietal olive oils with a chemical composition outside limits of trade standards. Today, cultivars whose chemical composition is within the limits of trade standards, if cultivated in the traditional producer countries, show a very different composition if grown in some of the new producer countries, to the point that they could be classified as nongenuine. The latitude, altitude, and climate of the new producing areas have played a dirty trick on farmers because those physical parameters affect biochemical cascades of some cultivars more than would be expected. The solution can come from decision trees that, using other chemical compounds, can act as a safeguard for the genuineness of those olive oils without modifying the current limits.

Successful control of the adulteration of olive oils by means of certain analytes (e.g., stigmastadienes) encouraged some researchers to believe that a strong mathematical relationship between two factors could prevail over the lack of scientific

evidence explaining whether that relationship was causal. The inappropriate application of statistical procedures, most of the time without the required validation tests, opens the door to overoptimistic conclusions that usually present umpteen exceptions in the validation step. New trade standards should be analyzed under a magnifying glass to prevent a casual relationship between chemical compounds and authenticity from being seen as causal. The paradigm is the implication of certain chemical compounds that, being related to temperature increases, are used to explain the natural oxidation process of lipids. The casual relationship between the increase in pyropheophytins (PPPs) with shelf life and the causal relationship between shelf life and organoleptic quality (rancid perception increases with time) is used to relate PPPs to sensory quality when there is currently no any scientific hypothesis that relates the concentration of PPPs to VOO flavor.

References

Albi T, Lanzón A, Cert A, Aparicio R (1990) Values of erythrodiol in samples of Andalusian virgin olive oils. Grasas Aceites 41:167–170

Alexa E, Dragomirescu A, Pop G, Jianu C, Dragoş D (2009) The use of FT-IR spectroscopy in the identification of vegetable oils adulteration. J Food Agric Environ 7:20–24

Al-Ismail KM, Alsaed AK, Ahmad R (2010) Detection of olive oil adulteration with some plant oils by GLC analysis of sterols using polar column. Food Chem 121:1255–1259

Alonso L, Fontecha J, Lozada L, Juarez M (1997) Determination of mixtures in vegetable oils and milk fat by analysis of sterol fraction by gas chromatography. J Am Oil Chem Soc 74:131–135

Amelio M, Rizzo R, Varazini F (1993) Separation of wax esters from olive oils by high-performance liquid chromatography. J Am Oil Chem Soc 70(8):793–796

Amelio M, Rizzo R, Varazini F (1998) Separation of stigmasta-3,5-diene, squalene isomers, and wax esters from olive oils by single high-performance liquid chromatography run. J Am Oil Chem Soc 75(4):527–530

Angerosa F, Camera L, Cumitini S, Gleixner G, Reniero F (1997) Carbon stable isotopes and olive oil adulteration with pomace oil. J Agric Food Chem 45:3044–3048

Angiuli M, Bussolino GC, Ferrari C, Matteoli E, Righetti MC, Salvetti G, Tombari E (2009) Calorimetry for fast authentication of edible oils. Int J Thermophys 30:1014–1024

Antoniosi Filho NR, Carrilho E, Lanças FM (1993) Fast quantitative analysis of soybean oil in olive oil by high temperature -capillary gas chromatography (HT-CGC). J Am Oil Chem Soc 70:1051–1053

Antoniosi Filho NR, Mendes OL, Lanças FM (1995) Computer prediction of triacylglycerol composition of vegetable oils by HRGC. Chromatographia 40:557–562

Aparicio R (1998) Oils and Fats. In: Lees M (ed) Food authenticity: issues and methodologies. Eurofins Scientific, Nantes

Aparicio R (2002) Chemometrics as an aid in authentication. In: Jee M (ed) Oils and fats authentication. CRC, Oxford, UK, pp 156–180

Aparicio R, Aparicio-Ruiz R (2000) Authentication of vegetable oils by chromatographic techniques. J Chromatogr A 881:93–104

Aparicio R, Aparicio-Ruiz R, García-González DL (2007) Rapid methods for testing authenticity: the case of olive oil. In: van Amerongen A, Barug DM, Lauwars M (eds) Rapid methods for food and feed quality determination. Wageningen Academic, Wageningen, pp 163–188

Aramendía MA, Marinas A, Moreno JM, Moalem M, Rallo L, Urbano FJ (2007) Oxygen-18 measurement of Andalusian olive oils by continuous flow pyrolysis/isotope ratio mass spectrometry. Rapid Commun Mass Spectrom 21:487–496

Association of Official Analytical Chemistry International (AOAC) (1995) Official methods of analysis, 16th edn. Cunniff PA (ed). AOAC International, Arlington

Association of Official Analytical Chemistry International (AOAC) (2002) AOAC official methods of analysis. Appendix D: guidelines for collaborative study procedures to validate characteristics of a method of analysis. AOAC International, Arlington

Aued-Pimentel S, Almeida MI, Mancini J (1993) The application of derivative spectrophotometry to evaluation of olive oil. Cient Tecnol Aliment 13:121–131

Baeten V, Aparicio R (1997) Possibilities offered by infrared and Raman spectroscopic techniques in virgin olive oil authentication. Olivae 69(12)

Bailey GF, Horvat RJ (1972) Raman spectroscopic analysis of the *cis/trans* isomer compositions of edible oils. J Am Oil Chem Soc 49:494–498

Baeten V, Hourant P, Morales MT, Aparicio R (1998) Oils and fats classification by FT-Raman spectroscopy. J Agric Food Chem 46:2638–2646

BaetenV MM, Morales MT, Aparicio R (1996) Detection of virgin olive oil adulteration by Fourier transform Raman spectroscopy. J Agric Food Chem 44:2225–2230

Baeten V, Pierna JAF, Dardenne P, Meurens M, García-González DL, Aparicio-Ruiz R (2005) Detection of the presence of hazelnut oil in olive oil by FT-Raman and FT-MIR spectroscopy. J Agric Food Chem 53:6201–6206

Barlett JG, Mahon JH (1958) Identification of oils and detection of oil adulteration by differential infrared spectroscopiy. J Assoc Off Anal Chem 41:450–459

Bianchi G (2002) Authentication of olive oil. In: Jee M (ed) Oils and fats authentication. CRC, Oxford, UK, pp 25–65

Biedermann M, Grob K, Mariani C (1995) On-line LC-UV-GC-FID for the determination of Δ^7- and $\Delta^{8(14)}$- seterols and its application for the detection of adulterated olive oils. Riv Ital Sost Grasse 72:339–344

Biedermann M, Grob K, Mariani C, Schmidt JP (1996) Detection of desterolized sunflower oil in olive oil through isomerized Δ^7-sterols. Z Lebensm Unter Forsch 202:199–204

Biedermann M, Haase-Aschoff P, Grob K (2008a) Wax ester fraction of edible oils: analysis by on-line LC-GC-MS and GC×GC-FID. Eur J Lipid Sci Technol 110:1084–1094

Biedermann M, Bongartz A, Mariani C, Grob K (2008b) Fatty acid methyl and ethyl esters as well as wax esters for evaluating the quality of olive oils. Eur Food Res Technol 228:65–74

Bortolomeazzi R, De Zan M, Pizzale L, Conte LS (2000) Identification of new steroidal hydrocarbons in refined oils and the role of hydroxy sterols as possible precursors. J Agric Food Chem 48:1101–1105

Bowadt S, Aparicio R (2003) The detection of the adulteration of olive oil with hazelnut oil: a challenge for the chemists. Inform 14:342–344

Bracci T, Busconi M, Fogher C, Sebastiani L (2011) Molecular studies in olive (*Olea europaea* L.): overview on DNA markers applications and recent advances in genome analysis. Plant Cell Rep 30:449–462

Brumley WC, Sheppard AJ, Rudolf TS, Shen CS, Yasaei P, Sphon JA (1985) Mass spectrometry and identification of sterols in vegetable oils as butyryl esters and relative quantitation by gas chromatography with flame ionization detection. J Assoc Off Anal Chem 68(4):701–709

Buchgraber M, Ulberth F, Emons H, Anklam E (2004) Triacylglycerol profiling by using chromatographic techniques. Eur J Lipid Sci Technol 106:621–648

Camin F, Larcher R, Perini M, Bontempo L, Bertoldi D, Gagliano G, Nicolini G, Versini G (2010) Characterisation of authentic Italian extra-virgin olive oils by stable isotope ratios of C, O and H and mineral composition. Food Chem 118:901–909

Calapaj R, Chiricosta S, Saija G (1993) Valutazione di resultanze analitiche gas-cromatografiche e spettrofotometriche nell'accertamento della presenza di oli di semi in campioni di olio di oliva. Riv Ital Sost Grasse 70:585–594

Carrasco-Pancorbo A, Cerretani L, Bendini A, Segura-Carretero A, Gallina-Toschi T, Fernández-Gutiérrez A (2005) Analytical determination of polyphenols in olive oils. J Sep Sci 28:837–858

Ceci LN, Carelli AA (2007) Characterization of monovarietal Argentinian olive oils from new productive zones. J Am Oil Chem Soc 84:1125–1136

Codex Alimentarius Commission (CAC) (2007) CCMAS 07/28/2: *guidance on measurement uncertainty*. FAO, Roma

Codex Alimentarius Commssion (CAC) (2009a) *Distribution of the report of the 21st session of the Codex Committee on fats and oils* (ALINORM 09/32/17). FAO, Roma

Codex Alimentarius Commission (2009b) *Report of the thirtieth session of the Codex Committee on methods of analysis and sampling. Appendix III. Draft guidelines on analytical terminology* (ALINORM 09/32/23). FAO, Roma

Codex Committee on Methods of Analysis and Sampling (CCMAS) (2007) Twenty-eigth session, Budapest, Hungary. FAO, Roma

Consolandi C, Palmieri L, Severgnini M, Maestri E, Marmiroli N, Agrimonti C, Baldoni L, Donini P, De Bellis G, Castiglioni B (2008) A procedure for olive oil traceability and authenticity: DNA extraction, multiplex PCR and LDR–universal array analysis. Eur Food Res Technol 227:1429–1438

Conte LS, Koprivnjak O, Fiorasi C, Pizzale L (1997) Solid-phase extraction applied to diacylglycerol determination in foods. Riv Ital Sostanze Grasse 74:411–414

Cortes HJ, Winniford B, Luong J, Pursch M (2009) Comprehensive two dimensional gas chromatography review. J Sep Sci 32:883–904

Cortesi N, Fedeli E (1983) Polar compounds of virgin olive oil. Note 1. Riv Ital Sost Grasse 60:341–351

Cortesi N, Rovellini P, Fedeli E (1990) I trigliceridi degli oli naturali. Nota I. Riv Ital Sost Grasse 67:69–73

Cossignani L, Luneia R, Damiani P, Simonetti MS, Riccieri R, Tisconia E (2007) Analysis of isomeric diacylglycerolic classes to evaluate the quality of olive oil in relation to storage conditions. Eur Food Res Technol 224:379–383

Cunha SC, Oliveira MBPP (2005) Discrimination of vegetable oils by triacylglycerols evaluation of profile using HPLC/ELSD. Food Chem 95:518–524

de Koning S, Kaal E, Janssen HG, van Platerink C, Brinkman UAT (2008) Characterization of olive oil volatiles by multi-step direct thermal desorption-comprehensive gas chromatography-time-of-flight mass spectrometry using a programmed temperature vaporizing injector. J Chromatogr A 1186:228–235

Damiani P, Santinelli F, Simonetti MS, Castellini M, Rosi M (1994) Comparison between 2 procedures for stereospecific analysis of triacylglycerols from vegetable oils-I: olive oil. J Am Oil Chem Soc 71:1157–1162

Damiani P, Cossignani L, Simonetti MS, Santinelli F (2000) Prediction of isocratic non-aqueous RP-HPLC retention parameters and response factors of triacylglycerols detected and response factors of triacylglycerols detected by an UV-diode array-evaporative light scattering on-line system. J Chromatogr Sci 38:195–199

DGF-Einheitsmethoden (2006) DGF *Standard Method C-VI 16 (06) Isomeric diacylglycerols. Determination of 1,2- and 1,3-diacylglycerols in vegetable edible oils.* Wissenschaftliche Verlagsgesellschaft mbH Stuttgart

Dionisi F, Prodolliet J, Tagliaferri E (1995) Assessment of olive oil adulteration by reversed phase high performance liquid chromatography/amperometric detection of tocopherols and tocotrienols. J Am Oil Chem Soc 72:1505–1511

El-Abassy RM, Donfack P, Materny A (2009) Visible Raman spectroscopy for the discrimination of olive oils from different vegetable oils and the detection of adulteration. J Raman Spectrosc 40:1284–1289

El-Hamdy AH, Perkins EG (1981) High-performance reversed-phase chromatography of natural triglyceride mixtures: critical pair separation. J Am Oil Chem Soc 58:867–872

El-Hamdy AH, El-Fizga NK (1995) Detection of olive oil adulteration by measuring its authenticity factor using reversed-phase high-performance liquid chromatography. J Chromatogr A 708:351–355

Ellison SLR, Rosslein M, Williams A (2000) Quantifying uncertainty in analytical measurement. EURACHEM/CITAC Guide CG 4, 2nd edn

European Communities (EC) (1987) Official Journal of the Commission of the European Communities. Regulation no 2658/87, L256, 9 Sept 1987
European Communities (EC) (1991) Official Journal of the Commission of the European Communities. Regulation no 2568/91, L248, 5 Sept 1991
European Communities (EC) (1995) Official Journal of the Commission of the European Communities. Regulation no 656/95, 29 Mar 1995
European Communities (EC) (1997) Official Journal of the Commission of the European Communities. Regulation no 2472/97, 11 Dec 1997
European Communities (EC) (2007) Official Journal of the European Union. Regulation no 702/2007, 22 June 2007
European Communities (EC) (2011) Official Journal of the European Union. Regulation no 61/2011, L 23, 27 Jan 2011
Fiebig HJ (1985) HPLC-Trennung von Triglyceriden. Fette Seifen Anstrichm 87:53–57
Flor RV, Hecking LT, Martin D (1993) Development of high performance liquid chromatography criteria for determination of grades of commercial olive oils. Part I. The normal ranges for the triacylglycerols. J Am Oil Chem Soc 70:199–203
Frankel EN (2010) Chemistry of extra virgin olive oil: adulteration, oxidative stability, and antioxidants. J Agric Food Chem 58:5991–6006
Frede E (1986) Improved HPLC of triglycerides by special tempering procedures. Chromatographia 21:29–36
Frega N, Bocci F, Lercker G (1993) High-resolution gas-chromatography determination of diacylglycerols in common vegetable oils. J Am Oil Chem Soc 70:175–177
Frega N, Bocci F, Lercker E (1990) The HRGC determination of triglycerides. Ital J Food Sci 4:257–264
Gallina-Toschi TG, Christie WW, Conte LS (1993) Capillary gas chromatography combined with high performance liquid chromatography for the structural analysis of olive oil triacylglycerols. J High Resolut Chromatogr 16:725–730
Gallina-Toschi T, Bendini A, Lercker G (1996) Evaluation of 3,5-stigmastadiene content of edible oils: comparison between the traditional capillary gas chromatographic method and the on-line high performance liquid chromatography-capillary gas chromatographic analysis. Chromatographia 43(3/4):195–199
García-González DL, Mannina L, D' Imperio M, Segre AL, Aparicio R (2004) Using ^{1}H and ^{13}C NMR techniques and artificial neural networks to detect the adulteration of olive oil with hazelnut oil. Eur Food Res Technol 219:545–548
García-González DL, Aparicio R (2006) Olive oil authenticity: the current analytical challenges. Lipid Technol 18(4):80–85
García-González DL, Viera M, Tena N, Aparicio R (2007) Evaluation of the methods based on triglycerides and sterols for the detection of hazelnut oil in olive oil. Grasas Aceites 58:344–350
García-González DL, Aparicio-Ruiz R, Aparicio R (2009) Olive oil. In: Moreau RA, Kamal-Eldin A (eds) Gourmet and health-promoting speciality oils. AOCS Press, Urbana, pp 33–72
Garcia-Pulido J, Aparicio R (1993) Triacylglycerol determination based on fatty acid composition using chemometrics. Anal Chim Acta 271:293–298
Gegiou D, Georgouli M (1983) A rapid argentation TLC method for detection of reesterified oils in olive oil and olive-pomace oil. J Am Oil Chem Soc 60:833–835
Gertz C (2008) New analytical possibilities to survey quality and identity of olive oils. In: 99th AOCS annual meeting, Seattle, 18th–21st May
Gertz C, Fiebig HJ (2005) Statement on the applicability of methods for the determination of pyropheophytin A and isomeric diacylglycerols in virgin olive oils. German Society for Fat Science (DGF). http://www.dgfett.de/meetings/archiv/hagenolive/Conclusions/Statement_Methods.pdf
Gertz C, Fiebig HJ (2006) Isomeric diacylglycerols – determination of 1,2- and 1,3 diacylglycerols in virgin olive oil. Eur J Lipid Sci Technol 108:1066–1069
Goodacre R, Kell DB, Bianchi G (1993) Rapid assessment of the authentication of virgin olive oils by other seed oils using pyrolysis mass spectrometry and articial neural networks. J Sci Food Agric 63:297–307

Grob K, Lanfranchi ML, Mariani C (1990) Evaluation of olive oils through the fatty alcohols, the sterols and their esters by coupled LC-GC. J Am Oil Chem Soc 67(10):626–634
Grob K, Artho A, Mariani C (1992) Determination of raffination of edible oils and fats by olefinic degradation products of sterols and squalene, using coupled LC-GC. Fat Sci Technol 10:394–400
Grob K, Bronz M (1994) Analytical problems in determining 3,5-stigmastadiene and campestadiene inedible oils. Riv Ital Sost Grasse 71:291–295
Grob K, Giuffré AM, Leuzzi U, Mincione B (1994a) Recognition of adulterated oils by direct analysis of the minor components. Fat Sci Technol 8:286–290
Grob K, Giuffré MA, Biedermann M, Bronz M (1994b) The detection of adulteration with desterolized oils. Fat Sci Technol 9:341–345
Guillén MD, Ruiz A (2003) Rapid simultaneous determination by proton NMR of unsaturation and composition of acyl groups in vegetable oils. Eur J Lipid Sci Technol 105:688–696
Guillou C, Lipp M, Radovic B, Reniero F, Schmidt M, Anklam E (1999) Use of pyrolysis–mass spectrometry in food analysis: applications in the food analysis laboratory of the European Commissions' Joint Research Centre. J Anal Appl Pyrolysis 49:329–335
Guimet F, Ferré J, Boqué R (2005a) Rapid detection of olive–pomace oil adulteration in extra virgin olive oils from the protected designation of origin "Siurana" using excitation–emission fluorescence spectroscopy and three-way methods of analysis. Anal Chim Acta 544:143–152
Guimet F, Ferré J, Boqué R, Vidal M, García J (2005b) Excitation-emission fluorescence spectroscopy combined with three-way methods of analysis as a complementary technique for olive oil characterization. J Agric Food Chem 53:9319–9328
Gurdeniz G, Tokatli F, Ozen B (2007) Differentiation of mixtures of monovarietal olive oils by mid-infrared spectroscopy and chemometrics. Eur J Lipid Sci Technol 109:1194–1202
Gurdeniz G, Ozen B (2009) Detection of adulteration of extra-virgin olive oil by chemometric analysis of mid-infrared spectral data. Food Chem 116:519–525
Gurr MI (1986) Trans-fatty acids. Metabolic and nutritional significance. BNF Nutr Bull 11:105–122
Hrncirik K, Fritsche S (2004) Comparability and reliability of different techniques for the determination of phenolic compounds in virgin olive oil. Eur J Lipid Sci Technol 106:540–549
Horwitz W (1988) Protocol for the design, conduct and interpretation of collaborative studies. Pure Appl Chem 60(6):855–864
Horwitz W (1995) Protocol for the design, conduct and interpretation of collaborative studies. Pure Appl Chem 67(2):331–343
Horwitz W, Kamps LR, Boyer KW (1980) Quality assurance in the analysis of foods for trace constituents. J Assoc Off Anal Chem 63:1344–1354
Hourant P, Baeten V, Morales MT, Meurens M, Aparicio R (2000) Oil and fat classification by selected bands of near-infrared spectroscopy. Appl Spectrosc 54:1168–1174
International Olive Council (IOC) (2006a) COI/T.20/Doc No 25. Global method for the detection of extraneous oils in olive oils. Madrid
International Olive Council (IOC) (2006b) COI/T.20/Doc No 23. Method for the determination of the percentage of 2-glyceryl-monopalmitate. Madrid
International Olive Council (IOC) (2006c) Method COI/T20/Doc No 18. Determination of wax content by capillary-column gas–liquid chromatography, Madrid
International Olive Council (IOC) (2009) COI/T.20/Doc No 29. Determination of biophenols in olive oils by HPLC. Madrid
International Olive Council (IOC) (2010) COI/T.15/NC No 3/Rev. 5. Trade standards applying to olive oils and olive-pomace oils. Madrid
International Organization of Standardization (ISO) (1994a) ISO 5725–1:1994, Accuracy (trueness and precision) of measurement methods and results – part 1: general principles and definitions. Geneva
International Organization of Standardization (ISO) (1994b) ISO 5725–2:1994, Accuracy (trueness and precision) of measurement methods and results –part 2: basic method for the determination of repeatability and reproducibility of a standard measurement method. Geneva

International Organization of Standardization (ISO) (1994c) SO 5725–3:1994, Accuracy (trueness and precision) of measurement methods and results – part 3: intermediate measures of the precision of a standard measurement method. Geneva
International Organization of Standardization (ISO) (1994d) ISO 5725–6:1994, Accuracy (trueness and precision) of measurement methods and results – part 6: use in practice of accuracy values. Geneva
International Organization of Standardization (ISO) (2001) ISO 5555, Animal and vegetable fats and oils – sampling. Geneva
International Organization of Standardization (ISO) (2003) ISO 661, Animal and vegetable fats and oils – preparation test sample. Geneva
International Organization of Standardization (ISO) (2005a) ISO 17025, General requirements for the competence of testing and calibration laboratories. Geneva
International Organization of Standardization (ISO) (2005b) ISO 9000, Quality management systems – fundamentals and vocabulary. Geneva
International Organization of Standardization (ISO) (2006a) ISO Guide 35, Reference materials – general and statistical principles for certification. Geneva
International Organization of Standardization (ISO) (2006b) ISO 3534–2, Statistics -vocabulary and symbols – part 2: applied statistics. Geneva
International Organization of Standardization (ISO) (2008) ISO 9001, Quality management systems – requirements. Geneva
International Organization of Standardization (ISO) (2009) ISO Guide 34, General requirements for the competence of reference material producers. Geneva
International Organization of Standardization (ISO) (2010) ISO 21748, Guidance for the use of repeatability, reproducibility and trueness estimates in measurement uncertainty estimation. Geneva
International Union of Pure and Applied Chemistry (IUPAC) (1997) Compendium of chemical terminology. In: McNaught AD, Wilkinson A (eds) Blackwell Scientific Publications, Oxford, on-line corrected version: http://goldbook.iupac.org (2006-) created by Nic M, Jirat J, Kosata B; updates compiled by Jenkins A
Kafatos A, Comas G (1991) Biological effects of olive oil on human health. In: Kiritsakis AK (ed) Olive oil. AOCS Press, Urbana, pp 157–181
Kapoulas VM, Passaloglou-Emmanouilidou S (1981) Detection of the adulteration of olive oil with seed oils by a combination of column and gas liquid chromatography. J Am Oil Chem Soc 58:694–697
Kapoulas VM, Andrikopoulos NK (1987) Detection of virgin olive oil adulteration with refined oils by second-derivative spectrophotometry. Food Chem 23:183–192
Kelly S, Parker I, Sharman M, Dennis J, Goodall I (1997) Assessing the authenticity of single seed vegetable oils using fatty acid stable carbon isotope ratios ($^{13}C/^{12}C$). Food Chem 59:181–186
Kumar S, Kalon T, Chaudhary S (2011) A rapid screening for adulterants in olive oil using DNA barcodes. Food Chem 127:1335–1341
Lai YW, Kemsley EK, Wilson RH (1995) Quantitative analysis of potential adulterants of extra virgin olive oil using infrared spectroscopy. Food Chem 53:95–98
Lanzón A, Albi T, Cert A (1989) Detección de la presencia de aceete de oliva refinado en el aceite de oliva virgen. Grasas Aceites 40:385–388
Lanzón A, Albi T, Cert A, Gracián J (1994) The hydrocarbon fraction of virgin olive oil and changes resulting from refining. J Am Oil Chem Soc 71:285–291
Lees M (1998) Introduction. In: Lees M (ed) Food authenticity: issues and methodologies. Eurofins Scientific, Nantes, pp 11–17
Lercker G, Moschetta M, Caboni MF, Frega N (1985) Determinazione degli oli esterificati negli oli provenienti dalla lavorazione delle olive. Riv Ital Sost Grasse 62:15–18
Lipp M, Anklam E (1998) Review of cocoa butter and alternative fats for use in chocolate-Part B. Analytical approachesfor identification and determination. Food Chem 62:99–108
Lisa M, Holĉapek M, Bohac M (2009) Statistical evaluation of triacylglycerol composition in plant oils based on high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry data. J Agric Food Chem 57:6888–6898

Mailer RJ, Ayton J, Graham K (2010) The influence of growing region, cultivar and harvest timing on the diversity of Australian olive oil. J Am Oil Chem Soc 87:877–884
Mannina L, D'Imperio M, Capitani D, Rezzi S, Guillou C, Mavromoustakos T, Vichez MDM, Fernandez AH, Thomas F, Aparicio R (2009) ^{1}H NMR-based protocol for the detection of adulterations of refined olive oil with refined hazelnut oil. J Agric Food Chem 57:11550–11556
Mariani C, Bondioli P, Venturini S, Fedeli E (1991) Sulla formazione di acidi grassi *trans* nel processo di reffinazione dell'olio di oliva lampante. Riv Ital Sost Grasse 68:455–459
Mariani C, Venturini S, Bondioli P, Fedeli E, Grob K (1992) Evaluation of the variations products by bleaching process on the more meaningful minor components, free and esterified in olive oil. Riv Ital Sost Grasse 69:393–399
Mariani C, Gasparoli A, Venturini S, Fedeli E (1993) Strutto vergine e strutto raffinato: Loro differenziazione analitica. Riv Ital Sost Grasse 70:275–278
Mariani C, Venturini S, Grob K (1995) Individuazione dell'olio di girasole alto oleico desterolato nell'olio d'oliva. Riv Ital Sost Grasse 72:473–482
Mariani C (1998) Sulla presenza di ergosterolo negli oli di oliva. Riv Ital Sost Grasse 75:3–10
Mariani C, Bellan G, Lestini E, Aparicio R (2006) The detection of the presence of hazelnut oil in olive oil by free and esterified sterols. Eur Food Res Technol 223:655–661
Marigheto NA, Kemsley EK, Defernez M, Wilson RH (1998) Comparison of mid-infrared and Raman spectroscopies for the authentication of edible oils. J Am Oil Chem Soc 75:987–992
Martens H, Naes T (1989) Multivariate calibration. Wiley, Chichester
Mavromoustakos T, Zervou M, Bonas G, Kolokouris A, Petrakis P (2000) A novel method to detect adulteration of virgin olive oil by other oils. J Am Oil Chem Soc 77:405–411
Montealegre C, Alegre MLM, García-Ruiz C (2010) Traceability markers to the botanical origin in olive oils. J Agric Food Chem 58:28–38
Montedoro GF, Servili M, Baldioli M, Miniati E (1992) Simple and hydrolyzable compounds in virgin olive oil. 1. Their extraction, separation and quantitative and semiquantitative evaluation by HPLC. J Agric Food Chem 40:1571–1576
Morchio G, Di Bello A, Mariani C, Fedeli E (1989) Individuazione di particolari oli rettificati in oli vergini di oliva. Riv Ital Sostanze Grasse 66:251–257
Moreda W, Pérez-Camino MC, Cert A (2003) Improved method for the determination of triacylglycerols in olive oils by high performance liquid chromatography. Grasas Aceites 54:175–179
Motta L, Brianza M, Stanga F, Amelotti G (1983) Analisi gascromatografica di gliceridi parziali dopo lipolisi con lipasi pancreatica. Riv Ital Sost Grasse 60:625–633
Muzzalupo I, Perri E (2002) Recovery and characterisation of DNA from virgin olive oil. Eur Food Res Technol 214:528–531
Nagy K, Bongiorno D, Avellone G, Agozzino P, Ceraulo L, Vekey K (2005) High-performance liquid chromatography-mass spectrometry based chemometric characterization of olive oils. J Chromatogr A 1078:90–97
Norme Grassi e Derivati (NGD) (2005) NGD C 87–05. Determinazione della composizione e del contenuto di digliceridi mediante GLC capillare. Stazione Sperimentale per le Industrie degli Oli e dei Grassi (SSOG), Milano
Ogrinc N, Košir IJ, Spangenberg JE, Kidrič J (2003) The application of NMR and MS methods for detection of adulteration of wine, fruit juices, and olive oil. A review. Anal Bioanal Chem 376:424–430
Ollivier D, Artaud J, Pinatel C, Durbec JP, Guérère M (2003) Triacylglycerol and fatty acid compositions of French virgin olive oils. Characterization by chemometrics. J Agric Food Chem 51:5723–5731
Özdemir D, Öztürk B (2007) Near infrared spectroscopic determination of olive oil adulteration with sunflower and corn oil. J Food Drug Anal 15:40–47
Ozen BF, Mauer LJ (2002) Detection of hazelnut oil adulteration using FT-IR spectroscopy. J Agric Food Chem 50:3898–3901
Öztürk B, Yalçin A, Özdemir D (2010) Determination of olive oil adulteration with vegetable oils by near infrared spectroscopy coupled with multivariate calibration. J Near Infrared Spectrosc 18:191–201

Pafundo S, Busconi M, Agrimonti C, Fogher C, Marmiroli N (2010) Storage-time effects on olive oil DNA assessed by amplified fragments length polymorphisms. Food Chem 123:787–793

Paganuzzi V (1997) Sulle attuali possibili sofisticazione dell'olio di oliva. Riv Ital Sost Grasse 74:49–58

Passaloglou-Emmanouilidou S (1990) A xomparative study of UV spectrophotometric methods for detection of olive oil adulteration by refined oils. Z Lebensm Unterz Forsch 191:132–134

Pérez-Camino MC, Moreda W, Cert A (1996) Determination of diacylglycerol isomers in vegetable oils by solid-phse extraction followed by gas chromatography on a polar phase. J Chromatogr A 721:305–314

Pérez-Camino MC, Moreda W, Mateos R, Cert A (2002) Determination of esters of fatty acids with low molecular weight alcohols in olive oils. J Agric Food Chem 50:4721–4725

Pérez-Camino MC, Moreda W, Mateos R, Cert A (2003) Simultaneous determination of long-chain aliphatic aldehydes and waxes in olive oils. J Chromatogr A 983:283–288

Pérez-Camino MC, Cert A, Romero-Segura A, Cert-Trujillo R, Moreda W (2008) Alkyl esters of fatty acids a useful tool to detect soft deodorized olive oils. J Agric Food Chem 56:6740–6744

Phillips FC, Erdahl WL, Nadenicek JD, Nutter LJ, Schmit JA et al (1984) Analysis of triglyceride species by high-performance liquid chromatography via a flame ionization detector. Lipids 19:142–150

Podlaha O, Toregard B (1982) A system for identification of triglycerides in RP-HPLC chromatograms based on equivalent carbon numbers. J High Resolut Chromatogr 5:553–558

Poulli KI, Mousdis GA, Georgiou CA (2006) Synchronous fluorescence spectroscopy for quantitative determination of virgin olive oil adulteration. Anal Bioanal Chem 386:1571–1575

Poulli KI, Mousdis GA, Georgiou CA (2007) Rapid synchronous fluorescence method for virgin olive oil adulteration assessment. Food Chem 105:369–375

Purcaro G, Morrison P, Moret S, Conte LS, Marriott PJ (2007) Determination of polycyclic aromatic hydrocarbons in vegetable oils using solid-phase microcomprenhensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry. J Chromatogr A 1161:284–291

Rivera del Alamo RM, Fregapane G, Gómez-Alonso S, Aranda F, Salvador MD (2004) Sterols and alcohols composition of Cornicabra virgin olive oil: the campesterol content exceeds the upper limit of 4% established by EU Regulations. Food Chem 84:533–537

Rohman A, Che Man YB (2010) Fourier transform infrared (FTIR) spectroscopy for analysis of extra virgin olive oil adulterated with palm oil. Food Res Int 43:886–892

Rossell JB (1986) Classical analysis of oils and fats. In: Hamilton RJ, Rossell JB (eds) Analysis of oils and fats. Elsevier Applied Science, London, pp 261–327

Sacchi R, Addeo F, Giudicianni I, Paolillo L (1992) Analysis of positional distribution of fatty acids in olive oil triacylglycerols by high resolution ^{13C}-NMR of the carbonyl region. Ital J Food Sci 4:117–123

Salivaras M, McCurdy AR (1992) Detection of olive oil adulteration with canola oil from triacylglycerol analysis by reversed-phase high-performance liquid chromatography. J Am Oil Chem Soc 69:935–939

Salvador MD, Aranda F, Fregapane G (1998) Chemical composition of comercial Cornicabra virgin olive oil from 1995/96 and 1996/97 crops. J Am Oil Chem Soc 75:1305–1311

Sánchez-Casas JS, Osorio-Bueno E, Montaño-García AM, Martinez-Cano M (2004) Sterol and erythrodiol + uvaol content of virgin olive oils from cultivars of Extremadura (Spain). Food Chem 87:225–230

Sayago A, García-González DL, Morales MT, Aparicio R (2007) Detection of the presence of refined hazelnut oil in refined olive oil by fluorescence spectroscopy. J Agric Food Chem 55:2068–2071

Serani A, Piacenti D, Staiano G (2001) Sistema analitico per l'identificazione di oli deodorati in oli vergini di oliva – Nota 2. Cinetica di isomerizzazione dei digliceride in oli vergine di oliva. Riv Ital Sost Grasse 78:525–528

Sinouri S, Frangiscos E, Cristopoulou E, Lazaraki M, Alexiou F (1995) Influence of certain cultivars on the composition of olive-pomace oils. Note I: triglycerides and fatty acids. Riv Ital Sost Grasse 72:483–491

Šmejkalová D, Piccolo A (2010) High-power gradient diffusion NMR spectroscopy for rapid assessment of extra-virgin olive oil adulteration. Food Chem 118:153–158

Tena N, Aparicio R, García-González DL (2009) Thermal deterioration of virgin olive oil monitored by ATR-FTIR analysis of trans content. J Agric Food Chem 57:10505–10511

Tranchida PQ, Purcaro G, Conte LS, Dugo O, Dugo G, Mondello L (2009a) Enhanced resolution comprehensive two-dimensional gas chromatography applied to the analysis of roasted coffee volatiles. J Chromatogr A 1216:7301–7306

Tranchida PQ, Purcaro G, Conte LS, Dugo O, Dugo G, Mondello L (2009b) Optimized use of a 50 μm internal diameter secondary column in a comprehensive two-dimensional gas chromatography system. Anal Chem 81:8529–8537

Vandeginste BGM, Massart DL, Buydens LMC, de Jong S, Lewi PJ, Smeyers-Verbeke J (1998) Handbook of chemometrics and qualimetrics: Part B. Elsevier, Amsterdam

van de Voort FR, Sedman J, Emo G, Ismail AA (1992) Rapid and direct value and saponification number determination of fats and oils by attenuated total reflectance/Fourier transform infrared spectroscopy. J Am Oil Chem Soc 69:1118–1123

van de Voort FR, Ismail AA, Sedman J (1995) A rapid, automated method for the determination of *cis* and *trans* content of fats and oils by Fourier transform infrared spectroscopy. J Am Oil Chem Soc 72:873–880

Vecchio S, Cerratini L, Bendini A, Chiavaro E (2009) Thermal decomposition study of monovarietal extra virgin olive oil by simultaneous thermogravimetry/differential scanning calorimetry: relation with chemical composition. J Agric Food Chem 57:4793–4800

Verleyen T, Szulczewska A, Verhe R, Dewettinck K, Huyghebaert A, De Greyt W (2002) Comparison of steradiene analysis between GC and HPLC. Food Chem 78:267–272

Vlachos N, Skopelitis Y, Psaroudaki M, Konstantinidou V, Chatzilazarou A, Tegou E (2006) Applications of Fourier transform-infrared spectroscopy to edible oils. Anal Chim Acta 573–574:459–465

Vlaeminck B, Harynuk J, Fievez V, Marriott P (2007) Comprehensive two-imensional gas chromatography for the separation of fatty acids in milk. Eur J Lipid Sci Technol 109:757–766

Wesley IJ, Barnes RJ, McGill AEJ (1995) Measurement of adulteration of olive oils by near-infrared spectroscopy. J Am Oil Chem Soc 72:289–292

Wesley IJ, Pacheco F, McGill AEJ (1996) Identification of adulterants in olive oils. J Am Oil Chem Soc 73:515–518

Woodbury SE, Evershed RP, Rossell JB (1998) Purity assessments of major vegetable oils based on $\delta^{13}C$ values of individual fatty acids. J Am Oil Chem Soc 75:371–379

Woolfe M, Primrose S (2004) Food forensics: using DNA technology to combat misdescription and fraud. Trends Biotechnol 22:222–226

Yang H, Irudayaraj J (2001) Comparison of near-infrared, Fourier transform-infrared, and Fourier transform-Raman methods for determining olive pomace oil adulteration in extra virgin olive oil. J Am Oil Chem Soc 78:889–895

Zamora R, Gómez G, Alba V, Hidalgo FJ (2001) Use of highresolution 13C nuclear magnetic resonance spectroscopy for the screening of virgin olive oil. J Am Oil Chem Soc 78:89–93

Zeitoun MAM, Neff WE, Selke E, Mounts TL (1991) Analyses of vegetable oil triglyceride molecular species by reversed phase high-performance liquid chromatography. J Liq Chromatogr 14:2685–2698

Zou MQ, Zhang XF, Xiao-Hua QI, Ma H-L, Dong Y, Chun-Wei LIU, Guo XUN, Wang H (2009) Rapid authentication of olive oil adulteration by Raman spectrometry. J Agric Food Chem 57:2001–6006

Chapter 17
Role of Lipids in Human Nutrition

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Contents

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties*,
DOI 10.1007/978-1-4614-7777-8_17, © Springer Science+Business Media New York 2013

17.1 Terminology and Structure of Lipids

Lipids are composed of a carbon skeleton with hydrogen and oxygen substitutions and tend to be insoluble in water; an exception are the short-chain fatty acids (FAs) (less than eight carbons), which are water-soluble. Simple lipids are esters of FAs with various alcohols such as glycerol or cholesterol. They include triacylglycerols (TAGs), waxes, cholesteryl esters and vitamin A and D esters. Compound lipids consist of esters of FAs in combination with both alcohols and other groups, and include phospholipids, glycolipids and lipoproteins, amongst others.

The main components of all dietary lipids are FAs, which are carboxylic acids with chain lengths of up to 30 carbons. Long-chain FAs (more than 14 carbons) are the main constituents of dietary fat. Saturated fatty acids (SFAs) contain no double bonds and are therefore 'saturated' with hydrogen. Unsaturated FAs contain one or more double bonds; monounsaturates contain one double bond, while polyunsaturates contain more than one. Double bonds can allow either a *cis* or a *trans* orientation. SFAs generally occupy less space than the equivalent chain-length unsaturated FAs.

The systematic name for a FA is derived from the number of carbons in the acyl chain (Table 17.1). For unsaturated FAs, the exact positions of double bonds in the acyl chain and their configurations are also reflected in the systematic nomenclature. Traditionally, the position of double bonds was identified by naming the carbon number (from carbon 1, the carboxyl carbon) on which each double bond occurs. Thus, octadecadienoic acid, an 18-carbon FA with *cis* double bonds between carbons 9 and 10 and carbons 12 and 13, is correctly denoted as *cis* 9, *cis* 12-octadecadienoic acid or as *cis*, *cis*, 9,12-octadecadienoic acid. More recently, an alternative shorthand notation for FAs has come into frequent use. Thus, octadecanoic acid is denoted by 18:0, indicating that it has an acyl chain of 18 carbons and contains no

Table 17.1 Fatty acid nomenclature

Trivial name	Systematic name	Shorthand symbol
Capric acid	Decanoic acid	10:0
Lauric acid	Dodecanoic acid	12:0
Myristic acid	Tetradecanoic acid	14:0
Palmitic acid	Hexadecanoic acid	16:0
Palmitoleic acid	9-Hexadecanoic acid	16:1
Stearic acid	Octadecanoic acid	18:0
Oleic acid	*Cis*-9-Octadecenoic acid	18:1n-9
Linoleic acid	*Cis,cis*-9,12-Octadecadienoic acid	18:2n-6
α-Linolenic acid	9,12,15-Octadecatrienoic acid	18:3n-3
γ-Linolenic acid	6,9,12-Octadecatrienoic acid	18:3n-6
Arachidonic acid	5,8,11,14-Icosatetraenoic acid	20:4n-6
Behenic acid	Docosanoic acid	22:0
Lignoceric acid	Tetracosanoic acid	24:0
Nervonic acid	*Cis*-15-Tetracosenoic acid	24:1
Eicosapentaenoic acid	(*Cis*)5,8,11,14,17-Eicosapentaenoic acid	20:5n-3

double bonds. Unsaturated FAs are named simply by identifying the number of double bonds and the position of the first double bond counted from the methyl terminus (with the methyl, or ω, carbon as number 1) of the acyl chain. The way the first double bond is identified is as ω-x, where x is the carbon number on which the double bond occurs. Therefore *cis*, *cis*, 9,12-octadecadienoic acid is also known as 18:2ω-6. The ω-x nomenclature is sometimes referred to as omega x (e.g. 18:2 omega 6) or *n*-x (e.g. 18:2*n*-6). In addition to these nomenclatures, FAs are often described by their common names (Table 17.1). In most polyunsaturated fatty acids (PUFAs) the double bonds are separated by a methylene ($-CH_2$) group. However, this is not always the case, and in some PUFAs the double bonds are conjugated (i.e. the two double bonds are separated by only a single carbon atom). Most common unsaturated FAs contain *cis* rather than *trans* double bonds. *Trans* double bonds do occur, however, as intermediates in the biosynthesis of FAs, in ruminant fats (e.g. cow's milk), in plant lipids and in some seed oils. *Cis*, but not *trans*, double bonds produce a kink in a molecule so that the molecular shape of unsaturated FAs containing *cis* double bonds is distinct from that of SFAs.

17.2 Lipids in Foods

FAs in fats, oils and foodstuffs are mainly esterified to glycerol, as TAGs, although some are present as esterified components of phospholipids, glycolipids and other lipids. The FA composition of cow's, sheep's and goat's milks is typically characterised by relatively high proportions of short- and medium-chain FAs and low proportions of PUFAs (Table 17.2). Ruminant milks also contain small quantities of a variety of branched and odd-numbered FAs. The proportions of different FAs can be markedly affected by the nature of the feed (Table 17.2). While eggs are rich in palmitic and oleic acids, the phospholipid fraction of yolk provides linoleic acid and other PUFAs (Table 17.2). Again, different feeding regimens alter the FA composition of eggs. While animal and poultry storage fats tend to be rich in SFAs and MUFAs, the muscle (i.e. meat) contains significant proportions of PUFA (Table 17.2). Fish can be classified into lean fish that store lipid as TAGs in the liver (e.g. cod) or fatty (oily) fish that store lipid as TAGs in the flesh (e.g. mackerel,

Table 17.2 Dietary sources of saturated and unsaturated fatty acids

Fatty acid	Rich dietary sources
Short/medium chain fatty acids	Cow's milk
Palmitic acid	Butter, lard, animal fat, palm oil, coconut oil
Oleic acid	Olive oil, rapeseed oil, palm oil
Linoleic acid	Corn oil, sunflower oil
α-Linolenic acid	Flaxseed oil (linseed oil), walnut oil
Eicosapentaenoic acid	Oily fish, fish oil
Docosahexaenoic acid	Oily fish, fish oil

herring. salmon, tuna). The oil obtained from fatty fish flesh or lean fish livers is termed fish oil and it has the distinctive characteristic of being rich in long-chain *n*-3 PUFAs (Table 17.2). Different oily fish (and so different fish oils) contain different amounts of *n*-3 PUFA (Table 17.2). This relates to the dietary habits and metabolic characteristics of the fish as well as to season, water temperature and so on.

The FA composition of lipids in plant membranes varies little between different types of leaves. Five FAs generally account for more than 90 % of total FAs: palmitic (ca. 13 %), palmitoleic (ca. 3 %), oleic (ca. 7 %), linoleic (ca.16 %) and α-linolenic (ca. 56 %). Thus, green leaves are an important source of the essential fatty acid (EFA) α-linolenic acid for herbivorous and omnivorous mammals. In contrast to the uniformity of the FA composition of plant leaves, seed oils exhibit a wide range of FA compositions, where one FA tends to predominate (Table 17.2), but, like plant leaves, they are important sources of EFAs (Table 17.2). Some seed oils contain moderate to high proportions of relatively unusual FAs. For example, borage (starflower) and evening primrose oil contains γ-linolenic aid and echium oil contains stearidonic acid (18:4*n*-3).

17.3 Intakes of Fatty Acids in Humans

Most fat consumed in the diet (90–95 %) is in the form of TAGs, although the diet also contains phospholipids, glycolipids, other complex lipids, and cholesterol. FAs are components of each of these structures, apart from cholesterol. There are significant differences in fat intake between countries, with average intakes among adults varying from less than 20 g/day in some developing countries to more than 150 g/day in some developed countries. The mix of FAs consumed also varies in accordance with the FA compositions of the fats and oils used in food preparation and of the foodstuffs eaten. Average fat consumption has changed over time and continues to do so. These time trends differ between countries. In many developing countries fat intake is increasing, while in developed countries fat intake has tended to decline over the last 30 years or so. For example, the average consumption of fat in the UK fell from 110 to 86 g/person/day between 1959 and 1990 (DH 1994a). The type of fat has also changed over time, such that the FA composition of the human diet has changed. For example, in the UK the consumption of SFAs as a percentage of food energy decreased from 20.3 % in 1975 to 16.6 % in 1990 (DH 1994a). The ratio of PUFAs to SFAs in the average UK diet increased from 0.17 in 1959 to 0.22 in 1979 and to 0.40 in 1990 (DH 1994a). Much of this change has been brought about by a change in consumption from butter to margarine and from animal fats to vegetable oils. The main PUFA in the diet is linoleic acid, followed by α-linolenic. On average, adult men in the UK consume about 13.5 and 1.7 g linoleic and α-linolenic acids, respectively, per day. Adult women in the UK consume about 9.3 and 1.2 g linoleic and α-linolenic acids, respectively, per day. A British Nutrition Foundation briefing paper on unsaturated FAs (Lunn and Theobald 2006) provides

details on the intakes of linoleic and α-linolenic acids among 14 Western European countries. Longer-chain PUFAs are consumed in lower amounts than linoleic and α-linolenic acids. Estimates of the intake of arachidonic acid in Western populations vary between 50 and 300 mg/day for adults. In the absence of fatty fish or fish oil consumption, α-linolenic acid is by far the principal dietary *n*-3 PUFA. Average intake of the long-chain *n*-3 PUFA in the UK is estimated at less than 250 mg/day (Lunn and Theobald 2006).

17.4 Lipids in the Body

The most common SFAs in the body are palmitic and stearic acids, which can be of dietary or endogenous origin. In the absence of dietary SFAs, the body would be able to synthesise adequate amounts to maintain normal function. However, during excessive intake, this class of FAs is associated with significantly increased risk of cardiovascular disease (see later section).

A range of FAs is required for membrane composition, integrity and function to be retained. This means that a supply of the correct balance of FAs to cells and tissues is essential for the optimal functioning of those cells and tissues. Furthermore, different cells and tissues may require a different balance of FAs (i.e. they may have different demands for FAs). Although many FAs can be synthesised in the human body, some cannot (linoleic and α-linolenic acids), and so these FAs must be consumed in the diet. In the absence of significant dietary intakes, synthesis of some other FAs (e.g. arachidonic acid) requires the provision of a preformed precursor FA (in this case linoleic acid). This means that dietary supply of some FAs is very important to meet the demands imposed by optimal cell and tissue function. Thus, an inadequate or unbalanced supply of FAs may impair cell and tissue function and lead to ill health and disease.

17.5 Essential Fatty Acids

Studies in the mid-twentieth century identified the effects, in rats, of EFA deficiency. Biochemically, the disease is characterised by changes in the FA compositions of many cell membranes whose functions are impaired. One of the striking features of EFA deficiency in rats is skin dermatitis and water loss. Epidermal lipids are rich in ceramides. The fatty acyl substituent in these is linoleic acid linked via its carboxylic acid group to the terminal methyl carbon of another FA (34:1*n*-9) to generate an extremely long chain (52 carbons) structure. These lipids form an intercellular matrix that prevents excessive water loss. In the absence of linoleic acid, the correct structure cannot be formed, and so there is breakdown of the water permeability barrier. Another feature of EFA deficiency is poor performance in converting

food energy into metabolic energy to sustain growth. This may be because the membranes of mitochondria from EFA-deficient animals have lower proportions of linoleic and arachidonic acids and higher proportions of oleic and Mead (20:3*n*-9) acids than those of healthy animals. This may explain why β-oxidation and oxidative phosphorylation are less efficient in the EFA-deficient animals. Other features of EFA deficiency most likely relate to structural requirements for certain PUFAs (e.g. in the brain, eye, testis and sperm) and the requirement for eicosanoid precursors to maintain physiological homeostasis. Linoleic acid is able to prevent or reverse the effects of EFA deficiency; α-linolenic acid is also able to do this, but it is less potent than linoleic acid. Some *n*-6 and *n*-3 FAs that are derivatives of either linoleic or α-linolenic acid are also able to reverse the effects of EFA deficiency (BNF 1992), suggesting that deficiency is, at least in part, due to insufficient supply of metabolic precursors. Nevertheless, linoleic and α-linolenic acids are the only true EFAs. However, circumstances may exist where other PUFAs are "conditionally essential" (Cunnane 2003). EFA deficiency is rare in humans, but it has been described in some clinical situations.

17.6 Biosynthesis of Fatty Acids

17.6.1 Biosynthesis of Saturated Fatty Acids

SFAs are built up by successive addition of 2-carbon units to a growing acyl chain. Full details of this pathway may be found elsewhere (Yaqoob et al. 2010). The principal product of FA synthesis is palmitic acid (16:0). However, enzymes exist in some tissues that act to release FAs of shorter chain length than palmitic acid. For example, in the mammary gland of some species are enzymes that are responsible for the release of the medium-chain SFAs such as caprylic acid (8:0) and capric acid (10:0), which are characteristic of the milks of those species. In eukaryotes, FA synthase produces 18:0 in most species, and elongation (catalysed by elongases) then generates very-long-chain FAs.

17.6.2 Biosynthesis of Monounsaturated Fatty Acids

The endoplasmic reticulum is a site for introduction of double bonds (desaturation) into FAs. Examples are the conversion of stearic acid (18:0) to oleic acid (18:1*n*-9) and of palmitic acid to palmitoleic acid (16:1*n*-7) by insertion of a *cis* double bond between carbons 9 and 10. Because the double bond is inserted between carbons 9 and 10 counting from the carboxyl end of the acyl chain, the desaturase enzyme is known as delta-9 desaturase (Δ^9-desaturase), although sometimes this enzyme is referred to as stearoyl CoA desaturase.

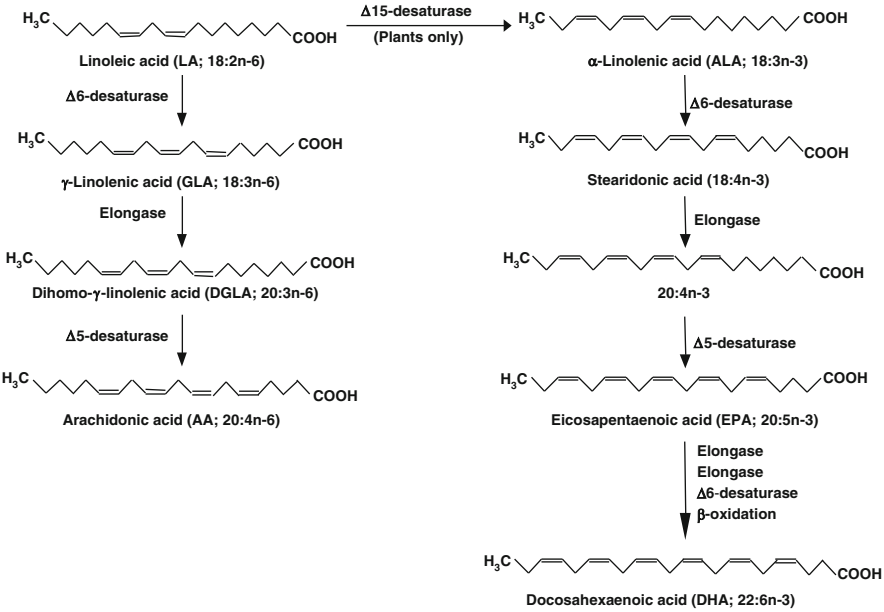

Fig. 17.1 Polyunsaturated fatty acid (PUFA) metabolism

17.6.3 Biosynthesis of Polyunsaturated Fatty Acids

All eukaryotes and some bacteria can produce PUFAs. Plant enzymes normally introduce a new double bond between an existing double bond and the terminal methyl group, whereas animal enzymes normally introduce a new double bond between an existing double bond and the carboxyl group. Insertion of a double bond between carbons 12 and 13 (counted from the carboxyl carbon) of oleic acid yields linoleic acid (18:2*n*-6). The enzyme that catalyses this reaction is called Δ^{12}-desaturase. Linoleic acid can be further desaturated by insertion of a double bond between carbons 15 and 16 (counted from the carboxyl carbon) by Δ^{15}-desaturase to yield α-linolenic acid (18:3*n*-3) (Fig. 17.1). Linoleic and α-linolenic acids are the simplest members of the *n*-6 and *n*-3 families of FAs, respectively. As indicated earlier, mammals lack the enzymes that introduce double bonds at carbon atoms beyond carbon 9 in the acyl chain (counting from the carboxyl carbon). Because these include the Δ^{12}- and Δ^{15}-desaturases, this means that mammals cannot synthesise linoleic and α-linolenic acids (Harwood 2005; 2007). Since these FAs are required by mammalian cells, they are termed EFAs, and there is a need for their consumption in the diet.

Although mammalian cells cannot synthesise linoleic and α-linolenic acids, they can metabolise them by further desaturation and elongation; desaturation occurs at carbon atoms below carbon number 9 (counting from the carboxyl carbon). Linoleic

acid can be converted to γ-linolenic (18:3*n*-6) by a Δ^6-desaturase and, subsequently, γ-linolenic can be elongated to dihomo-γ-linolenic (20:3*n*-6) acid (Fig. 17.1). Dihomo-γ-linolenic can be further desaturated by Δ^5-desaturase to yield arachidonic acid (20:4*n*-6) (Fig. 17.1). Using the same series of enzymes as those used to metabolise *n*-6 PUFA, α-linolenic acid is converted to eicosapentaenoic acid (20:5*n*-3; EPA) (Fig. 17.1). In mammals the pathway of desaturation and elongation occurs mainly in the liver.

It is evident from the pathway shown in Fig. 17.1 that there is competition between the *n*-9, *n*-6 and *n*-3 FA families for metabolism. The Δ^6-desaturase reaction is rate limiting in this pathway (BNF 1992). The preferred substrate for Δ^6-desaturase is α-linolenic acid, followed by linoleic acid, followed by oleic acid (BNF 1992). However, because linoleic acid is much more prevalent in most human diets than α-linolenic acid, metabolism of *n*-6 FAs is quantitatively more important. In the absence of intake of linoleic and α-linolenic acids, metabolism of oleic acid is enhanced, resulting in accumulation of Mead acid (20:3*n*-9), which is normally only found in tissues in trace amounts. The appearance and accumulation of Mead acid are taken to indicate dietary EFA deficiency (BNF 1992).

Further conversion of EPA to docosahexaenoic acid (22:6*n*-3; DHA) involves addition of two carbons to form docosapentaenoic acid (22:5*n*-3), two further carbons to produce 24:5*n*-3 and desaturation at the Δ^6 position to form 24:6*n*-3 (Fig. 17.1). Then two carbons are removed from 24:6*n*-3 by limited β-oxidation to yield DHA (Fig. 17.1). Arachidonic acid can be metabolised by the same series of enzymes to yield, in turn, 22:4*n*-6, 24:4*n*-6, 24:5*n*-6 and 22:5*n*-6.

17.7 Digestion, Absorption and Transport of Dietary Fat

The typical daily intake of fat in Western diets is between 50 and 100 g and supplies 35–40 % of energy intake. Digestion occurs in three phases: the gastric, duodenal and ileal phases. It has been suggested lingual lipase is secreted in the mouth before the first phase of digestion, although its contribution is minor; at most it may travel to the stomach and contribute to some fat hydrolysis there. The gastric phase involves mechanical digestion and some emulsification in the stomach. Pancreatic lipase, which hydrolyses TAG by cleavage of bonds between the FAs and the glycerol backbone, is introduced in the duodenum, together with bile salts, which solubilise the fat. TAG hydrolysis occurs in a sequential fashion, with removal of FAs from positions 1 and 3, generating a 2-monoacylglycerol. The majority of fat absorption occurs in the ileum. FAs of short and medium chain length (more than 12 carbons) are absorbed directly into the portal circulation and are rapidly oxidized by the liver. Long-chain FAs, on the other hand, undergo more complex processing within the enterocytes in the intestinal wall. Absorption into the enterocyte was originally thought to be a passive process but is now known to occur by facilitated diffusion, assisted by a FA binding protein (FABP) within the cell membrane. Once inside the enterocyte, FAs are rapidly re-esterified into 2-monoacylglycerols and subsequently to TAGs. Since TAGs are not water-soluble, they cannot be

transported free in the blood. Instead, they are packaged into specialised structures known as lipoproteins, whose function is to transport exogenous lipids (synthesised in the gut from dietary fat) and endogenous lipids (synthesised in the liver) to peripheral sites of utilisation and storage. Lipoproteins have a hydrophobic core of TAGs and cholesterol esters and a hydrophilic surface consisting of phospholipids and free cholesterol, which interacts with the aqueous environment of the blood and lymph. Lipoproteins also contain specific proteins, apolipoproteins, which, in addition to being essential for maintaining the structure and solubility of the particle, determine how the lipoprotein is metabolised. Apolipoproteins recognise and interact with specific receptors on the cell surface, and the receptor–lipoprotein complex is internalised into the cell by the process of endocytosis. Apolipoproteins also determine the activities of a range of proteins, including hydrolysing enzymes (lipases), receptors and lipid transfer proteins, which are involved in all stages of lipoprotein metabolism. In the small intestine, the lipoproteins are particularly large and known as chylomicrons, while those produced by the liver are termed very-low-density lipoproteins (VLDLs). Chylomicrons leave the enterocyte by exocytosis through the basement membrane and into the lacteal, which delivers the chylomicrons into the lymphatic vessels and subsequently into the bloodstream via the thoracic duct. The number and size of chylomicrons entering the bloodstream increases following a meal, and the nature of FAs in chylomicrons reflects that of the meal. Postprandial lipaemia (the appearance of dietary fat in the bloodstream) tends to peak approximately 3–4 h after a meal and subsides after approximately 6 h.

The TAG in circulating chylomicrons is hydrolysed by an enzyme called lipoprotein lipase (LPL), which is tethered to the endothelial lining of blood vessels in peripheral tissues. LPL releases FAs from the TAG within chylomicrons, and they are then taken up by tissues for utilisation or storage. The process of chylomicron hydrolysis is rapid, with 50 % of chylomicron TAG being removed within the first 2–3 min of entry into the bloodstream. Following a number of cycles through the tissues and the eventual removal of a large fraction of the TAG, a portion of cholesterol, and the surface phospholipids and apolipoproteins, a smaller, more cholesterol-ester-enriched particle termed a chylomicron remnant remains. The production and metabolism of chylomicrons is termed the exogenous pathway for lipoprotein metabolism, reflecting the fact that the fat was originally of dietary origin.

17.7.1 Lipoprotein Classes and Their Apolipoproteins

To aid description, lipoproteins have traditionally been classified according to their density into four main subgroups: chylomicrons, VLDLs, low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs). Chylomicrons formed in the gut and VLDLs formed in the liver are the main transporters of TAGs to the tissues and are referred to as triacylglycerol-rich lipoproteins (TRLs). They are the least dense of the lipoproteins as they contain the lowest protein:lipid ratio. ApoB48 and apoB100 represent the main protein components of chylomicrons and VLDLs respectively, with both proteins encoded for by the same gene; apoB48 represents the N-terminal

domain (48 %) of apoB100. Only one molecule of apoB protein is present per lipoprotein particle. ApoE and apoC are smaller apolipoproteins, which are passed from one particle to another, and the transfer of these molecules from HDLs is an important mediator of TAG metabolism.

The smaller, denser LDLs and HDLs are involved in the transport of cholesterol to and from cells, with approximately 70 % of total cholesterol present in LDLs. LDLs are derived from the metabolism of VLDLs in the circulation and therefore also contain apoB100 as its main apoprotein. HDLs, which are originally synthesised in the gut and the liver, are responsible for the removal of excess cholesterol from peripheral tissues and its return to the liver, a process called reverse cholesterol transport. These particles are relatively small and contain a high protein:lipid ratio (50:50) and are therefore more dense than chylomicrons, VLDLs or LDLs. The apoA series are the main proteins of HDL.

17.7.2 Endogenous Lipoprotein Pathway: VLDL Metabolism

VLDLs transport TAGs from the liver to the tissues via the endogenous lipoprotein pathway. The sources of FAs for liver TAG synthesis include FAs returned to the liver by chylomicron remnants, LDLs or HDLs, FAs delivered bound to albumin, and FAs formed by de novo synthesis from carbohydrates in the liver. This latter source is thought to be small in a typical Western diet but may become more significant in a high-carbohydrate diet. ApoB100 is the main structural and functional protein of VLDLs. Following secretion into the bloodstream, VLDLs acquire apoC2 from HDLs, which facilitates TAG hydrolysis by LPL, and the resulting FAs are accumulated by tissues in a manner similar to those from chylomicrons. However, mainly due to their greater surface area, chylomicrons are thought to provide a better substrate for LPL and are hydrolysed preferentially when both particles are present in the postprandial (fed) state. In the fasting state, when few chylomicrons are present, VLDLs are hydrolysed more rapidly.

VLDL remnants, known as intermediate-density lipoproteins (IDLs), have two metabolic fates. Approximately 40–50 % are taken up by the liver by receptor-mediated endocytosis, with both the apoB100 and apoE acting as ligands. The remaining 50–60 % lose all surface components except for a layer of phospholipids, free cholesterol and apoB100 and become LDLs, the major carrier of cholesterol in the blood. An increased secretion or delayed clearance of TAG-rich lipoproteins (VLDLs and chylomicrons) is a significant risk factor for coronary heart disease.

17.7.3 Endogenous Lipoprotein Pathway: LDL Metabolism

The role of LDLs formed from VLDLs is to transport cholesterol to the peripheral tissues and regulate de novo synthesis of cholesterol at these sites. On arrival at the

cell surface, the apoB100 component of LDL is recognised by the LDL receptor. Following internalisation of the LDL-receptor complex, the vesicle fuses with lysosomes, which contain a variety of degradative enzymes. The apoB100 protein is hydrolysed into free amino acids and the cholesterol esters into free cholesterol. The majority of the LDL receptor is returned to the cell surface unaltered, with a round trip time of approximately 10 min; the receptor is thought to have a lifespan of approximately 1 day. The released cholesterol can be used immediately for incorporation into cell membranes or synthesis of steroid hormones. Alternatively, the cholesterol can be re-esterified and stored within the cell. Cellular cholesterol is derived from both extracellular sources (LDL) and synthesised in the cell. The process of cellular cholesterol metabolism is tightly regulated.

The physiological importance of the LDL receptor in cholesterol homeostasis is demonstrated in the condition familial hypercholesterolaemia (FH), in which there is an absence or deficiency of functional LDL receptors. Marked elevations in circulating LDL levels are evident, which leads to deposition of cholesterol in a variety of tissues, including the artery walls, thus contributing to atherogenesis.

A number of additional receptors which recognise LDLs, one class of which is known as the scavenger receptors, have been identified. These receptors, which are present in large numbers on the surface of macrophages, do not bind to native LDLs but only LDLs that have been chemically modified, e.g. oxidised. Unlike LDL receptors, scavenger receptors are not subject to downregulation, and therefore macrophages can take up LDL indefinitely until they become lipid laden, when they are known as foam cells. This process forms the basis of the lipid accumulation which occurs in the development of atherosclerosis, and the process is accelerated in people with high circulating LDL levels.

17.7.4 Reverse Cholesterol Transport: HDL Metabolism

Excessive accumulation of cholesterol in tissues is toxic because the cell cannot break down cholesterol. This excess cholesterol is transported in the form of HDL to the liver, where it can be excreted in the bile or transported to other cells. Low HDL levels are an independent risk factor for cardiovascular disease (CVD).

17.8 Influence of Diet on Blood Lipids and Lipoproteins

Raised blood TAGs and cholesterol, particularly LDL cholesterol, is a well-established risk factor for CVD, while HDL cholesterol is inversely associated with risk. Over 40 years ago, it was reported that variation in the concentration of blood cholesterol was positively related to the intake of saturated fat and negatively related to that of polyunsaturated fat (Hegsted et al. 1965; Keys et al. 1965). These researchers formulated equations to predict, in quantitative terms, the relative effects of

different types of dietary FAs on blood cholesterol levels. These have been supported, to a large extent, by studies demonstrating that SFAs with chain lengths of 12–16 carbon atoms increase both LDL and HDL cholesterol, stearic acid (which is also saturated, but 18 carbon atoms in length) is neutral with respect to blood cholesterol, MUFAs decrease LDL cholesterol and increase HDL cholesterol, *n*-6 PUFAs decrease LDL cholesterol but may have a tendency to increase HDL cholesterol, and *n*-3 PUFAs have no overall effect on blood cholesterol but do lower levels of TAG at high doses. The mechanisms for these effects are described in detail elsewhere (Lin et al. 2005; Fernandez and West 2005). High levels of intake of *trans* FAs (greater than 7 % of energy) are associated with increased LDL cholesterol and decreased HDL cholesterol (Booker and Mann 2008), but since intakes in Western diets do not normally exceed 3–4 % of energy intake, this may not be a major cause for concern (Lunn and Theobald 2006). Perhaps surprisingly, reducing total fat intake without altering the composition of dietary FAs does not appear to have a dramatic impact on blood lipids (Lichtenstein 2006). Furthermore, isocaloric substitution of MUFAs and PUFAs for carbohydrate does not affect fasting TAG levels (Lichtenstein 2006). Thus the consensus of opinion is that both dietary MUFAs and PUFAs are good candidates for substitution for dietary SFAs (Mensink et al. 2003). However, on occasion, MUFAs have been preferentially recommended over *n*-6 PUFAs as substitutes for SFAs on the basis of the potential undesirable reduction in HDL cholesterol by *n*-6 PUFAs (Lunn and Theobald 2006).

17.9 Functions of Dietary Fat

Fats perform a range of essential functions within the body, including the provision of energy, structural and specific functional roles in cell membranes and hormone-like activities.

17.9.1 Energy Storage

Due to its energy density, fat is the nutrient of choice to act as a long-term fuel reserve for the organism. The majority of fat is stored as TAG in adipose tissue. Some fat is also stored in other cells in the body, such as liver and muscle cells, although excessive accumulation can have pathological consequences. There is a relationship between the FA composition of the adipose tissue and long-term dietary intake (e.g. an individual eating PUFAs will have a greater proportion in their adipose tissue), although in general, FAs stored in the adipocyte as TAGs tend to be more saturated than the FAs in cell membrane phospholipids.

Following a meal, ingested fat which is not required by the body tissues for immediate use is transported to the adipose tissue in lipoproteins. The FAs are hydrolysed from the TAGs in circulating lipoproteins by the enzyme lipoprotein

lipase, taken up by the adipose tissue and re-esterified into TAGs. When dietary energy is limited (e.g. after an overnight fast), the fat is mobilised and FAs are released from the adipocyte into the circulation, bound to serum albumin.

In addition to its role as an energy reserve, subcutaneous adipose tissue is important in the maintenance of body temperature, whereas internal fat (visceral fat) protects the vital organs such as the kidney and spleen. Accumulation of excessive visceral fat (abdominal obesity) is a risk factor for heart disease and diabetes and is linked with insulin resistance.

17.9.2 Structural Functions: Fats as Components of Cell Membranes

Fats form an integral part of cell membranes, which form a barrier between the cell and the external environment. Intracellular membranes compartmentalise different areas within the cell. The basic structural unit of most biological membranes is phospholipids, which, like TAGs, have FAs esterified at carbons 1 and 2 of glycerol. Carbon 3 is esterified to a phosphate group, which in turn is esterified to one of a variety of bases (e.g. choline, ethanolamine, serine, inositol); this contributes to the amphiphilic nature of membrane lipids providing both hydrophobic (fat-soluble) and hydrophilic (water-soluble) regions (Gunstone et al. 2007). In mammalian tissues the most common base is choline, and phosphatidylcholine is the main membrane phospholipid.

Lipids based on a sphingosine rather than a glycerol backbone (sphingolipids) are also widespread in membranes and are particularly abundant in the brain and nervous system (de Chaves and Sipione 2010). In sphingomyelin the amino group of the long unsaturated hydrocarbon chain of sphingosine is linked to a FA and the hydroxyl group is esterified to phosphoryl choline, yielding a molecule with a conformation similar to that of phosphatidylcholine. Glycolipids, as their name implies, contain carbohydrate. They consist of a sphingomyelin backbone and a FA unit bound to the amino group, with one or more sugars attached to the hydroxyl group. The simplest is cerebroside, which contains a single sugar, either glucose or galactose.

In the membrane, phospholipids and sphingolipids arrange themselves in a lipid bilayer with the hydrophobic FA tails facing inwards and the hydrophilic head interacting with the aqueous environment of the cytosol (at the inner face) and the extracellular fluid at the outer face. The chain length and degree of unsaturation of the FAs within the bilayer has an impact on the physical properties of the membrane, altering membrane fluidity and, therefore, function. Dietary FA intake affects membrane composition to a limited extent. The presence of lipid-soluble antioxidants, such as α-tocopherol, within the membrane serves to minimise oxidation of the unsaturated FAs.

Cholesterol, which is almost entirely absent from plant tissues, is the most common sterol found in animal tissues. It inserts itself into the lipid bilayer, where its hydrophobic interactions with FAs are essential to maintain membrane structure

and fluidity. In a diet rich in PUFAs, an increase in the cholesterol to phospholipid ratio serves to maintain membrane order.

In addition to lipids, membranes contain a variety of proteins: enzymes, receptors and transporters. The protein content is variable and reflects the function of the cell. Myelin, whose function is mainly to provide electrical insulation in nerve fibres, contains only about 18 % protein, whereas highly active membranes such as mitochondrial membranes have the highest protein content, typically 75 %.

17.9.3 Role of Lipids in Cell Signalling

Various lipids are involved in cell signalling and the conversion of extracellular signals into intracellular ones. The discovery of the phosphatidylinositol bisphophate cycle indicated that membrane inositol phospholipids are important mediators of hormone and neurotransmitter action. The binding of a hormone to membrane receptor proteins activates the enzyme phospholipase C, which hydrolyses the phosphatidylinositol molecule to diacylglycerol and inositol-1,4,5-triphosphate (IP_3). Both products activate protein kinases and act as secondary messengers involved in the regulation of cellular processes such as smooth muscle contraction, glycogen metabolism and cell proliferation and differentiation.

In addition to inositol phospholipids, sphingolipids are important modulators of membrane receptor activity in all stages of the cell cycle including apoptosis (cell death) and in inflammation. Intact membrane sphingolipids act as a ligand for receptors on nearby cells and modulate the activity of receptors and membrane-associated proteins in the same cell (Fantini and Barrantes 2009). Hydrolysis of sphingolipids can give rise to a variety of second messengers, such as ceramide, lactosylceramide, glycosylceramide and sphingosine, via the sphingomyelin cycle. Ceramide is the best known of the sphingoid signalling molecules. Activation of membrane-bound sphingomyelinase releases ceramide from membrane sphingolipids. Ceramide activates protein kinases involved in various metabolic processes within the cell, including cell growth and death, and inflammatory responses. For further insight into the roles of lipids in cell signalling, readers are referred to reviews by Wymann and Schnieter (2008) and Yaqoob (2003).

17.9.4 Lipids as Precursors of Eicosanoids

Membrane unsaturated FAs, in particular the C20 and C22 PUFAs, are the precursors of a variety of hormone-like compounds known collectively as eicosanoids, which mediate a variety of cellular functions, including smooth muscle contraction and blood clotting. They act locally to their site of synthesis and are metabolised very rapidly. It is largely this role of FAs as precursors of eicosanoids which underlies the essentiality of linoleic and α-linolenic acids since these two FAs, which cannot be synthesised in the body, are the precursors of the C20 and C22 PUFAs.

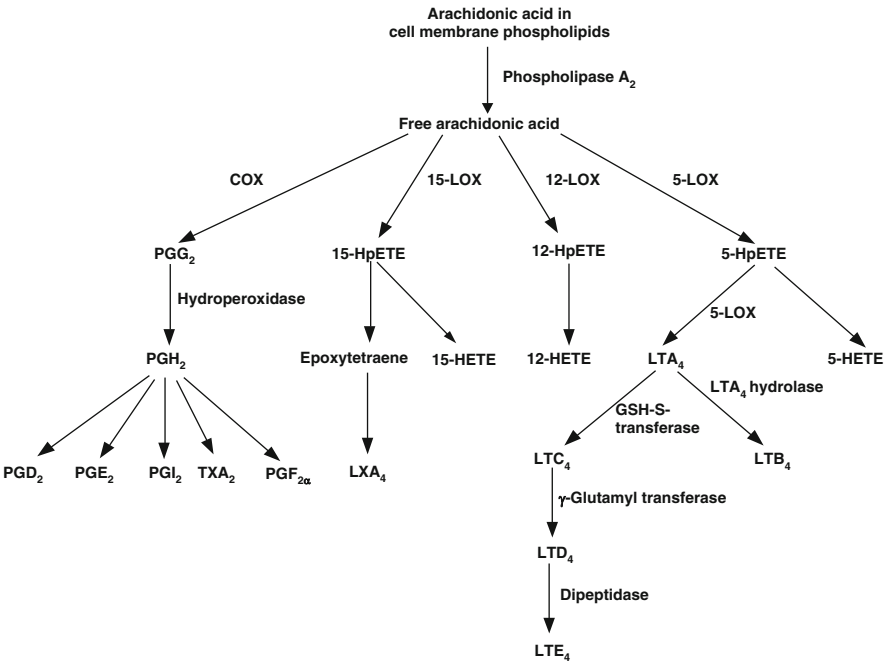

Fig. 17.2 Synthesis of eicosanoids from arachidonic acid

The main precursor for the synthesis of eicosanoids is arachidonic acid (20:4 *n-6*), which can be released from membrane phospholipids by phospholipase A following an appropriate stimulus and metabolised by lipoxygenases or by cyclo-oxygenase (Fig. 17.2). Metabolism by lipoxygenases gives rise to leukotrienes, lipoxins and hydroxy FAs, while metabolism by cyclo-oxygenase gives rise to prostaglandins, thromboxanes and prostacyclin. The range of biological activities of the eicosanoids is enormous and varies from tissue to tissue (Funk 2001). In fact, the full extent of their identities, their functions and the degree to which different eicosanoids interact with each other has not yet been fully characterised, largely because they are produced locally and are so short-lived.

Although arachidonic acid is regarded as the main precursor of eicosanoids, a separate family of eicosanoids is derived from the 20-carbon *n*-3 PUFA EPA. Oily fish and fish oils are rich sources of dietary EPA, which is readily incorporated into biological membranes and can replace arachidonic acid to some degree. This has two consequences. First, the replacement of arachidonic acid in the membranes of eicosanoid-synthesising cells by EPA results in a decrease in the production of arachidonic-acid-derived eicosanoids (Calder 2010). Second, there appears to be production of selected EPA-derived eicosanoids (Serhan et al. 2002). The physiological significance of the *n*-3 PUFA-derived eicosanoids is of considerable interest but is relatively poorly understood (Serhan 2010). Some studies have demonstrated that the EPA-derived eicosanoids are less potent than those derived from arachidonic acid. For example, leukotriene C_4, derived from arachidonic acid, is a

chemotactic factor with approximately ten-fold higher activity than leukotriene C_5, which is derived from EPA (Calder 2010). This type of observation has formed the basis of suggestions that the *n*-3 PUFAs possess anti-inflammatory and immunomodulatory properties (Calder 2010). However, the full range of biological activities of these compounds has not yet been investigated, and new eicosanoids such as the resolvins, protectins, docosatrienes and maresins are continually being discovered.

17.10 Dietary Fats in Health and Disease

17.10.1 Cardiovascular Disease

CVD is one of the leading causes of mortality and morbidity worldwide. Established risk factors include age, sex, ethnicity, hypertension, abnormal blood lipids (dyslipidaemia), diabetes, obesity, physical inactivity and smoking. Emerging risk factors, such as inflammation and endothelial dysfunction, may also play a role (BNF 2005). A number of these risk factors are potentially modifiable through diet. Evidence from cross-cultural comparisons, prospective studies, case–control studies and intervention trials suggests that the amount and type of fat in the diet can influence risk of both CVD and its associated risk factors. It is well accepted that diets high in SFAs increase risk of both heart disease and stroke. The influence of unsaturated FAs has emerged more recently.

There is epidemiological evidence for an inverse association between MUFA intake and death from CVD (e.g. Hu et al. 1997), although not all studies support this protective effect (e.g. Posner et al. 1991). Epidemiological data on *n*-6 PUFAs are lacking, but it is suggested that diets rich in *n*-6 PUFAs and low in SFAs are protective (Hu et al. 2001). A relatively large body of evidence reports an inverse association between intakes of long-chain *n*-3 PUFA and risk of CVD (Bjerregaard and Dyerberg 1998; Hu et al. 2002). This is supported by secondary prevention studies, such as the GISSI study (GISSI 1999), which reported a reduction in sudden cardiac death by 45 % and all-cause mortality by 20 % after supplementation with fish oil at a dose of just under 1 g/day *n*-3 PUFAs for 2 years (GISSI 1999). However, while much of the evidence relating to cardioprotective effects of *n*-3 PUFA is positive, there are still uncertainties regarding mechanisms involved, optimum doses required, impact of use alongside traditional medical treatments in high-risk patients and effects on arrhythmias (Saravanan et al. 2010).

Since many individuals do not consume oily fish and have low intakes of *n*-3 PUFA, there is interest in whether α-linolenic acid, which is from plant-based sources, is also associated with reduced risk of CVD. However, the findings have been inconsistent and suggest that α-linolenic acid does not have the same cardioprotective effects as the longer-chain *n*-3 PUFA (Lunn and Theobald 2006).

17.10.2 Stroke

Relatively few studies exist which examine the influence of dietary fat on risk of stroke. There is, however, some evidence of reduced risk with MUFA and *n*-6 PUFA (Ricci et al. 1997; Iso et al. 2001). For *n*-3 PUFA, the issue is more complex because there is evidence for an inverse association with total stroke and ischaemic stroke (Keli et al. 1994; He et al. 2004; Iso et al. 2002) but a positive association with haemorrhagic stroke (Bjerregaard and Dyerberg 1988). This may be due to the relatively potent inhibitory effects of *n*-3 PUFA on the blood-clotting cascade through modulation of eicosanoid metabolism. Further research is required to evaluate the risk versus benefit with respect to stroke.

17.10.3 Cardiovascular Disease Risk Factors

The effects of dietary fat on blood lipids were described in a previous section. Elevated blood pressure is another established risk factor for CVD and stroke. In healthy, normotensive individuals, MUFAs have little effect on blood pressure, and there is insufficient evidence regarding *n*-6 PUFA (Lunn and Theobald 2006). However, there is some evidence of a blood-pressure-lowering effect of *n*-3 PUFA in hypertensive subjects at high doses (Geleijnse et al. 2002).

17.10.4 Arrhythmia

Arrhythmias are irregular or abnormal heart rhythms caused by irregular electrical activity within the heart. They are often the cause of sudden cardiac death. There is some evidence from large intervention studies, such as the GISSI study (GISSI 1999), that *n*-3 PUFAs reduce rates of sudden death substantially. This has often led to the assumption that *n*-3 PUFAs are anti-arrhthymic. However, recent trials have failed to support this notion and in some cases demonstrate a pro-arrhythmic effect (Saravanan et al. 2010). Further research is required in this area. There is no information available at present regarding the influence of other dietary FAs on arrhythmias.

17.10.5 Diabetes

Individuals with diabetes have a two- to four-fold increased risk of CVD, so dietary management and the influence of dietary fat intake is especially pertinent in this group. Furthermore, excessive intake of fat can lead to accumulation of TAGs in many tissues, which can result in metabolic disturbances and hyperglycaemia. Recently, research has focussed on whether the FA profile of the diet can influence

insulin sensitivity. It has been suggested that a low-fat diet and an isocaloric high-MUFA diet are equally effective at maintaining glycaemic control (Lunn and Theobald 2006). However, there is a need for more research to determine whether the ratio of unsaturated FAs is an important consideration when recommending low fat diets to individuals with diabetes.

17.10.6 Cancer

Diet is considered to be an important determinant of human cancers; diet, lifestyle and physical activity are commonly suggested to account for approximately 30 % of all cancers, but particularly for colorectal, breast and prostate cancers (Doll and Peto 1981). Epidemiological studies suggest an association between dietary fat intake and these cancers, but it is a complex disease involving many factors over a long period of time, and there is considerable uncertainty regarding the relationship (WCR 2007). Low rates of colorectal, breast and prostate cancers in the Mediterranean region have often led to the suggestion that MUFA are protective (Keys et al. 1986). However, the Mediterranean diet consists of a number of components and lifestyle factors apart from MUFA which could be protective, e.g. fruits, vegetables, and whole grains. For PUFAs there is even less certainty; reviews and meta-analyses generally conclude that there is no influence, but there is some concern that high intakes of PUFAs could increase the risk of cancers based on laboratory-based studies showing tumour-promoting effects (Zock and Katan 1998). Regarding the *n*-3 PUFA, cancer incidence rates in countries with high fish consumption are lower, but while this has been linked with a protective effect of *n*-3 PUFA, there are many factors inherent in these comparative studies which cannot be properly accounted for. It is notable that the WCR (2007), the UK Department of Health (DH 1994b), and the World Health Organization/Food and Agricultural Organization (WHO/FAO 2003) conclude that there is insufficient evidence to make any recommendations regarding the intake of specific FAs as a means of preventing cancer.

17.10.7 Inflammatory Conditions

Inflammation involves redness, swelling, heat and often pain and serves to protect tissues from damage. However, in situations where it becomes excessive, for example in chronic inflammatory diseases, it can be very harmful. There is potential for some of these conditions to be alleviated by dietary modification, particularly with *n*-3 PUFA, which are precursors for the synthesis of eicosanoids (see earlier section) and are reported to have anti-inflammatory properties (Calder 2010). However, for most conditions, including asthma and inflammatory bowel disease, there is insufficient conclusive evidence to warrant dietary recommendations for the use of

n-3 PUFA in the management of these conditions. For rheumatoid arthritis, a number of clinical studies support the therapeutic effects of fish oil at high doses, and some studies show evidence that fish reduces the need for anti-inflammatory drugs. James et al. (2010) note that the benefits of fish oil in rheumatoid arthritis extend beyond symptom alleviation because patients have a two-fold increased risk of mortality, mainly due to CVD, and the cardioprotective effects of *n*-3 PUFA are therefore also relevant. Since the increased CVD risk is not attributable to traditional risk factors, it has been suggested that increased systemic inflammation, an emerging risk factor for CVD, is a contributor. In addition, some non-steroidal anti-inflammatory drugs (NSAID) are thought to increase CVD risk, so long-term use may also be a contributory factor and, conversely, the NSAID-sparing effect of fish oil a protective factor.

17.11 Conclusions and Future Perspectives

Dietary advice tends to stress the importance of fat reduction, yet fat has important and diverse roles in the body, from providing an energy source to structural and functional roles. The debate about the influence of different classes of FAs on human health continues. Current consensus suggests that both dietary MUFAs and PUFAs are good candidates for substitution of dietary SFA in terms of blood lipids, but detailed comparisons of the effects of MUFAs and PUFAs on a wide range of cardiovascular risk factors, and indeed in other conditions, including diabetes and cancer, are lacking. It remains to be seen whether MUFAs will be preferentially recommended over *n*-6 PUFA as substitutes for SFAs in the future.

References

Bjerregaard P, Dyerberg J (1988) Mortality from ischemic heart disease and cerebrovascular disease in Greenland. Int J Epidemiol 17:514–519

Booker CS, Mann JI (2008) Trans fatty acids and cardiovascular health: translation of the evidence base. Nutr Metab Cardiovasc 18:448–456

BNF (British Nutrition Foundation) (1992) Unsaturated fatty acids: nutritional and physiological significance. Report of the British Nutrition Foundation's task force. Chapman and Hall, London

BNF (British Nutrition Foundation) (2005) Cardiovascular disease: diet, nutrition and emerging risk factors. Report of the British Nutrition Foundation's task force Stanner S (ed). Blackwell, London

Calder PC (2010) The 2008 ESPEN Sir David Cuthbertson lecture: fatty acids and inflammation-from the membrane to the nucleus and from the laboratory bench to the clinic. Clin Nutr 29:5–12

Cunnane SC (2003) Problems with essential fatty acids: time for a new paradigm? Progress in Lipid Research 42:544–568

De Chaves EP, Sipione S (2010) Sphingolipids and gangliosides of the nervous system in membrane function and dysfunction. FEBS Lett 584:1748–1759
DH (Department of Health) (1994a) Nutritional aspects of cardiovascular disease. HMSO, London
DH (Department of Health) (1994b) Nutritional aspects of the development of cancer. HMSO, London
Doll R, Peto R (1981) The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. J Natl Cancer Inst 66:1191–1308
Fantini J, Barrantes FJ (2009) Sphingolipid/cholesterol regulation of neurotransmitter receptor conformation and function. BBA Biomembr 1788:2345–2361
Fernandez ML, West KL (2005) Mechanisms by which dietary fatty acids modulate plasma lipids. J Nutr 135:2075–2078
Funk CD (2001) Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294:1871–1875
Geleijnse JM, Giltay EJ, Grobbee DE (2002) Blood pressure response to fish oil supplementation: metaregression analysis of randomized trials. J Hypertens 20:1493–1499
GISSI (1999) Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Lancet 354:447–455
Gunstone FD, Harwood JL, DijKstra AJ (2007) The lipid handbook, 3rd edn. Taylor and Francis, Boca Raton
Harwood JL (2005) Fatty acid biosynthesis. In: Murphy DJ (ed) Plant lipids – biology, utilisation and manipulation. Blackwell, Oxford, UK, pp 27–66
Harwood JL (2007) Lipid metabolism. In: Gunstone FD, Harwood JL, Dijkstra AJ (eds) The lipid handbook. Taylor and Francis, Boca Raton, pp 637–699
He K, Song Y, Daviglus ML (2004) Fish consumption and incidence of stroke: a meta-analysis of cohort studies. Stroke 35:1538–1542
Hegsted DM, McGandy RB, Myers ML, Stare FJ (1965) Quantitative effects of dietary fat on serum cholesterol in man. Am J Clin Nutr 17:281–295
Hu FB, Bronner L, Willett WC (2002) Fish and omega-3 fatty acid intake and risk of coronary heart disease in women. J Am Med Assoc 287:1815–1821
Hu FB, Manson JE, Willett WC (2001) Types of dietary fat and risk of coronary heart disease: a critical review. J Am Coll Nutr 20:5–19
Hu FB, Stampfer MJ, Manson JE (1997) Dietary fat intake and the risk of coronary heart disease in women. N Eng J Med 337:1491–1499
Iso H, Rexrode KM, Stampfer MJ (2001) Intake of fish and omega-3 fatty acids and risk of stroke in women. J Am Med Assoc 285:304–312
Iso H, Sato S, Umemura U (2002) Linoleic acid, other fatty acids, and the risk of stroke. Stroke 33:2086–2093
James M, Proudman S, Clelnad L (2010) Fish oil and rheumatoid arthritis: past, present and future. Proc Nutr Soc 69:316–323
Keli SO, Feskens EJ, Kronhout D (1994) Fish consumption and risk of stroke. The Zutphen study. Stroke 25:328–332
Keys A, Anderson JT, Grande F (1965) Serum cholesterol response to changes in the diet, IV: particular saturated fatty acids in the diet. Metabolism 14:776–787
Keys A, Menotti A, Karvonen MJ (1986) The diet and 15-year death rate in the seven countries study. Am J Epidemiol 124:903–915
Lichtenstein A (2006) Dietary fat, carbohydrate and protein: effects on plasma lipoprotein patterns. J Lipid Res 47:1661–1667
Lin J, Yang R, Tarr PT, Wu P-H, Handschin C et al (2005) Hyperlipidemic effects of dietary saturated fats mediated through PGC-1β coactivation of SREBP. Cell 120:261–273
Lunn J, Theobald HE (2006) The health effects of dietary unsaturated fatty acids, British Nutrition Foundation briefing paper. British Nutrition Foundation, London
Mensink RP, Zock PL, Kester AD (2003) Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. Am J Clin Nutr 77:1146–1155

Posner BM, Cobb JL, Belanger AJ (1991) Dietary lipid predictors of coronary heart disease in men. The Framingham study. Arch Intern Med 151:1181–1187
Ricci S, Celani MG, Righetti E (1997) Fatty acid dietary intake and the risk of ischemic stroke: a multi-centre case–control study. J Neurol 244:360–364
Saravanan P, Davidson NC, Schmidt EB, Calder PC (2010) Cardiovascular effects of marine omega-3 fatty acids. Lancet 376:540–550
Serhan CN (2010) Resoltuion phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways. Annu Rev Immunol 25:101–137
Serhan CN, Hong S, Gronert K, Colgan SP, Devchand PR (2002) Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals. J Exp Med 196:1025–1037
WCR (World Cancer Research) (2007) Food, nutrition, physical activity and the prevention of cancer; a global perspective. World Cancer Research Fund report
WHO/FAO (World Health Organization/Food and Agriculture Organization) (2003) Global report on diet, nutrition and prevention of chronic diseases. Technical report 916. WHO, Geneva
Wymann MP, Schneiter R (2008) Lipid signalling in disease. Nat Rev Mol Cell Biol 9:162–176
Yaqoob P (2003) Fatty acids as gatekeepers of immune cell regulation. Trends Immunol 24:639–645
Yaqoob P, Minihane AM, Williams CM (2010) Fats. In: C G, Powers HJ (eds) Human nutrition & dietetics, 12th edn. Elsevier Science, Edinburgh
Zock PL, Katan MB (1998) Linoleic acid intake and cancer risk: a review and meta-analysis. Am J Clin Nutr 68:142–153

Chapter 18
Olive Oil as a Functional Food: Nutritional and Health Benefits

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties*,
DOI 10.1007/978-1-4614-7777-8_18, © Springer Science+Business Media New York 2013

18.1 Introduction

The purpose of the diet has in the past been viewed as being to supply the nutrients needed for an individual to survive and remain healthy. In recent years, however, it has become clear that, in addition to providing basic nutrients, some foods have beneficial effects that improve physical and mental health and reduce the risk of disease, and this has led to the development of the idea of functional foods. A functional food is defined as a food consumed as part of the normal diet that promotes health or reduces disease risk in addition to supplying nutrients (Roberfroid 2002). The active components of functional foods may be vitamins, minerals, fatty acids, antioxidants, and others, which are commonly found in traditional foodstuffs such as fruit, vegetables, and whole grains.

The concept of the Mediterranean diet is now well known to the general public as well as health professionals. This diet is believed to be associated with numerous health benefits that lead to increased longevity and a lower incidence of chronic diseases, including cardiovascular disease (CVD), cancer, and neurodegenerative conditions (Huang and Sumpio 2008; Lairon 2007; Pérez-Jiménez et al. 2007). Although the components of the diet vary somewhat between different cultures in the various Mediterranean countries, olive oil is an important common factor, with approximately 90% of world production of olive oil originating in this geographical area (Huang and Sumpio 2007). Investigation of the contribution of olive oil to the health-promoting effects of the Mediterranean diet has demonstrated that it has a beneficial influence on a wide range of processes and risk factors (Table 18.1) (Huang and Sumpio 2007; Lairon 2007; Serra-Majem et al. 2004), and thus it is recognized as a key element in this respect.

Approximately 85% of the fat content of the Mediterranean diet is provided by olive oil, which contains mostly monounsaturated fatty acid (MUFA) in the form of oleic acid (Huang and Sumpio 2007; Pérez-Jiménez et al. 2007). It has been known for many years that replacing saturated fatty acid (SFA) in the diet with MUFA reduces the risk of heart disease, and more recently a range of other health benefits of MUFA consumption have been discovered (Table 18.1) (Covas 2007; Covas et al. 2009). Certain seed oils rich in oleic acid developed by modern technology (Pérez-Jiménez et al. 2007) can provide an alternative source of dietary MUFA. However, since virgin olive oil (VOO), like fruit juice, is extracted only by pressing the fruit, it has the advantage of retaining a wide variety of potentially beneficial micronutrients, including vitamins, carotenoids, squalene, and phenolic compounds, which are lost during the refining of seed oils. These minor components, together with the high content of MUFA, make olive oil the quintessential functional food.

18.2 Olive Oil and the Mediterranean Diet

The Mediterranean basin is a broad geographical region bordering the Mediterranean Sea that has been the settlement of some of the oldest civilizations in the world. Ancient Greeks and Romans created a culinary culture that lasted for centuries,

Table 18.1 Health benefits of olive oil

Benefit	Disease affected	Active components	References
Lower cardiovascular mortality	Atherosclerosis, CVD	MUFA, phenolic compounds	Covas et al. (2009), Lairon (2007)
Improved blood lipid profile	atherosclerosis	MUFAs	Covas et al. (2009), Huang and Sumpio (2007), Covas (2007), Pérez-Jiménez et al. (2007)
Reduced blood pressure	Hypertension, CVD, particularly stroke	MUFAs, phenolic compounds	Covas (2007), Pérez-Jiménez et al. (2007)
Reduced inflammation	Atherosclerosis, rheumatoid arthritis, asthma	MUFAs, phenolic compounds, oleuropein, oleocanthal, α-tocopherol, β-sitosterol, oleanolic acid	Covas et al. (2009), Covas (2007), Sales et al. (2009), Pérez-Jiménez et al. (2007), Perona et al. (2006)
Reduced oxidative damage	Atherosclerosis, CVD, NAFLD (Non-alcoholic fatty liver disease) and NASH (Non-alcoholic steatohepatitis), cancer	MUFAs, phenolic compounds, α-tocopherol	Reaven and Witztum (1996), Bester et al. (2010), Raederstorff (2009), Assy et al. (2009), Fito and de la Torre (2007)
Reduced hemostasis	Thrombosis, CVD	MUFAs, phenolic compounds	Huang and Sumpio (2007), Covas (2007), López-Miranda et al. (2007), Pérez-Jiménez et al. (2007)
Reduced risk of neurodegenerative diseases	Alzheimer's disease, Parkinson's disease	MUFAs, phenolic compounds	López-Miranda et al. (2010), Del Parigi et al. (2006)
Reduced cancer risk	Breast, ovarian, colorectal, prostate, and upper aero-digestive tract cancers	MUFAs, squalene	Bosetti et al. (2009), Waterman and Lockwood (2007), Escrich et al. (2007)
Increased life span	CVD, cancer, neurodegenerative diseases	–	Pérez-López et al. (2009), Pérez-Jiménez (2005), Trichopoulou and Vasilopoulou (2000)

determined by the local availability of specific foods. This culture allows the combination and preparation of available foods in ways that best preserve health. The Ancient Greeks used olives instead of meat as their main source of fat because they believed that animal fat was unhealthy. In contrast, the so-called barbarians ate more meat and dairy products because they were nomadic and had less opportunity to grow olive trees or to prepare olive oil. The Mediterranean diet is based on products derived from wheat, olives, and grapes, which constitute the Mediterranean triad of bread, oil, and wine, although it has been suggested that legumes should be added to form a tetrad. Although the types of foods available differ substantially among different regions of the Mediterranean basin, even when they are in close proximity, olive oil is the common element, and it differentiates this diet from other models. It is noteworthy that in the Italian and Spanish traditional diets, the daily dietary intake of olive oil is approximately 15–20 g, but in the Cretan diet it is much higher, reaching 70 g (Zampelas et al. 2004).

In the 1950s, it was reported that populations throughout the Mediterranean region had a lower risk of heart disease (Keys et al. 1966, 1986). The World Health Organization (WHO) sponsored the Seven Countries Study, regarding the dietary habits of people from Greece, Italy, Yugoslavia, Holland, Finland, the USA, and Japan, that lasted for 30 years and involved approximately 13,000 subjects. The main finding was that people from the Greek island of Crete had exceptionally low death rates from heart disease, despite their moderate to high intake of fat (Keys et al. 1966), and this was attributed to their diet and lifestyle. When these studies were carried out, the Mediterranean region was an economically depressed area, and most people had a relatively restricted diet and did hard physical work, hence rates of obesity were very low.

The Mediterranean culinary culture, regarded as the Mediterranean diet, is not really a diet as this term is understood in Western countries but rather a dietary pattern. In addition to social, political, and economic differences among the Mediterranean countries, the concept of the Mediterranean diet is based on dietary habits more like those of the 1960s than the present day. It typically emphasizes fresh fruits, cooked vegetables and legumes, grains, and, in moderation, wine, nuts, fish, and dairy products, with large amounts of olive oil.

VOO is obtained directly from pressed fruits of the olive tree (*Olea europaea*), and its consumption can provide antiatherosclerotic, anti-inflammatory, antithrombotic, and antihypertensive vasodilatory effects that are beneficial for cardiovascular health (Esposito et al. 2004; Pérez-Jiménez et al. 2007; Covas 2007). In contrast to some other refined seed oils, olive oil is a natural juice rich in microcomponents of biological significance, such as carotenes, tocopherols, phytosterols, phenolic compounds, and terpenic compounds. However, benefits cannot be expected from just adding quantities of olive oil to an unhealthy diet. A high intake of fresh fruit and vegetables has been shown to protect against both heart disease and cancer, and this has been attributed to the antioxidant content of these foods. Tomatoes have received particular attention because they are an important component of the Mediterranean diet. The major benefits of tomato ingestion are related to lycopene, a lipophilic antioxidant pigment that gives the red color to tomatoes (Kavanaugh

et al. 2007). Oily fish are a source of very-long-chain n-3 polyunsaturated fatty acids (PUFAs), which appear to be particularly beneficial to heart health because of the anti-inflammatory and vasodilatory properties of their metabolites. Nuts are rich in MUFAs and PUFAs and have been reported to reduce cardiovascular risk, ameliorate lipid profile and triglyceride (triacylglycerol) levels, and decrease inflammatory adhesion molecules in patients with hypercholesterolemia (Kris-Etherton 1999; Hu and Willett 2002). Walnuts are particularly rich in 18:3, n-3 (α-linolenic acid), which is a precursor of very-long-chain n-3 PUFA.

Although the Mediterranean diet is still the model for southern Mediterranean populations, many individuals of the northern regions have changed their lifestyle and food habits. Published evidence indicates that there are heterogeneous degrees of adherence to the diet, and this prevents a strict delineation of the influence of gender on the association of its consumption with various morbid conditions. The combination of healthy weight, varied natural and fresh products, physical activities, a relaxed, well-ordered life, and a small dose of sunlight – all together – may reduce cardiovascular events, metabolic syndrome, insulin resistance, diabetes, cancer, and other chronic diseases.

18.3 Active Components of Olive Oil

18.3.1 Major Components

According to EC Regulation 1638/98 OJEC 210 of 28/7/98, VOOs are those oils obtained from the fruit of the olive tree by mechanical processes or other physical processes, in conditions, especially thermal ones, that do not cause alterations in the oil. Oil obtained in this way will be a natural, fresh, and aromatic juice with different flavors and slightly different compositions.

Different processing methods produce VOO, pomace olive oil, or ordinary (common) olive oil. VOO is produced by direct pressing or centrifugation of the olive and is considered "extra" when the free acidity, expressed as oleic acid, is lower than 1 g/100 g and organoleptic characteristics (flavor and color) are excellent. VOO has a maximum acidity of 2 g/100 g. Oils below that standard are called lampante and need to be refined and blended with VOO before being marketed. Virgin and refined oils differ little in their fatty acid composition but have important differences in minor components.

Olive oil components can be divided into two fractions according to their ability to form soaps when it is treated with a strong base. The saponifiable fraction constitutes 98–99 % of the oil and is composed mainly of triacylglycerols (TAGs). The minor components are found in the unsaponifiable fraction, and although it constitutes only a small proportion of the total, this fraction confers important biological activities on the oil. The minor components of VOO, classified in increasing order of polarity, are hydrocarbons, tocopherols, fatty alcohols, triterpenic alcohols,

4-methylesterols, sterols, triterpenic dialcohols, polar-colored pigments (chlorophylls and pheophitins), and polyphenols.

The MUFA, oleic acid (18:1n-9), is the main component of olive oil, ranging from 55 to 83% depending on the maturation stage, the variety of olive tree, and the growing conditions (Boskou 2000). The palmitic acid (16:0) and linoleic acid (18.2, n-6) content range from 7.5 to 20% and from 3.5 to 21%, respectively. These fatty acids are esterified to form TAGs. According to the fatty acid composition of olive oil, more than 70 molecular species of TAGs are possible, but in fact, only a few major ones are found. The most abundant one is triolein (trioleoyl-glycerol), accounting for approximately 40–60% of total TAGs, followed by dioleoyl-palmitoyl-glycerol (12–20%) and palmitoyl-oleoyl-linoleoyl-glycerol (5.5–7.0%). Usually, unsaturated fatty acids, like oleic and linoleic acids, are preferentially found at the sn-2 position of the TAG molecule, although because of its high oleate content olive oil contains this acid at all three positions. These features are extremely important for all the processes related to the digestion, absorption, transport, and accumulation of olive oil TAGs.

The digestion and absorption of the saponifiable fraction of olive oil, as dietary TAGs, is a dynamic, complex, and very efficient process that is only partially understood at the molecular level. The hydrophobicity of lipids is a limiting factor for digestion because of the hydrophilic character of lipases (Konturek et al. 1998). Digestion begins with the formation of an initial emulsion (chyme) to solubilize the oil. Lingual and gastric lipases can hydrolyze partially emulsified TAGs and have a preference for short- and medium-chain molecules (Ramirez et al. 2001). The hydrolysis products of these lipases, as well as those of pancreatic lipase, are 1,2- and 2-3-diacylglycerols and free fatty acids. Diacylglycerols are then further hydrolyzed to 2-monoacylglycerols and free fatty acids. The positional distribution of fatty acids in dietary oils determines which fatty acids are absorbed as free fatty acids or 2-monoacylglycerols. The fatty acid at the sn-2 is conserved throughout the absorption and remaining metabolic processes (Renaud et al. 1995). Once in the enterocyte, fatty acids are sequentially transferred to 2-monoacylgycerols by monoacylglycerol-acyltransferases (MGAT) and to diacylglycerols by diacylglycerol-acyltransferases (DGAT) to resynthesize TAGs. These enzymes form a complex called TAG synthetase (Lehner and Kuksis 1995).

18.3.2 Minor Components

Olive oil is not, however, merely a MUFA fat; it contains other minor components with important biological properties (Covas et al. 2006). Thus, the unsaponifiable fraction of olive oil contains highly bioactive compounds that are present in minor concentrations, usually in the range of milligrams per kilogram (parts per million). It is important to note that only VOO (and not refined olive oil) contains minor compounds since most of them are removed as a result of the refining processes (Rastrelli et al. 2002). Among the minor components with a higher biological

activity, α-tocopherol (with vitamin E activity), β-carotene (which functions as vitamin A), and phenolics (antioxidant activity), have received the most attention.

One of the main differences between VOO and other edible oils is the hydrocarbon composition (Lanzon et al. 1994). The most abundant of these in VOO is squalene (Ginda et al. 1996), a polyunsaturated triterpene constituted by condensation of six isoprene units. Its concentration varies from 1.2 to 7.5 g/kg oil and it is a precursor in the biosynthesis of cholesterol and steroid hormones. Other olive oil components in this group include carotenes such as β-carotene and lycopene, which are found in amounts lower than 1 mg/kg (Su et al. 2002). β-carotene plays an important role as precursor of vitamin A, and lycopene is a potent antioxidant. Both compounds contribute to the yellowish color of the oil. α-tocopherol, with vitamin E activity, is the most abundant tocopherol in olive oil, although it is found in lower concentrations compared to other seed oils (Herrera and Barbas 2001). Chlorophylls *a* and *b* and their oxidation products, pheophytins *a* and *b*, are naturally present in olive oil and are responsible for the greenish color. In VOO from mature olives, chlorophyll levels vary from approximately 1 to 10 mg/kg, while those of pheophytins are in the range of 0.2–24 mg/kg (Psomiadou and Tsimidou 2001).

Phytosterols comprise a major proportion of the unsaponifiables in all vegetable oils, including olive oil, in which its content varies, but is always below 2,600 mg/kg. The main sterol found in VOO is β-sitosterol (approximately 95%), but there are other species present, like Δ^5-avenasterol, campesterol, Δ^7-stigmastenol, stigmasterol, and campestanol. The β-sitosterol content in VOO ranges from 683 to 2,600 mg/kg (Benítez-Sánchez et al. 2003).

In 1962, Martel and Gracián isolated a compound in the unsaponifiable fraction of second pressing oil, which was identified as homo-olestranol, now known as erythrodiol, with the structure of a triterpenic dialcohol. This compound is found in the skin of the fruit in amounts fluctuating between 100 and 120 mg/kg oil, but in VOOs the concentration is usually as low as 6–10 mg/kg. Maslinic and oleanolic acids are also pentacyclic triterpenes present in the skin of olive fruits with higher concentrations in olive-pomace oil than in VOO. In olive fruits maslinic and oleanolic acids were found at concentrations of 227 and 356 mg/kg, respectively, in the Picual variety (Perez-Camino and Cert 1999).

Phenolic compounds are rarely determined in routine analysis because of their solubility in water; thus they are the 'polar fraction' in VOO, which explains their absence in both unsaponifiable and glyceridic fractions. Four groups of polyphenols are present in VOO: simple phenolic acids (homovainillinic, gentisic, p-hydrobenzoic, and syringic acids), cinnamic acids (p-cumaric, sinapic, and caffeic acids), oleuropein derivatives (hydroxytyrosol and tyrosol), and flavonoids (lutein and apigenin) (Akasbi et al. 1993; Tuck and Hayball 2002). These substances prevent VOO autooxidation and underlie its exceptional thermal stability (Gutfinger 1981; Tsimidou et al. 1992), as well as contributing to its characteristic flavor and taste. The concentration of these phenols in olive oil depends on many factors, including the species, location, climate, and maturation of the olives (Kiritsakis and Markakis 1987), being highest in the first pressed extra VOO. The most abundant phenolic compounds in VOO are oleuropein- and ligstroside-aglycones and their derivatives,

which are formed during ripening of olive fruits by enzymatic removal of glucose from their respective oleuropein and ligstroside glycosides. Further degradation of the aglycones generates the simple phenolic compounds hydroxytyrosol and tyrosol, respectively (Owen et al. 2000a). Tyrosol, hydroxytyrosol, and their secoiridoid derivatives make up around 90% of the total phenolic content of a VOO.

After olive oil ingestion, tyrosol and hydroxytyrosol, as well as oleuropein, undergo rapid hydrolysis under gastric conditions, resulting in significant increases in the amount of tyrosol and hydroxytyrosol free forms in the small intestine (Corona et al. 2006). Simple phenols can be absorbed in the intestine, but few data on the phenolic derivatives are available. In the process of crossing epithelial cells of the gastrointestinal tract, phenolic compounds from olive oil are subject to a biotransformation phase. The structure of phenolic derivatives and their transformation into glycosylated and esterified compounds have a major impact on the mechanisms of intestinal absorption. In addition, they are degraded by microorganisms in the colon, which results in an overestimation of the absorbed amount when fecal excretion is measured (Scalbert and Williamson 2000).

18.4 Olive Oil and Inflammation

Inflammation occurs as the response of the body to harmful conditions, such as infection by pathogens or tissue injury. Acute inflammation is characterized by a local increase in temperature, reddening of the skin, swelling, and pain (hence the name) but is of relatively short duration, and its function is to start the healing process, enabling infections to be eliminated and damaged tissue to be repaired. In contrast, chronic inflammation is associated with long-term malfunction of the body's homeostatic mechanisms, which triggers prolonged active inflammation and leads to tissue damage (Medzhitov 2008). This persistent inflammation has been linked to a number of chronic human diseases, including atherosclerosis, rheumatoid arthritis, asthma, and multiple sclerosis. Current evidence for a role for olive oil in reducing inflammatory processes is, in the main, related to atherosclerosis (Perona et al. 2006; Covas 2007; Covas et al. 2009), although there is some evidence that it may reduce the risk of the development of rheumatoid arthritis (Sales et al. 2009), and a few very recent reports suggest that it might also help to protect against asthma (Wood et al. 2010) and multiple sclerosis (Materljan et al. 2009).

The understanding that prolonged inflammation plays an important role in atherosclerosis has evolved over the past two decades. It is now clear that inflammatory processes are involved in the initiation, progression, and final stages of the disease, which result in cardiovascular events (Libby 2002; Libby et al. 2002). Risk factors such as hypercholesterolemia, hyperlipidemia, and hypertension cause activation of the endothelium, leading to the release of inflammatory mediators such as prostaglandin E_2 (PGE_2) and thromboxane, which influence vasoreactivity, and cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β, which upregulate the expression of adhesion molecules, including intracellular (ICAM-1) and

vascular (VCAM-1) adhesion molecules and P- and E-selectins. In addition, the secretion of chemoattractants such as leukotriene B_4 (LTB_4) and the monocyte chemoattractant protein-1 (MCP-1) is stimulated (Perona et al. 2006; Libby et al. 2002).

These changes activate leukocytes and promote their adherence to the endothelium and migration into the artery wall, where they differentiate into macrophages that scavenge lipoproteins, particularly oxidized low-density lipoprotein (LDL), that have become trapped in the subendothelial space. The cells become engorged with lipid, forming foam cells, aggregates of which cause fatty streaks, the first visible atherosclerotic lesions (Libby 2002; Albertini et al. 2002). Activated monocytes and macrophages also contribute to the inflammatory response in several ways: they enhance LDL oxidation by producing reactive oxygen species (ROSs); they secrete inflammatory chemokines and cytokines including TNF-α, IL-1β, and MCP-1; and they produce IL-6, which stimulates the release by the liver and adipose tissue of C-reactive protein (CRP), an established marker of vascular inflammation, although it appears not to have a direct causal role in atherosclerosis (Covas 2007; Genest 2010).

Inflammatory processes contribute to the progression of atherosclerosis via the release of fibrogenic factors that promote the formation of the dense extracellular matrix and fibrous cap seen in advanced lesions (Libby et al. 2002). The final stage of atherosclerosis is rupture of the fibrous cap, causing thrombosis and acute myocardial infarction, and inflammation also plays a role here. Activated macrophages produce proteolytic enzymes, which can weaken the fibrous cap and secrete tissue factor, which triggers coagulation and thromobosis (Libby 2002; Libby et al. 2002).

A number of studies have shown that consumption of olive oil has anti-inflammatory effects (Table 18.2). When food consumption was assessed in 772 participants at high cardiovascular risk, Salas-Salvado et al. (2008b) found that subjects with the highest consumption of olive oil and nuts had the lowest plasma levels of VCAM-1, ICAM-1, IL-6, and CRP, and these finding have been supported by several intervention studies in similar types of patients. In a cohort of patients with metabolic syndrome, subjects following a Mediterranean-style diet containing olive oil (n = 90) as compared to a control diet (n = 90) were found to have lower serum concentrations of CRP, IL-6, and other inflammatory cytokines after 2 years (Esposito et al. 2004), and three shorter-term studies (1–3 months) with high CVD risk patients (approximately 900 in total) or hypercholesterolemic men also showed decreased blood levels of ICAM-1, VCAM-1, P-selectin, IL-6, and CRP in subjects consuming a Mediterranean-style diet supplemented with VOO in comparison to a low-fat diet (Mena et al. 2009; Estruch et al. 2006; Fuentes et al. 2001). Benefits have also been reported in healthy individuals. Human LDL has been shown to be enriched in oleic acid after olive oil consumption (Tsimikas et al. 1999) and to have reduced ability to induce monocyte chemotaxis and monocyte adhesion to the endothelium (Covas 2007). In addition, the surface expression of ICAM-1 on peripheral blood mononuclear cells (PBMC) has been reported to be reduced in middle-aged men after consumption of an olive-oil-enriched diet for 2 months (Yaqoob et al. 1998).

Other investigations in humans have focused on the postprandial phase since postprandial lipemia is known to have inflammatory effects, as genes involved in

Table 18.2 Anti-inflammatory effects of olive oil

Effect	Types of study	Active ingredients	References
Reduced levels/expression of adhesion molecules	Epidemiological Human intervention, including postprandial Cultured human endothelial cells	Oleic acid Phenolic compounds	Mena et al. (2009), Fito et al. (2008), Fuentes et al. (2001, 2008), Pacheco et al. (2008), Salas-Salvado et al. (2008b), Cortés et al. (2006), Bellido et al. (2004, Esposito et al. (2004), Yaqoob et al. (1998)
Decreased plasma levels of inflammatory cytokines, prostaglandins, and leukotrienes	Human intervention, including postprandial	Oleic acid Phenolic compounds Hydroxytyrosol	Fito et al. (2008), Bogani et al. (2006), Léger et al. (2005)
Decreased production of inflammatory cytokines, prostaglandins, and leukotrienes by vascular cells	Human intervention, including postprandial, coupled with isolated or cultured cells in vitro	Oleic acid Phenolic compounds Long-chain fatty alcohols	Dell'Agli et al. (2010), Fernández-Arche et al. (2009), Jiménez-Gómez et al. (2009), Zhang et al. (2009), Perona et al. (2006), De la Puerta et al. (2004), Yaqoob and Calder (1995)
Decreased plasma C-reactive protein	Epidemiological Human intervention	Not identified	Mena et al. (2009), Salas-Salvado et al. (2008b), Estruch et al. (2006), Esposito et al. (2004)
Reduced monocyte activation/ migration	Human postprandial Cultured cells in vitro	Oleic acid Phenolic compounds	Covas (2007), Perona et al. (2006), Bellido et al. (2004)
Decreased ROS production	Endothelial cells in vitro	Oleic acid, oleuropein, hydroxytyrosol Oleanolic acid	Covas (2007), De la Puerta et al. (2004), Perona et al. (2006), Simon et al. (1992)
Inhibition of COX-2 activity/ expression	Cultured cells in vitro	Oleocanthal Oleanolic acid	Beachamp et al. (2005), Ringbom et al. (1998)
Decreased NF-κB activation	Human postprandial Cultured human endothelial cells	Oleic acid Phenolic compounds	Dell'Agli et al. (2010), Brunelleschi et al. (2007), Covas (2007), Perona et al. (2006), Bellido et al. (2004)

this response are activated by the prooxidative state induced (Perez-Jiménez et al. 2007). In these studies, volunteers are typically given a fat meal containing olive oil or a control fat, sometimes following a short period (1–4 weeks) on a diet with a similar fat composition, and measurements are made up to 9 h postprandially. Decreases in serum concentrations of adhesion molecules, including ICAM-1, VCAM-1, and E-selectin, have been found after a meal containing olive oil, either compared to baseline or to an alternative fat (butter, high palmitic sunflower oil, or n-3 PUFA) in several studies with healthy, hypercholesterolemic, and hypertriglyceridemic subjects (Bellido et al. 2004; Cortés et al. 2006; Fuentes et al. 2008; Pacheco et al. 2008), although Tousoulis et al. (2010) did not detect any effect on blood ICAM-1 levels. In addition, a meal containing extra VOO, but not refined olive oil or corn oil, has been demonstrated to cause a significant decrease in blood levels of thromboxane B_2 and LTB_4 (Bogani et al. 2006). Furthermore, Jiménez-Gómez et al. (2009) have reported that induction of the expression of mRNA for TNF-α in PBMCs isolated postprandially was lower in healthy subjects given a breakfast meal containing olive oil or walnuts as compared to butter, although mRNA expression for IL-6 was higher after the olive oil breakfast than after the walnut test meal. Plasma levels of TNF-α and IL-6, however, were unaffected. It has also been shown that the nuclear factor-κB (NF-κB), a transcription factor that controls the expression of genes for a number of proinflammatory factors, including TNF-α, IL-6, and MCP-1 (de Winther et al. 2005), in monocytes is activated after a meal containing butter or walnuts, while a meal containing olive oil does not cause this effect (Bellido et al. 2004).

In addition to human studies, animal and in vitro work have contributed important evidence for the anti-inflammatory effects of olive oil. In rats, a diet containing 20% olive oil has been reported to decrease natural killer cell activity (Covas 2007); supplementation of the diet with 10% olive oil reduces lipoprotein oxidative susceptibility in hypercholesterolemic animals (El Seweidy et al. 2005); the production of ROS and PGE_2 has been shown to decrease in neutrophils after feeding 15% olive oil rather than a low-fat diet for 2 months (de la Puerta et al. 2004). Moreover, the secretion of inflammatory mediators such as TNF-α, IL-1β, IL-6, and PGE_2 by LPS-stimulated murine peritoneal macrophages isolated from mice fed 15–20% olive oil for 8 weeks has been found to decrease (de la Puerta et al. 2009; Yaqoob and Calder 1995).

In vitro studies have investigated the modulation of inflammatory processes by oleic acid in cultured human endothelial cells, and reduced levels of intracellular ROS, lowered expression of adhesion molecules, and decreased transcriptional activation of NF-κB on the addition of oleic acid to the cultures have been demonstrated, in addition to decreased induction of the expression of VCAM-1 and E-selectin by oxidized LDL isolated from subjects consuming a diet supplemented with olive oil as compared to SFA (Covas 2007; Pérez-Jiménez et al. 2007; Perona et al. 2006). Moreover, monocyte chemotaxis and adhesion to endothelial cells were increased on exposure to liposomes containing linoleic acid, but this effect was almost abolished when linoleic acid was replaced with oleic acid. Another recently reported anti-inflammatory effect of oleic acid is the reversal of the inhibitory effect of TNF-α on insulin production in the rat pancreatic B cell line, INS-1 (Vassiliou et al. 2009).

The anti-inflammatory action of oleic acid has been attributed to its low oxidizability in comparison to linoleic acid, which it is likely to replace in lipoproteins in individuals consuming a diet rich in olive oil (Tsimikas et al. 1999). Lee et al. (1998), however, have demonstrated that increasing the liposome content of oleic acid in the presence of a constant level of linoleic acid decreases the susceptibility of the particles to oxidation, suggesting that oleic acid may also have a direct antioxidant effect. Current evidence suggests that this may occur by suppression of intracellular ROS production or by quenching of ROSs after their formation (Perona et al. 2006). It is clear from many recent studies, however, that in addition to oleic acid, the minor components of olive oil, including phenolic compounds, and triterpenes, tocopherols, and plant sterols, which are found in the unsaponifiable fraction, make an important contribution to its anti-inflammatory properties (Covas et al. 2009; Covas 2007; Pérez-Jiménez 2007; Perona et al. 2006).

Intervention studies in humans have shown that a diet enriched with VOO as compared to refined olive oil is associated with reduced serum thromboxane B_2, LTB_4, ICAM-1, and VCAM-1 levels in patients with hyperlipidemic and stable heart disease, in healthy subjects in the fasting state (Covas 2007; Fito et al. 2008), and with lowered postprandial plasma concentrations of these inflammatory markers in hypertriacylglycerolemic and normal individuals (Bogani et al. 2006; Pacheco et al. 2007). In addition, in postmenopausal women consuming a diet containing VOO as compared to oleic acid-rich sunflower oil, the production of inflammatory prostaglandins was decreased (Bogani et al. 2006; Covas 2007). Many authors have attributed these effects to phenolic compounds, including oleuropein and its metabolites hydroxytyrosol and tyrosol, since they are abundant in VOO but absent after refining. Considerable evidence has accumulated from studies with cell types such as leukocytes and endothelial cells to indicate that these compounds have anti-inflammatory properties via inhibition of LTB_4 and cytokine and eicosanoid production and metalloproteinase expression, and via a decrease in monocyte adhesion due to downregulation of VCAM-1, ICAM-1, and E-selectin expression (Dell'Agli et al. 2010; Zhang et al. 2009; Perona et al. 2006), and that some of these effects may be mediated by suppression of the activation of NF-κB (Dell'Agli et al. 2010; Brunelleschi et al. 2007; Perona et al. 2006). Moreover, a minor component of the olive oil phenolic fraction, oleacanthal [the dialdehydic form of (−)deacetoxy-ligstroside aglycone], has been found to have an action similar to that of the anti-inflammatory drug ibuprofen in inhibiting the activity of cyclooxygenase enzymes, which are involved in the biosynthesis of inflammatory prostaglandins (Beachamp et al. 2005). The idea that phenolic compounds contribute to the anti-inflammatory effects of a diet rich in olive oil is also supported by an intervention study in which diabetic patients showed decreased serum TBX_2 levels after consuming olive mill waste rich in hydroxytyrosol for 4 days (Léger et al. 2005) and by a recently published nutrigenomic study in which consumption of VOO rather than washed VOO (which differed only in having a lower phenolic compound content) was found to result in a decrease in the profile of expression of inflammatory genes (Konstantinidou et al. 2010).

Compounds present in the unsaponifiable fraction of VOO may also contribute to its anti-inflammatory properties. Perona et al. (2006) have demonstrated that the

induction of endothelial cell production of inflammatory prostaglandins by postprandial TRLs from healthy subjects was reduced when the VOO in the test meal was enriched with its unsaponifiable fraction. Potential active components of this fraction include tocopherols (vitamin E), plant sterols, long-chain fatty alcohols, and triterpenoids. α-Tocopherol and the plant sterol β-sitosterol have been reported to have anti-inflammatory effects (Perona et al. 2006) and the triterpenoid oleanolic acid, which is present in significant amounts in pomace oil but not VOO, has also been shown to suppress prostaglandin production via inhibition of COX-2 and to reduce ROS generation in leukocytes (Simon et al. 1992; Ringbom et al. 1998). In addition, a recent report has suggested that long-chain fatty alcohols found in pomace oil reduce inflammatory cytokine and prostaglandin secretion by macrophages and neutrophils (Fernández-Arche et al. 2009).

Evidence suggests that the Mediterranean diet can reduce pain and stiffness in the joints as well as disease activity in patients with rheumatoid arthritis (Sales et al. 2009). Berbet et al. (2005) have reported that the improvements brought about by a dietary supplement of fish oil are enhanced when used in combination with olive oil, and Linos et al. (1999) found that consumption of olive oil is inversely related to the risk of development of the disease. The components of VOO that are believed to contribute to these beneficial effects include oleic acid, phenolic compounds, and oleocanthal (Sales et al. 2009; Iacono et al. 2010). Asthma is an allergic inflammatory condition characterized by raised eosinophil numbers in the blood and lungs and increased secretion of proinflammatory cytokines such as IL-4, IL-5, and IL-13 (Wood et al. 2010). It was reported recently that a dietary supplement containing olive oil in addition to marine oils reduced the influx of eosinophils into bronchoalveolar lavage fluid (Wood et al. 2010) and that increased consumption of olive oil by the mother during pregnancy decreases wheezing due to asthma in the first year of life (Castro-Rodríguez et al. 2010). Finally, from the results of a recent epidemiological study, Materljan et al. (2009) have suggested that olive oil may protect against inflammation in mutiple sclerosis by inhibiting COX enzymes involved in demyelination, possibly due to the effects of oleocanthal.

18.5 Olive Oil and Longevity

It was first noticed in the 1950s that the population of the Greek island of Crete had long life spans, and it was suggested that this was due to their Mediterranean diet, high in olive oil, fruits and vegetables, and fish (Pérez-López et al. 2009). More recently, mortality statistics collected by the WHO between 1960 and 1990 also indicated that life expectancy was increased in Mediterranean countries compared to that in more developed Western countries, despite the fact that the health care available was poorer and smoking was more prevalent during that period (Trichopoulou and Vasilopoulou 2000). The first direct evidence of a link between reduced mortality and the Mediterranean diet came from a study by Trichopoulou et al. (1995) that used data from 183 elderly Greeks to show that adherence to the

diet was inversely related to overall mortality, and similar results were obtained in subsequent studies in Denmark (Osler and Scroll 1997), Australia (Kouris-Blazos et al. 1999), the USA (Mitrou et al. 2007) Finland, Italy, the Netherlands (Knoops et al. 2004), Spain (Lasheras et al. 2000), and in the European Prospective Investigation into Cancer and Nutrition (EPIC) Elderly Study, which covered nine European countries (Trichopoulou et al. 2005; Bamia et al. 2007), although one study covering seven European countries found no significant correlation (Haveman-Nies et al. 2002). Adherence to the Mediterranean diet also showed to be positively related to lifespan in the EPIC Study, with good adherence found to be associated with an increase of 1 year in males aged 60 (although in some countries the association was not significant), and in a metaanalysis involving more than 1.5 million subjects, Sofi et al. (2008) found that the risk of early death from all causes was reduced by 9% when a Mediterranean diet was followed.

The increased longevity associated with the Mediterranean diet is mainly due to reduced mortality from cardiovascular disease, cancer, Alzheimer's disease, and Parkinson's disease (Sofi et al. 2008). Since consumption of olive oil has been found to be beneficial for all these conditions (see Sect. 18.6, below) and is a major feature of all Mediterranean diets, it has been suggested that it is an important contributory factor to this effect, perhaps because of the many micronutrients that it contains (Pérez-Jiménez 2005; Pérez-López et al. 2009). This idea is supported by data from the Italian and Greek cohorts of the EPIC Study, which showed that high consumption of olive oil, as well as fruit, nuts, and vegetables, was inversely related to overall mortality (Masala et al. 2007; Trichopoulou et al. 2009). Thus, there is strong and consistent evidence to indicate that adherence to the Mediterranean diet is associated with increased lifespan, and it is highly likely that olive oil is at least partly responsible; however, other components of the Mediterranean diet (for example, high intake of fruit, and vegetables) could play a part, and further investigation is needed to establish the mechanisms by which olive oil or other constituents of the diet help to extend life.

18.6 Olive Oil and Disease Risk

18.6.1 Cardiovascular Disease

CVD, whose main forms are coronary heart disease (CHD) and stroke, is a major cause of mortality in the Western world, accounting for 30–40% of total deaths per annum (Allender et al. 2008; Lloyd-Jones et al. 2009). Moreover, CHD is the single most common individual cause of death, killing 9–16% of the men who die each year and 9–12% of women. A link between Mediterranean diet and mortality from CHD was first uncovered by the Seven Countries Study. The traditional Cretan diet was found to be associated with the lowest rates of deaths from heart disease (Aravanis et al. 1970), and more recently in the Spanish cohort of the EPIC Study, Moreno-Iribas and coworkers (Guallar-Castillón et al. 2010) reported that both the

traditional diet as consumed in the Mediterranean region in the 1960s and the modern version (the evolved Mediterranean diet, which includes more red meat, saturated fats, and simple carbohydrates and less whole grain cereals and legumes) lowered the risk of CHD. The Mediterranean diet was also found to be effective in secondary prevention of CHD in the Lyon Diet Heart Study, which compared the effects of a Mediterranean-style diet rich in olive oil with a control diet formulated following the recommendations of the American Heart Association in more than 600 patients who had suffered one myocardial infarction over a period of 4 years. The results showed a very substantial reduction of approximately 65% in CHD mortality in the subjects given the test diet (de Lorgeril et al. 1999).

The cardioprotective effects of the Mediterranean diet were linked to olive oil consumption in a 15-year follow-up study to the Seven Countries Study, showing that the mortality rate from CHD of men aged 40–59 was negatively correlated with the MUFA content of the diet (Keys et al. 1986), and a Spanish case–control study found that the olive oil content of the diet was negatively correlated with the risk of a first nonfatal myocardial infarction. However, a population-based prospective investigation involving more than 22,000 Greek adults found no significant correlation between consumption of olive oil or other individual food group from the Mediterranean diet, although a higher degree of adherence to the diet was associated with a reduction in overall total mortality (Covas 2007). Despite this, there is a wealth of evidence to indicate that dietary olive oil has a beneficial effect on a number of contributory causes of myocardial infarction, including atherosclerosis development, hypertension, and hemostasis (Covas 2007; Covas et al. 2009; López-Miranda et al. 2007; Huang and Sumpio 2007; Bester et al. 2010), and that these benefits are due not only to the high MUFA content but also to the many micronutrients present in the oil.

18.6.2 Atherosclerosis Development

In addition to its anti-inflammatory effects (Sect. 18.4), olive oil and its components help to protect against atherosclerosis development by lowering plasma cholesterol levels and by decreasing the oxidation of LDL.

It has been recognized since the 1950s that raised plasma cholesterol levels contribute to the development of atherosclerosis, and by the mid-1960s it was clear from the work of Keys and coworkers that this risk factor is modulated by the type of fat consumed in the diet. The initial studies indicated that dietary SFA increased blood cholesterol concentrations, while dietary PUFA has a hypocholesterolemic effect (Keys 1965). It soon became clear, however, that the increased risk of atherogenesis is associated with cholesterol carried in LDL, while increased levels of cholesterol in HDL are beneficial (Miller 1982), and in 1985, Mattson and Grundy reported that a diet rich in MUFA (oleic acid) as compared to PUFA (linoleic acid) was as effective in reducing LDL cholesterol in patients and also decreased HDL cholesterol less frequently. Similar results were obtained in later studies, and meta-analyses have confirmed that dietary MUFA and PUFA have comparable cholesterol-lowering effects (Gardner and Kraemer 1995; Covas 2007; Pérez-Jiménez 2007).

In addition, postprandial lipemia, which is also a risk factor for atherosclerosis, has been found to be decreased by diets supplemented with MUFA as compared to SFA (Bravo et al. 2010). Clearly, therefore, diets rich in olive oil are hypocholesterolemic and thus protect against atherosclerosis.

Although LDL is strongly implicated in the early events in atherosclerosis development, its damaging effects are greatly enhanced by oxidative modification of the particles (Albertini et al. 2002). Oxidative stress results in the increased production of ROS, thus leading to increased LDL oxidation. A number of studies in humans and animals have suggested that consumption of olive oil reduces oxidative stress, and this has been partly attributed to its high oleic acid content. As the single double bond of MUFA makes conjugated diene formation less favorable, oleate-rich LDL would be expected to be more resistant to oxidation than PUFA-rich LDL (Fito and de la Torre 2007), and dietary supplementation studies have generally supported this view (Bester et al. 2010; Fitó et al. 2007). For example, in a recent study with 200 healthy subjects, Cicero et al. (2008) found that supplementation of the diet with 25 mL olive oil/day increased the oleic acid content of LDL and that this was associated with a reduction in lipid oxidative damage. Other work has also shown that an olive oil, as compared to an SFA-rich diet, reduces the uptake of oxidized LDL by macrophages, thereby reducing foam cell formation, an early event in atherogenesis (Moreno et al. 2008).

In addition to MUFA, VOO contains many minor components with antioxidant properties, and considerable attention has been paid to the potential role of olive oil phenolic compounds in protecting against atherosclerosis. Experiments in vitro and with animal models have supported the idea that they protect LDL from oxidation (Covas 2007; Bester et al. 2010; Raederstorff 2009). Moreover, human intervention studies have shown that these compounds reduce the concentration of oxidized LDL and markers of oxidative stress in the blood (Raederstorff 2009), and in the recent EUROLIVE Study, levels of phenols in LDL after consumption of VOO as compared to refined olive oil were found to be increased and inversely correlated to the degree of LDL oxidation in healthy men (de la Torre-Carbot et al. 2010). Among the individual olive oil phenolic compounds, hydroxytyrosol and oleuropein have been reported to work dose-dependently and synergistically to inhibit LDL oxidation (Bester et al. 2010). There is some evidence to suggest that other minor components of olive oil that have antioxidant properties, including squalene (Covas 2007; Bester et al. 2010), triterpenoids (Covas 2007), and plant sterols (Chan et al. 2007), may also help to protect against oxidative stress and atherosclerosis development, but further work is needed to establish their effects.

18.6.3 Hypertension

In hypertension (high blood pressure), systemic arterial blood pressure is chronically raised. The condition is diagnosed when the diastolic blood pressure is greater than or equal to 90 mmHg or when the systolic blood pressure is greater than or equal to 140 mmHg consistently after three or more visits to the doctor. Secondary hypertension, due to diseases of the kidney, heart, or endocrine system, accounts for

only 5–10% of cases, leaving 90–95% as primary hypertension for which no medical cause can be found (Carretero and Oparil 2000). Hypertension is common in Western countries, with the age- and sex-adjusted prevalence being reported to be 28% for North America and 44% for European countries in a study published in 2003 (Wolf-Maier et al. 2003); it is an important risk factor for CVD and stroke in particular (Carretero and Oparil 2000; Wolf-Maier et al. 2003).

Consumption of the Mediterranean diet has been found to be associated with reduced blood pressure in a number of studies (Waterman and Lockwood 2007; Covas 2007; Pérez-Jiménez et al. 2007; Pérez-López et al. 2009). In the Greek cohort of the EPIC Study, adherence to the Mediterranean diet and olive oil consumption were inversely related to both systolic and diastolic blood pressure (Psaltopoulou et al. 2004), and high intake of olive oil was also inversely associated with diastolic blood pressure in the Italian (Florence) cohort of the same study (Masala et al. 2008). Systolic blood pressure in patients at high risk for CVD has also been shown to be reduced by consumption of a Mediterranean diet enriched with VOO for 3 months in the PREDIMED Study (Barceló et al. 2009).

Evidence suggests that the effects of olive oil in reducing hypertension are related to both its high MUFA content and its minor components. Diets high in MUFA (almost always from olive oil), as compared to SFA, have been shown to lower blood pressure (Covas 2007), and Ferrara et al. (2000) reported that patients given sunflower oil (a rich source of PUFA) in the diet needed more medication to control their hypertension than those given an equivalent amount of olive oil. In hypertensive women, however, VOO was found to be more effective in reducing blood pressure than high-oleic-acid sunflower oil, which has similar MUFA content (Ruíz-Gutiérrez et al. 1996; Perona et al. 2004), suggesting that the micronutrients in olive oil also play a part in its beneficial effects on hypertension. This is supported by the finding that systolic blood pressure was decreased after consumption of olive oil with a high, as compared to low, content of phenolic compounds in hypertensive patients with stable CHD (Fitó et al. 2005).

The mechanisms by which olive oil reduces blood pressure are not yet understood, but they may involve correction of the structural and functional alterations in erythrocyte membranes that occur in hypertension (Perona et al. 2004; Barceló et al. 2009) and changes in the fatty acid composition of the aorta (Ferrara et al. 2000). The antioxidant properties of both MUFA and the minor components such as phenolic compounds may also decrease ROS production and increase the availability of nitric oxide, leading to improved endothelial function (Covas 2007; Waterman and Lockwood 2007; Bester et al. 2010). In addition, studies in normotensive rats have suggested that olive oil may act as a calcium channel agonist (Gilani et al. 2005).

18.6.4 Hemostasis

Hemostasis is the process by which bleeding is stopped after damage to a blood vessel, and in a healthy individual a balance is maintained between the formation of the clot, or thrombus, and its breakdown by fibrinolysis after the injury is healed.

In pathological conditions, however, the regulatory mechanisms fail and thrombosis may result. Thrombosis is responsible for a considerable proportion of the morbidity and mortality associated with CVD and is also the second most common cause of death in cancer patients (Furie and Furie 2008).

Thrombus formation is initiated when coagulation factor VII binds to tissue factor, which, along with collagen, is exposed to the blood after damage to the endothelium. Platelets become activated and accumulate at the injury site, initially via binding to von Willebrand factor, and this leads to the conversion of fibrinogen to fibrin via the blood-clotting cascade, and the formation of thrombin is triggered. The breakdown of the clot, or fibrinolysis, requires the activation of the plasma protein plasminogen to the proteolytic enzyme plasmin by plasminogen activator secreted by the vascular endothelium, and the breakdown process is regulated by the balance between plasminogen activator and plasminogen activator inhibitors (PAI), particularly PAI-1 (Furie and Furie 2008). A possible beneficial effect of an olive-oil-rich Mediterranean diet on hemostasis was reported as early as 1986 (Sirtori et al. 1986). Moreover, it is now thought that there is chronic activation of the mechanisms of thrombosis in subjects at high risk of CVD, creating a 'prothrombotic environment'. Thus, in the past 25 years there has been considerable interest in exploring the potential to modify this by simple dietary changes. As a result, there is now evidence to indicate that olive oil may improve defenses against thrombosis in a number of ways, including inhibiting platelet aggregation, decreasing plasma levels of blood coagulation factors, and promoting fibrinolysis (López-Miranda et al. 2007).

Consumption of olive oil, as compared to SFA-rich fats, was found in a number of studies to be associated with reduced platelet aggregation, and this effect has also been found with other MUFA-rich oils such as high-oleic sunflower oil, suggesting that MUFA is at least partly responsible (López-Miranda et al. 2007). In addition, MUFAs, as compared to SFA or carbohydrate-rich diets, have been reported to lower serum levels of von Willebrand factor and TBX_2, another promoter of platelet aggregation, although the effects on von Willebrand factor have not been found consistently (López-Miranda et al. 2007). Minor components of olive oil may also be important for its beneficial effects on platelet aggregation. There is good evidence from both human and animal studies to indicate that phenolic compounds, including hydroxytyrosol, oleuropein aglycone, quercitin, and luteolin, play a significant part via modulation both of the aggregation process and of serum TBX_2 levels (López-Miranda et al. 2007; Dell'Agli et al. 2008; González-Correa et al. 2008; Correa et al. 2009; Cicerale et al. 2010). Additionally, compounds present in the unsaponifiable fraction may also have a role (Perona et al. 2004; López-Miranda et al. 2007), although more work is needed to establish the specific effects and components involved.

The effects of olive-oil- and SFA-rich diets on coagulation factors have been compared, but still some controversy remains. López-Miranda et al. (2007) found that an olive oil diet reduced plasma levels of activated factor VII (FVIIa). However, whereas Junker et al. (2001) reported a reduction in FVII coagulant activity (FVIIc) (and also factors XIIa, XIIc, and Xc) in healthy men after diets rich in olive oil, others have found increments (Sanders 2001). Blood concentrations of FVIIa and

FVIIc are known to rise after a fat meal as part of a postprandial procoagulant tendency, whatever the type of fat consumed. Recent work by Delgado-Lista et al. (2008), however, has shown that the increase in FVIIc after a test meal was reduced when healthy men were given a test meal containing MUFA rather than SFA or carbohydrate and n-3 PUFA, and this is consistent with a number of other studies that suggest that olive oil may provide some protection against the postprandial procoagulation environment (López-Miranda et al. 2007).

Although a few studies have suggested that diets rich in olive oil, as compared to SFA, are associated with lower basal levels of fibrinogen, this effect has not been shown consistently (López-Miranda et al. 2007). Current evidence, however, does support the idea that consumption of MUFAs rather than SFAs lowers blood concentrations of PAI-1, which would promote fibrinolysis (López-Miranda et al. 2007; Delgado-Lista et al. 2008), although in this case low-fat and PUFA-rich diets are likely to have a similar effect.

18.6.5 Obesity, Metabolic Syndrome, and Diabetes

Metabolic syndrome is considered a clustering of metabolic alterations that seems to affect around 25% of the population (Alvárez-León et al. 2003; Ford et al. 2004; Athyros et al. 2005). Since Reaven (2005) described metabolic syndrome for the first time in 1988, diagnostic criteria have been systematically updated by expert groups (WHO 1999; Alberti et al. 2006), but no general consensus regarding the definition of metabolic syndrome has been reached.

The lack of a universal definition of metabolic syndrome hinders the determination of the true global prevalence both locally and internationally. While the clustering of the components that define the different versions of metabolic syndrome is similar, each places a slightly different emphasis on the metabolic phenotype (Table 18.3). The WHO criteria focus on risk for diabetes centered around impaired glucose tolerance (Alberti and Zimmet 1998). The International Diabetes Federation (IDF) definition focuses on central adiposity, whereas the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults [Adult Treatment Panel (ATP) III, 2001] assigns no priority to any of the criteria. Therefore, estimates of the prevalence of metabolic syndrome will depend on the definition used. Using the NCEP definition, the prevalence of metabolic syndrome is estimated to be 25% of the general population, with no gender differences (Ford et al. 2002).

Epidemiological studies suggest that Western-style dietary patterns promote metabolic syndrome, while diets rich in fruits, vegetables, grains, fish, and low-fat dairy products, the paradigm of the Mediterranean diet, have a protective role (Esmaillzadeh et al. 2007; Lutsey et al. 2008). Apart from a cross-sectional study by Alvárez-León et al. (2003) that did not find a relationship between the adherence to the Mediterranean diet pattern and prevalence of metabolic syndrome, studies that have analyzed this relationship support a beneficial effect (Williams et al. 2000).

Table 18.3 Diagnostic criteria for metabolic syndrome[a]

Marker	WHO	EGIR	NCEP (ATP III)	IDF	AHA
Insulin resistance	Positive	Positive			
Fasting plasma glucose (mmol/L)	≥6.1	≥6.1	≥6.1	≥5.6 or treatment	≥5.6 or treatment
Obesity (waist, cm)	WHR > 0.9 men	≥94 men	≥102 men	≥94 men	≥120 men
	WHR > 0.85 women	≥80 women	≥88 women	≥80 women	≥88 women
Obesity (BMI, kg/cm^2)	>30				
Blood pressure SBP/DBP (mmHg)	≥140/90	≥140/90 or treatment	≥130/85	≥130/85or treatment	≥130/85 or treatment
Triacylglycerols(mmol/L)	≥1.7	≥2.0 or treatment	≥1.7	≥1.7 or treatment	≥1.7 or treatment
HDL-cholesterol (mmol/L)	<0.9 men	<1.0	<1.04 men	<1.03 men	<0.9 men
	<1.0 women		<1.29 women	<1.29 women	<1.0 women
Number of components	FPG or IR plus ≥ 2 others	IR plus ≥ 2 others	≥3	Central obesity plus ≥ 2 others	≥3

[a]*WHO* World Health Organization (1999)

EGIR European Group for the Study of Insulin Resistance (Balkau and Charles 1999), *NCEP* (*ATP III*) National Cholesterol Education Program (Adult Treatment Panel III) (Grundy et al. 2004), *IDF* International Diabetes Federation (IDF 2006), *AHA* American Heart Association (Grundy et al. 2005), *BMI* body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *HDL* high-density lipoprotein, *FPG* fasting plasma glucose, *IR* insulin resistance; treatment, under treatment of pathology stated in first column

Four feeding trials assessed the effect of dietary patterns on metabolic syndrome status to date (Esposito et al. 2004; Orchard et al. 2005; Azadbakht et al. 2005; Salas-Salvado et al. 2008a). In a cross-sectional substudy of the PREDIMED intervention trial, an inverse relationship between the score of adherence to the Mediterranean diet and the prevalence of metabolic syndrome of a cohort was observed (Babio et al. 2009). Esposito et al. (2004) showed that at the end of 2 years' follow-up, only 44% patients on a Mediterranean diet had features of metabolic syndrome compared to 87% patients in the control group. It was noteworthy that energy intake was reduced, especially in the Mediterranean diet intervention group, and substantial weight loss was achieved. However, a high-fat Mediterranean diet, as traditionally followed in the Mediterranean countries, has only been tested within the PREDIMED Study, which compared the effects of two high-fat Mediterranean diets, supplemented with VOO or mixed nuts, to a low-fat diet in volunteers at high risk for CVD. After 1 year, the prevalence of metabolic syndrome was reduced by 6.7, 13.7, and 2.0% in the MedDiet + VOO, MedDiet + Nuts, and control diet groups, respectively. However, incident metabolic syndrome rates were not significantly different among groups (22.9, 17.9, and 23.4%, respectively) (Salas-Salvado et al. 2008a).

The prevalence of obesity is one of the greatest public health problems in the industrialized world and is associated with the rapid adaptation to the current lifestyle, the reduction in physical exercise, and the increased consumption of fatty, affordable food. The human genotype has remained unchanged for thousands of years, and the increase in the prevalence of obesity is considered to be the result of the interaction between this genotype and the changing lifestyle of the industrialized countries (Neel 1999). The increase in obesity has arisen not only in the USA (Stunkard 1996) but also in most European countries, especially those around the Mediterranean basin (Papandreou et al. 2008). Since the Seven Countries Study (Keys et al. 1986), the patterns of the Mediterranean diet have changed substantially. Spain, Italy, and Greece have undergone very important changes in lifestyle, eating habits, and physical activity, with an increase in the dietary consumption of energy, animal proteins, and saturated fats. Obesity increases the risk of diabetes, hypertension, coronary disease, and nonalcoholic hepatic steatosis, either independently or within the context of metabolic syndrome (Kopelman 2000; Friedman 2004). Central adiposity, specifically visceral obesity, has been thought to be one of the key factors in the physiopathology of insulin resistance and all the other components of metabolic syndrome. Several components of the Mediterranean diet have been inversely associated with body mass index (BMI) or waist circumference. This is the case for whole grains and fiber (Liese et al. 2004).

Traditionally, nutritional advice for treating obesity has emphasized reducing all kinds of dietary fat and replacing them with carbohydrate. However, there is increasing evidence showing that in Mediterranean countries, restricted energy diets that were relatively high in fat from olive oil may be effective alternatives to the traditional low-fat diet for initial weight loss in obese persons (Schroder et al. 2004). Additionally, usually the Mediterranean diet shows better palatability and compliance, which helps to maintain weight loss (Shai et al. 2008). In contrast to fast food

and unhealthy dietary patterns, which have been consistently associated with a higher risk of weight gain and obesity (Pereira et al. 2005; Bes-Rastrollo et al. 2006), olive oil consumption was associated with a nonsignificant lower likelihood of weight gain in a large Mediterranean cohort of 7,368 individuals, who were followed for a median period of 28.5 months (Bes-Rastrollo et al. 2006). The EPIC Study suggested that adhering to the Mediterranean diet was not associated with becoming overweight. Therefore, promoting eating habits consistent with traditional Mediterranean diet patterns may play a useful part in efforts to combat obesity (Méndez et al. 2006). Moreover, the PREDIMED Study showed weight loss with high MUFA diets after a 3-month intervention (Estruch et al. 2006), which was attributed to a satiating effect of olive oil intake. In this regard, recent experimental evidence suggests that mobilization of intestinally derived oleoylethanolamide, a lipid messenger of satiety, is enabled by uptake of dietary oleic acid (Schwartz et al. 2008).

BMI and waist circumference correlate positively with insulin resistance (Parker et al. 1993; Clausen et al. 1996). Actually, waist circumference has been singled out as the most important risk factor in the new IDF definition of metabolic syndrome. Insulin resistance is one of the key contributors to metabolic syndrome and the development of type-2 diabetes.

There is little evidence that low-fat, high-carbohydrate diets can really improve insulin sensitivity (Reaven 2005). In developed countries, low-fat diets are usually rich in refined carbohydrates, which increase plasma glucose and insulin concentrations. The quality of dietary fat seems to be determinant in the effect of diet on insulin sensitivity. Diets high in SFAs consistently impair both insulin sensitivity and blood lipids, while substituting carbohydrates or MUFAs for SFAs reverts these abnormalities in both healthy (Salas et al. 1999; Vessby et al. 2001) and diabetic cohorts (Parillo et al. 1992). Postprandial lipemia and glucose homeostasis are also improved after meals containing MUFA from olive oil compared to meals rich in SFA (Paniagua et al. 2007).

Back in 1988, a study in patients with type-2 diabetes mellitus demonstrated that a high-fat, MUFA-enriched diet (33% of total energy) resulted in lower insulin requirements and lower plasma glucose concentrations compared to a low-fat (25% total energy), high-carbohydrate diet (60% total energy) (Garg et al. 1988). However, subsequent studies failed to show positive effects of dietary MUFA on fasting insulin in diabetics (Garg 1998). Olive oil, as a single component of the Mediterranean diet, has been studied in less detail. In a cross-sectional study in southern Spain, levels of insulin resistance were found to be lower in people who used olive oil than in those who used sunflower oil (Soriguer et al. 2004). The sectional Pizarra Study showed that dietary MUFA from olive oil and PUFA contributed to the variability of β-cell function (Rojo-Martínez et al. 2006). Ryan et al. (2000) examined the relationship between changes in membrane fatty acid composition and glucose transport and found a reduction in insulin resistance when a linoleic-acid-rich diet was changed to an oleic-acid-rich diet. This was attributed to a reduction in its fluidity when the membrane was enriched in oleic acid.

After a comprehensive review, Ros (2003) concluded that natural foods and olive oil as the main source of MUFAs provided a similar degree of glycemic control. Nevertheless, high-MUFA diets generally had more favorable effects on

proatherogenic alterations associated with the diabetic status, such as dyslipidemia, postprandial lipemia, small LDL, lipoprotein oxidation, inflammation, thrombosis, and endothelial dysfunction. Of particular interest was the ability of an olive-oil-rich Mediterranean diet to improve mild systemic inflammation in subjects with metabolic syndrome in the study of Esposito et al. (2004) and in the PREDIMED Study (Estruch et al. 2006).

The role of minor components has not yet been sufficiently addressed. Phenolic compounds are important in regard to insulin resistance and metabolic syndrome because they inhibit LDL cholesterol oxidation, platelet aggregation, and thromboxane production and stimulate anti-inflammatory components and increase nitric oxide production (Serra-Majem et al. 2003).

18.6.6 Alzheimer's Disease and Parkinson's Disease

Most age-related diseases have been associated with low-grade inflammation triggered and sustained by oxidative stress. Results from animal studies suggest that MUFA-enriched membranes are more resistant to oxidation, protecting the aged cell, mitochondrial structure, and DNA stability (Bello et al. 2006; Quiles et al. 2006). In the last decade, a number of studies have investigated the relationship between adherence to a Mediterranean diet and the development of Alzheimer's and Parkinson's diseases. In a metaanalysis of 12 prospective studies by Sofi et al. (2008), a two-point increase in a score of Mediterranean diet adherence was associated with a significant reduction in the incidence of these conditions.

Alzheimer's disease, the most common form of dementia, is a neurogenerative disease causing cognitive and memory decline, usually in elderly individuals, which is progressive and ultimately fatal (Sofi et al. 2010). Its incidence is increasing in developed countries, but currently there is no cure and little preventive treatment available. In recent years, however, evidence has begun to emerge that suggests that modifying lifestyle factors such as diet may be effective in delaying the onset of the disease and retarding its progression (Pasinetti and Eberstein 2008). Consumption of the Mediterranean diet has been shown to be associated with slower cognitive decline and a reduced risk of mild cognitive impairment and subsequent progression to the full disease (Feart et al. 2010).

There is evidence to suggest that olive oil may contribute to the beneficial effects of the Mediterranean diet on Alzheimer's disease development. High intake of MUFAs, mainly from olive oil, has been identified as being inversely related to cognitive decline in an elderly Italian population, and this effect was particularly marked in people with a low educational level (Solfrizzi et al. 1999; Del Parigi et al. 2006; López-Miranda et al. 2010). In addition, a recent study by Berr et al. (2009), which followed the olive oil intake and cognitive ability of nearly 7,000 elderly subjects, found that moderate (used for cooking or as a dressing) or intensive (used for both) consumption, as compared to no use of the oil, was associated with lower risk of decline in verbal fluency and visual memory. It has been suggested that MUFAs may help to maintain neuronal membrane structure and increase the

fluidity of synaptosomal membranes and, thus, aid neuronal transmission (López-Miranda et al. 2010).

The brain has a low level of endogenous antioxidants and is, therefore, particularly susceptible to oxidative damage, and oxidative stress is thought to play an important role in the development of Alzheimer's disease (Darvesh et al. 2010; Ramesh et al. 2010). For this reason, there is considerable interest in the possible role of dietary antioxidants as a potential therapeutic approach. Plant polyphenols have been the most widely studied, and compounds found in tea, red wine, and grape seed extract have been shown to have beneficial effects on neurodegeneration (Ramesh et al. 2010). Moreover, it was reported in two recent studies that oleocanthal, a minor component of the polyphenol fraction of VOO, may protect against neurodegeneration (Li et al. 2009; Pitt et al. 2009). No studies to date have specifically addressed the effects of the major olive oil polyphenols on Alzheimer's disease, but the relatively high content of these compounds and other antioxidants in VOO may be a factor in its reported effects in reducing cognitive decline (Berr et al. 2009).

Parkinson's disease is one of the most common age-related neurodegenerative disorders and is characterized clinically by resting tremor, rigidity, bradykinesia, and postural instability. Dietary fatty acids have been studied in association with disease risk, but the results have been inconsistent (Logroscino et al. 1996; Anderson et al. 1999; Chen et al. 2003; de Lau et al. 2005). Omega-3 PUFAs have been suggested to be neuroprotective in rat models of the disease (Delattre et al. 2010). In addition, the Rotterdam Study evaluated the association between intake of unsaturated fatty acids (by dietary assessment) and its incidence. After a 6-year follow-up, intakes of total fat, MUFAs, and PUFAs were associated with a lower risk (de Lau et al. 2005). However, a case–control study carried out in Japan, involving 249 cases of Parkinson's disease, found no decrease in risk with consumption of total fat, SFAs, MUFAs, and n-6 and n-3 PUFAs (Miyake et al. 2010).

Gao et al. (2007) examined the associations between dietary patterns, including the Mediterranean diet, and risk of Parkinson's disease in the Health Professionals Follow-Up Study and the Nurses' Health Study. Compared to Western diets, a "healthy diet" rich in fruit, vegetables, and fish with a low intake of saturated fat and a moderate intake of alcohol was inversely associated with that risk. In spite of this result and that the effect of olive oil was not specifically addressed in those studies, in these cohorts, the intake of vitamin E and carotenoids was not associated with a reduction in the risk of Parkinson's disease (Zhang et al. 2002). However, a subsequent metaanalysis of observational studies investigating the effect of vitamin C, vitamin E, and β-carotene intake on the risk of the disease found a neuroprotective effect of vitamin E (Etminan et al. 2005).

18.6.7 Cancer

A high number of cancers in humans are induced by carcinogenic factors present in our environment, including our food. Actually, it is estimated that approximately

one-third of all cancer deaths are related to dietary factors and reduced physical activity. The incidence of all kinds of cancer in Mediterranean countries is lower than in the rest of Europe and the USA. This is mostly accounted for by the lower incidence of large-bowel, breast, endometrial, and prostate cancers, which have been linked to dietary factors, particularly low consumption of vegetables and fruit and, to a certain extent, high consumption of meat. Additionally, epidemiological data suggest an inverse correlation between regular consumption of olive oil and cancer risk (Levi et al. 1999; Calza et al. 2001). This hypothesis has been supported by animal studies that showed a protective effect of olive oil against the UV-induced damage of the skin (Budiyanto et al. 2000) and its ability to prevent the colon crypt aberrant foci growth and colon carcinoma in rats (Bartoli et al. 2000).

It has been estimated that up to 25% of colorectal, 15% of breast, and 10% of prostate, pancreas, and endometrial cancers could be prevented by shifting to a healthy Mediterranean diet (Trichopoulou et al. 2000). Long-term consumption of olive oil, but not n-6 PUFAs, has been reported to be associated with decreased risk of breast cancer (Martin-Moreno et al. 1994; Trichopoulou 1995; Hunter et al. 1996; Holmes et al. 1999), but there are also contradictory results showing no beneficial effects derived from olive oil intake (Yu et al. 1990; Gaard et al. 1995). All efforts to achieve a relationship between dietary fatty acids and colorectal cancer, however, have failed. A combined analysis of 13 case–control studies indicated that there was essentially no association of intake of total, saturated, monounsaturated, or polyunsaturated fats with the risk of colorectal cancer (Howe et al. 1997).

In contrast, a series of epidemiological studies conducted in Italy indicated that there was an inverse relation between breast and ovarian cancer risk with the intake of olive oil, but not of butter or margarine (Lipworth et al. 1997; Bosetti et al. 2002b). However, the most consistent evidence for a favorable role of olive oil came for upper digestive and respiratory tract cancers. High intake of olive oil has been associated with significantly lowered risk of pharyngeal, laryngeal, and esophageal cancers compared to seed oils, margarine, and butter (Franceschi et al. 1999; Bosetti et al. 2000, 2002a), but no association with colorectal cancer risk was found in these studies (Galeone et al. 2007). Nevertheless, it is likely that the observed associations with olive oil are due not only to its specific components but to the fact that higher consumption of olive oil is an indicator of healthier dietary habits, with a more frequent consumption of vegetables, and possibly of other beneficial lifestyle factors.

The beneficial effect of olive oil against cancer has been attributed to its antioxidant properties due to the presence of oleic acid and minor components with biological activity, such as vitamin E, sterols, and polyphenols (Owen et al. 2000b). Carcinogens present in our diet can damage DNA directly by forming covalent adducts with DNA or indirectly after being activated from inactive procarcinogens or via their induction of ROS production. Other carcinogens are not genotoxic but stimulate cell proliferation, thereby increasing the probability of spontaneous occurrence of errors during DNA replication. Phenolic compounds can directly scavenge radical species by acting as chain-breaking antioxidants and suppress lipid peroxidation by recycling other antioxidants, such as α-tocopherol, by donating a hydrogen atom to the tocopherol molecule. However, there is little experimental evidence

indicating a potential role of VOO phenolics against cancer (Ragione et al. 2000; Babich and Visioli 2003). β-sitosterol may be protective against breast cancer cells, as it has been shown to inhibit tumor cell invasion and cell growth by 70% compared with controls (Awad et al. 2001). α-tocopherol has also been shown to inhibit the growth of human prostate and colon cancer cells by 86% in the androgen-independent prostate cancer cell line DU-145, 74% in the androgen-dependent prostate cancer cell line LNCaP, and 64% in human colon adenocarcinoma (Caco-2) cells compared to control (Gysin et al. 2002).

In the search for antitumor promoters from natural sources, the anticancer effects of pentacyclic triterpenes from olive oil have attracted attention (Hsu et al. 1997; Ukiya et al. 2002). Triterpenic acids are potent inducers of apoptosis in human colon (Juan et al. 2006; Reyes et al. 2006) and brain cancer cells (Martín et al. 2007). In human HT-29 colon cancer cells, oleanolic acid showed moderate antiproliferative activity and moderate cytotoxicity at high concentrations (greater than or equal to 250 μmol/L). In contrast to maslinic acid, oleanolic acid, which lacks a hydroxyl group at the carbon 2 position, failed to activate caspase-3 as a prime apoptosis protease. Induction of apoptosis by maslinic acid was confirmed microscopically by an increase in plasma membrane permeability and detection of DNA fragmentation (Juan et al. 2008). Astrocytomas are among the most common and aggressive types of primary malignant tumors in the neurological system that lack effective treatments. In the human 1321N1 astrocytoma cell line, the triterpenic diols, erythrodiol and uvaol, effectively modulated cell proliferation and the apoptotic response, promoting nuclear condensation and fragmentation. At the molecular level, changes in the expression of surface proteins associated with adhesion or death processes were also observed. Moreover, triterpene exposure correlated with the activation of c-Jun N-terminal kinases (JNK) (Martín et al. 2009).

Most of the aforementioned effects may be associated with reduced ROS production. ROS concentrations are lower when dietary n-6 PUFA are replaced by VOO and, in turn, there is a decrease in the level of exocyclic DNA adducts (Weisburger 1997; Hong et al. 2000; Owen et al. 2000c).

18.7 Future Trends and Perspectives

The Mediterranean diet is believed to be associated with numerous health benefits, with the consequence of increased longevity and lower incidence of chronic diseases, including cardiovascular disease, cancer, and neurodegenerative conditions. Olive oil has a crucial role in these health-promoting effects, but its benefits are due not only to its high MUFA content but also to the many micronutrients present in the oil.

Consumption of olive oil has anti-inflammatory effects by lowering plasma levels of cytokines and adhesion molecules and by reducing the induction of monocyte adhesion to the endothelium. In addition to its anti-inflammatory properties, it helps to protect against cardiovascular disease by reducing plasma cholesterol levels, protecting against the oxidation of LDL, lowering blood pressure, and attenuating

platelet aggregation. In addition, there is increasing evidence showing that in Mediterranean countries, diets relatively high in fat from olive oil may be effective alternatives to the traditional low-fat diet for initial weight loss in obese persons, which is attributed to a satiating effect of olive oil intake.

The mechanisms by which olive oil exerts its health-promoting effects are not yet fully understood, but it is now clear that the MUFA content alone is not sufficient and that minor components must also be considered. However, the role of these minor constituents has not yet been addressed in detail. Phenolic compounds have been the most widely investigated, and it is now known that they can inhibit LDL cholesterol oxidation, platelet aggregation, and thromboxane production and that they stimulate anti-inflammatory components and increase nitric oxide production. However, there is still a great deal of work to do regarding the components of the unsaponifiable fraction of olive oil. These include compounds such as phytosterols and tocopherols, which, apart from their well-known hypocholesterolemic and antioxidant effects, respectively, have also been shown to be active against inflammation. Another group of components that has been receiving attention lately is the terpenoids, encompassing acids and alcohols, which are found in low concentrations in olive oil. It has been claimed that these components not only may have anti-inflammatory properties but can be also beneficial against blood pressure and carcinogenesis.

Further investigations of the properties of the multitude of minor compounds found in olive oil, therefore, are likely help to explain not only some of the classic beneficial effects of the Mediterranean diet but also some of the more recently discovered effects on the pathophysiology of atherosclerosis, metabolic syndrome, diabetes, cancer, and neurodegenerative disorders.

References

Akasbi M, Shoeman CW, Csallany AS (1993) High-performance liquid-chromatography of selected phenolic-compounds in olive oils. J Am Oil Chem Soc 70:367–370

Alberti KG, Zimmet PZ (1998) Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med 15:539–553

Alberti KG, Zimmet P, Shaw J (2006) Metabolic syndrome a new world-wide definition. A consensus statement from the International Diabetes Federation. Diabet Med 23:469–480

Albertini R, Moratti R, DeLuca G (2002) Oxidation of low-density lipoprotein in atherosclerosis from basic biochemistry to clinical studies. Curr Mol Med 2:579–592

Allender S, Scarborough P, Peto V, Rayner M, |Leal J et al (2008) European cardiovascular disease statistics. European Heart Network. Available from http://www.ehnheart.org/

Alvárez-León EE, Ribas-Barba L, Serra-Majem L (2003) Prevalence of the metabolic syndrome in the population of Canary Islands, Spain. Med Clin (Barc) 120:172–174

Anderson C, Checkoway H, Franklin GM, Beresford S, Smith-Weller T, Swanson PD (1999) Dietary factors in Parkinson's disease: the role of food groups and specific foods. Mov Disord 14:21–27

Aravanis C, Corcondilas A, Dontas AS, Lekos D, Keys A (1970) Coronary heart disease in seven countries. IX. The Greek islands of Crete and Corfu. Circulation 41(4 Suppl):I88–I100

Assy N, Nassar F, Nasser G, Grosovski M (2009) Olive oil consumption and non-alcoholic fatty liver disease. World J Gastroenterol 15:1809–1815

Athyros VG, Ganotakis ES, Elisaf M, Mikhailidis DP (2005) The prevalence of the metabolic syndrome using the National Cholesterol Educational Program and International Diabetes Federation definitions. Curr Med Res Opin 21:1157–1159

Awad AB, Williams H, Fink CS (2001) Phytosterols reduce in vitro metastatic ability of MDA-MB-231 human breast cancer cells. Nutr Cancer 40:157–164

Azadbakht L, Mirmiran P, Esmaillzadeh A, Azizi T, Azizi F (2005) Beneficial effects of a dietary approach to stop hypertension eating plan on features of the metabolic syndrome. Diabetes Care 28:2823–2831

Babich H, Visioli F (2003) In vitro cytotoxicity to human cells in culture of some phenolics from olive oil. Farmaco 58:403–407

Babio N, Bullo M, Basora J, Martınez-Gonzalez MA, Fernandez-Ballart J et al (2009) Adherence to the Mediterranean diet and risk of metabolic syndrome and its components. Nutr Metab Cardiovasc Dis 19:563–570

Balkau B, Charles MA (1999) Comment on the provisional report from the WHO consultation. European Group for the Study of Insulin Resistance (EGIR). Diabet Med 16:442–443

Bamia C, Trichopoulos D, Ferrari P, Overvad K, Bjerregaard L et al (2007) Dietary patterns and survival of older Europeans: the EPIC-Elderly study (European prospective investigation into cancer and nutrition). Public Health Nutr 10:590–598

Barceló F, Perona JS, Prades J, Funari SS, Gomez-Gracia E et al (2009) Mediterranean-style diet effect on the structural properties of the erythrocyte cell membrane of hypertensive patients: the Prevencion con Dieta Mediterranea Study. Hypertension 54:1143–1150

Bartoli R, Fernandez-Banares F, Navarro E, Castella E, Mane J et al (2000) Effect of olive oil on early and late events of colon carcinogenesis in rats: modulation of arachidonic acid metabolism and local prostaglandin E(2) synthesis. Gut 46:191–199

Beachamp GK, Keast RS, Morel D, Lin J, Pika J et al (2005) Phytochemistry: ibuprofen-like activity in extra virgin olive oil. Nature 437:45–46

Bellido C, López-Miranda J, Blanco-Colio LM, Pérez-Martínez P, Muriana FJ et al (2004) Butter and walnuts, but not olive oil, elicit postprandial activation of nuclear transcription factor-κB in peripheral blood mononuclear cells from healthy men. Am J Clin Nutr 80:1487–1491

Bello RI, Gómez-Díaz C, Burón MI, Navas P, Villalba JM (2006) Differential regulation of hepatic apoptotic pathways by dietary olive and sunflower oils in the aging rat. Exp Gerontol 41:1174–1184

Benítez-Sánchez PL, León-Camacho M, Aparicio R (2003) A comprehensive study of hazelnut oil composition with comparisons to other vegetable oils, particularly olive oil. Eur Food Res Technol 18:13–19

Berbet AA, Kondo CR, Almendra CL, Matsuo T, Dichi I (2005) Supplementation of fish oil and olive oil in patients with rheumatoid arthritis. Nutrition 21:131–136

Berr C, Portet F, Carriere I, Akbaraly TN, Feart C et al (2009) Olive oil and cognition: results from the three-city study. Dement Geriatr Cogn Disord 28:357–364

Bes-Rastrollo M, Sanchez-Villegas A, de la Fuente C, de Irala J, Martinez JA et al (2006) Olive oil consumption and weight change: the SUN prospective cohort study. Lipids 41:249–256

Bester D, Esterhuyse AJ, Truter EJ, Van Rooyen J (2010) Cardiovascular effects of edible oils: a comparison between four popular edible oils. Nutr Res Rev 23:334–348

Bogani P, Galli C, Villa M, Visioli F (2006) Postprandial anti-inflammatory and antioxidant effects of extra virgin olive oil. Atherosclerosis 190:181–186

Bosetti C, La Vecchia C, Talamini R, Simonato L, Zambon P et al (2000) Food groups and risk of squamous cell esophageal cancer in northern Italy. Int J Cancer 87:289–294

Bosetti C, La Vecchia C, Talamini R, Negri E, Levi F et al (2002a) Food groups and laryngeal cancer risk: a case–control study from Italy and Switzerland. Int J Cancer 100:355–360

Bosetti C, Negri E, Franceschi S, Talamini R, Montella M et al (2002b) Olive oil, seed oils and other added fats in relation to ovarian cancer (Italy). Cancer Causes Control 13:465–470

Bosetti C, Pelucchi C, La Vecchia C (2009) Diet and cancer in Mediterranean countries: carbohydrates and fats. Public Health Nutr 12:1595–1600
Boskou D (2000) Olive oil. World Rev Nutr Diet 87:56–77
Bravo E, Napolitano M, Botham KM (2010) Postprandial lipid metabolism: the missing link between life-style habits and the increasing incidence of metabolic diseases in western countries? Open Trans Med J 2:1–13
Brunelleschi S, Bardelli C, Amoruso A, Gunella G, Ieri F et al (2007) Minor polar compounds extra-virgin olive oil extract (MPC-OOE) inhibits NF-kappa B translocation in human monocyte/macrophages. Pharmacol Res 56:542–549
Budiyanto A, Ahmed NU, Wu A, Bito T, Nikaido O et al (2000) Protective effect of topically applied olive oil against photocarcinogenesis following UVB exposure of mice. Carcinogenesis 21:2085–2090
Calza S, Ferraroni M, La Vecchia C, Franceschi S, Decarli A (2001) Low-risk diet for colorectal cancer in Italy. Eur J Cancer Prev 10:515–521
Carretero OA, Oparil S (2000) Essential hypertension. Part I: definition and etiology. Circulation 101:329–335
Castro-Rodríguez JA, García-Marcos L, Sánchez-Solis M, Pérez-Fernández V, Mártinez-Torres A et al (2010) Olive oil during pregnancy is associated with reduced wheezing during the first year of life of the offspring. Pediatr Pulmonol 45:395–402
Chan YM, Demonty I, Pelled D, Jones PJ (2007) Olive oil containing fatty acid esters of plant sterols and dietary diacylglycerol reduces low-density lipoprotein cholesterol and decreases the tendency for peroxidation in hypercholesterolemic patients. Br J Nutr 98:563–570
Chen H, Zhang SM, Hernán MA, Willett WC, Ascherio A (2003) Dietary intakes of fat and risk of Parkinson's disease. Am J Epidemiol 157:1007–1014
Cicerale S, Lucas L, Keast R (2010) Biological activities of phenolic compounds present in virgin olive oil. Int J Mol Sci 11(2):458–479
Cicero AF, Nascetti S, López-Sabater MC, Elosua R, Salonen JT et al (2008) Changes in LDL fatty acid composition as a response to olive oil treatment are inversely related to lipid oxidative damage: the EUROLIVE study. J Am Coll Nutr 27:314–320
Clausen JO, Borch-Johnsen K, Ibsen H, Bergman RN et al (1996) Insulin sensitivity index, acute insulin response, and glucose effectiveness in a population-based sample of 380 young healthy Caucasians. Analysis of the impact of gender, body fat, physical fitness, and life-style factors. J Clin Invest 98:1195–1209
Corona G, Tzounis X, Assunta-Dessa M, Deiana M, Debnam ES et al (2006) The fate of olive oil polyphenols in the gastrointestinal tract: implications of gastric and colonic microflora-dependent biotransformation. Free Radic Res 40:647–658
Correa JA, López-Villodres JA, Asensi R, Espartero JL, Rodríguez-Gutiérez G et al (2009) Virgin olive oil polyphenol hydroxytyrosol acetate inhibits in vitro platelet aggregation in human whole blood: comparison with hydroxytyrosol and acetylsalicylic acid. Br J Nutr 101:1157–1164
Cortés B, Núñez I, Cofán M, Gilabert R, Pérez-Heras A et al (2006) Acute effects of high-fat meals enriched with walnuts or olive oil on postprandial endothelial function. J Am Coll Cardiol 48:1666–1671
Covas MI (2007) Olive oil and the cardiovascular system. Pharmacol Rev 55:175–186
Covas MI, Ruiz-Gutiérrez V, de la Torre R, Kafatos A, Lamuela-Raventós RM et al (2006) Minor components of olive oil: evidence to date of health benefits in humans. Nutr Rev 64(Suppl 1):20–30
Covas MI, Konstantinidou V, Fito M (2009) Olive oil and cardiovascular health. J Cardiovasc Pharmacol 54:477–482
Darvesh AS, Carroll RT, Bishayee A, Geldenhuys WJ, Van der Schyf CJ (2010) Oxidative stress and Alzheimer's disease: dietary polyphenols as potential therapeutic agents. Expert Rev Neurother 10:729–745
de la Puerta R, Martínez-Domínguez E, Sánchez Perona J, Ruiz-Gutiérrez V (2004) Effects of different dietary oils on inflammatory mediator generation and fatty acid composition in rat neutrophils. Metabolism 53:59–65

de la Puerta R, Márquez-Martin A, Fernández-Arche A, Ruiz-Gutiérrez V (2009) Influence of dietary fat on oxidative stress and inflammation in murine macrophages. Nutrition 25:548–554
de la Torre-Carbot K, Chávez-Servín JL, Jaúregui O, Castellote AI, Lamuela-Raventós RM et al (2010) Elevated circulating LDL phenol levels in men who consumed virgin rather than refined olive oil are associated with less oxidation of plasma LDL. J Nutr 140:501–508
de Lau LM, Bornebroek M, Witteman JC, Hofman A, Koudstaal PJ et al (2005) Dietary fatty acids and the risk of Parkinson disease: the Rotterdam study. Neurology 64:2040–2045
de Lorgeril M, Salen P, Martin JL, Monjaud I, Delaye J et al (1999) Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyon Diet Heart Study. Circulation 99:779–785
de Winther MP, Kanters E, Kraal G, Hofker MH (2005) Nuclear factor kappa B signaling in atherogenesis. Arterioscler Thromb Vasc Biol 25:904–914
Del Parigi A, Panza F, Capurso C, Solfrizzi V (2006) Nutritional factors, cognitive decline and dementia. Brain Res Bull 69:1–19
Delattre AM, Kiss A, Szawka RE, Anselmo-Franci JA, Bagatini PB et al (2010) Evaluation of chronic omega-3 fatty acids supplementation on behavioral and neurochemical alterations in 6-hydroxydopamine-lesion model of Parkinson's disease. Neurosci Res 66:256–264
Delgado-Lista J, Lopez-Miranda J, Cortés B, Perez-Martinez P, Lozano A et al (2008) Chronic dietary fat intake modifies the postprandial response of hemostatic markers to a single fatty test meal. Am J Clin Nutr 87:317–322
Dell'Agli M, Fagnani R, Galli GV, Maschi O, Gilardi F et al (2010) Olive oil phenols modulate the expression of metalloproteinase 9 in THP-1 cells by acting on nuclear factor-kappaB signaling. J Agric Food Chem 58:2246–2252
Dell'Agli M, Maschi O, Galli GV, Fagnani R, Dal Cero E et al (2008) Inhibition of platelet aggregation by olive oil phenols via cAMP-phosphodiesterase. Br J Nutr 99:945–951
El Seweidy MM, El-Swefy SE, Abdallah FR, Hashem RM (2005) Dietary fatty acid unsaturation levels, lipoprotein oxidation and circulating chemokine in experimentally induced atherosclerotic rats. J Pharm Pharmacol 57:1467–1474
Esmaillzadeh A, Kimiagar M, Mehrabi Y, Azadbakht L, Hu FB et al (2007) Dietary patterns, insulin resistance, and prevalence of the metabolic syndrome in women. Am J Clin Nutr 85:910–918
Esposito K, Marfella R, Ciotola M, Di Palo C, Giugliano F et al (2004) Effect of a mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: a randomized trial. JAMA 292:1440–1446
Estruch R, Martínez-González MA, Corella D, Salas-Salvadó J, Ruiz-Gutiérrez V et al (2006) Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial. Ann Intern Med 145:1–11
Escrich E, Moral R, Grau L, Costa I, Solanas M (2007) Molecular mechanisms of the effects of olive oil and other dietary lipids on cancer. Mol Nutr Food Res 51:1279–1292
Etminan M, Gill SS, Samii A (2005) Intake of vitamin E, vitamin C, and carotenoids and the risk of Parkinson's disease: a meta-analysis. Lancet Neurol 4:362–365
Feart C, Samieri C, Barberger-Gateau P (2010) Mediterranean diet and cognitive function in older adults. Curr Opin Clin Nutr Metab Care 13:14–18
Fernández-Arche A, Márquez-Martín A, de la Puerta VR, Perona JS, Terencio C et al (2009) Long chain fatty alcohols from pomace olive oil modulate the release of proinflammatory mediators. J Nutr Biochem 20:155–162
Ferrara LA, Raimondi AS, d'Episcopo L, Guida L, Dello Russo A et al (2000) Olive oil and reduced need for antihypertensive medications. Arch Intern Med 160:837–842
Fitó M, Cladellas M, de la Torre R, Martí J, Alcántara M et al (2005) Antioxidant effect of virgin olive oil in patients with stable coronary heart disease: a randomized, crossover, controlled, clinical trial. Atherosclerosis 181:149–158
Fitó M, Guxens M, Corella D, Sáez G, Estruch R et al (2007) Effect of a traditional Mediterranean diet on lipoprotein oxidation: a randomized controlled trial. Arch Intern Med 167:1195–1203
Fito M, de la Torre R (2007) Olive oil and oxidative stress. Mol Nutr Food Res 51:1215–1224

Fito M, Cladellas M, de la Torre R, Marti J, Muñoz D et al (2008) Anti-inflammatory effect of virgin olive oil in stable coronary disease patients: a randomized, crossover, controlled trial. Eur J Clin Nutr 62:570–574

Ford ES, Giles WH, Dietz WH (2002) Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. JAMA 287:356–359

Ford ES, Giles WH, Mokdad AH (2004) Increasing prevalence of the metabolic syndrome among US adults. Diabetes Care 27:2444–2449

Franceschi S, Favero A, Conti E, Talamini R, Volpe R et al (1999) Food groups, oils and butter, and cancer of the oral cavity and pharynx. Br J Cancer 80:614–620

Friedman JM (2004) Modern science versus the stigma of obesity. Nat Med 10:563–569

Fuentes F, López-Miranda J, Sánchez E, Sánchez F, Paez J et al (2001) Mediterranean and low-fat diets improve endothelial function in hypercholesterolemic men. Ann Intern Med 134:1115–1119

Fuentes F, López-Miranda J, Pérez-Martínez P, Jiménez Y, Marín C et al (2008) Chronic effects of a high-fat diet enriched with virgin olive oil and a low-fat diet enriched with alpha-linolenic acid on postprandial endothelial function in healthy men. Br J Nutr 100:159–165

Furie B, Furie BC (2008) Mechanisms of thrombus formation. N Engl J Med 359:938–949

Gaard M, Tretli S, Løken EB (1995) Dietary fat and the risk of breast cancer: a prospective study of 25,892 Norwegian women. Int J Cancer 63(1):13–17

Galeone C, Talamini R, Levi F, Pelucchi C, Negri E et al (2007) Fried foods, olive oil and colorectal cancer. Ann Oncol 18:36–39

Gao X, Chen H, Fung TT, Logroscino G, Schwarzschild MA et al (2007) Prospective study of dietary pattern and risk of Parkinson disease. Am J Clin Nutr 86:1486–1494

Gardner CD, Kraemer HC (1995) Monounsaturated versus polyunsaturated dietray fat and serum lipids. A meta-anaylsis. Arterioscler Thromb Vasc Biol 15:1917–1927

Garg A (1998) High-monounsaturated-fat diets for patients with diabetes mellitus: a meta-analysis. Am J Clin Nutr 67:577S–582S

Garg A, Bonanome A, Grundy SM, Zhang ZJ, Unger RH (1988) Comparison of a high-carbohydrate diet with a high- monounsaturated-fat diet in patients with non-insulin- dependent diabetes mellitus. N Engl J Med 319:829–834

Genest J (2010) C-reactive protein: risk factor, biomarker and/or therapeutic target? Can J Cardiol 26(Suppl A):41A–44A

Gilani AH, Khan AU, Shah AJ, Connor J, Jabeen Q (2005) Blood pressure lowering effect of olive is mediated through calcium channel blockade. Int J Food Sci Nutr 56:613–620

Ginda A, Lanzón A, Albi T (1996) Differences in hydrocarbons of virgin olive oils obtained from several olive varieties. J Agric Food Chem 44(7):1723–1726

González-Correa JA, Navas MD, Muñoz-Marín J, Trujillo M, Fernández-Bolaños J et al (2008) Effects of hydroxytyrosol and hydroxytyrosol acetate administration to rats on platelet function compared to acetylsalicylic acid. J Agric Food Chem 56:7872–7876

Grundy SM, Brewer B Jr, Cleeman JI, Smith SC Jr, Lenfant C (2004) Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. Circulation 109:433–438

Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH et al (2005) Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung and Blood Institute scientific statement. Circulation 112:2735–2752

Guallar-Castillón P, Rodríguez-Artalejo F, Tormo MJ, Sánchez MJ, Rodríguez L et al (2010) Major dietary patterns and risk of coronary heart disease in middle-aged persons from a Mediterranean country: the EPIC-Spain cohort study. Nutr Metab Cardiovasc Dis 22:129–199

Gutfinger T (1981) Plyphenols in olive oils. J Am Oil Chem Soc 58:966–968

Gysin R, Azzi A, Visarius T (2002) Gamma-tocopherol inhibits human cancer cell cycle progression and cell proliferation by down-regulation of cyclins. FASEB J 16:1952–1954

Haveman-Nies A, de Groot LP, Burema J, Cruz JA, Osler M et al (2002) Dietary quality and lifestyle factors in relation to 10-year mortality in older Europeans: the SENECA study. Am J Epidemiol 156:962–968

Herrera E, Barbas C (2001) Vitamin E: action, metabolism and perspectives. J Physiol Biochem 57:43–56
Holmes MD, Hunter DJ, Colditz GA, Stampfer MJ, Hankinson SE et al (1999) Association of dietary intake of fat and fatty acids with risk of breast cancer. JAMA 281:914–920
Hong MY, Lupton JR, Morris JS, Wang N, Carrol RJ et al (2000) Dietary fish oil reduces O^6-methylguanine DNA adduct levels in rat colon in part by increasing apoptosis during tumour initiation. Cancer Epidemiol Biomarkers Prev 9:819–826
Howe GR, Aronson KJ, Benito E, Castelleto R, Cornée J et al (1997) The relationship between dietary fat intake and risk of colorectal cancer: evidence from the combined analysis of 13 case–control studies. Cancer Causes Control 8:215–228
Hsu HY, Yang JJ, Lin CC (1997) Effects of oleanolic acid and ursolic acid on inhibiting tumor growth and enhancing the recovery of hematopoietic system postirradiation in mice. Cancer Lett 111:7–13
Hu FB, Willett WC (2002) Optimal diets for prevention of coronary heart disease. JAMA 288:2569–2578
Huang CL, Sumpio BE (2007) Olive oil, the Mediterranean diet, and cardiovascular health. J Am Coll Surg 207:407–416
Hunter DJ, Spiegelman D, Adami HO, Beeson L, van den Brandt PA et al (1996) Cohort studies of fat intake and the risk of breast cancer-a pooled analysis. N Engl J Med 334:356–361
Iacono A, Gomez R, Sperry J, Conde J, Bianco G et al (2010) Effect of oleocanthal and its derivatives on inflammatory response induced by lipopolysaccharide in a murine chondrocyte cell line. Arthritis Rheum 62:1675–1682
IDF consensus worldwide definition of the metabolic syndrome (2006) Available http://www.idf.org/webdata/docs/MetS_def_update2006.pdf
Jiménez-Gómez Y, López-Miranda J, Blanco-Colio LM, Marín C, Pérez-Martínez P et al (2009) Olive oil and walnut breakfasts reduce the postprandial inflammatory response in mononuclear cells compared with a butter breakfast in healthy men. Atherosclerosis 204:70–76
Juan ME, Wenzel U, Ruiz-Gutiérrez V, Daniel H, Planas JM (2006) Olive fruit extracts inhibit proliferation and induce apoptosis in HT-29 human colon cancer cells. J Nutr 136: 2553–2557
Juan ME, Planas JM, Ruiz-Gutierrez V, Daniel H, Wenzel U (2008) Antiproliferative and apoptosis-inducing effects of maslinic and oleanolic acids, two pentacyclic triterpenes from olives, on HT-29 colon cancer cells. Br J Nutr 100:36–43
Junker R, Kratz M, Neufeld M, Erren M, Nofer JR et al (2001) Effects of diets containing olive oil, sunflower oil, or rapeseed oil on the hemostatic system. Thromb Haemost 85:280–286
Kavanaugh CJ, Trumbo PR, Ellwood KC (2007) The U.S. food and drug administration's evidence-based review for qualified health claims: tomatoes, lycopene, and cancer. J Natl Cancer Inst 99:1074–1085
Keys A (1965) Effect of dietary fats on plasma lipid levels. Lancet 17:237–242
Keys A, Aravanis C, Blackburn HW, Van Buchem FS, Buzina R et al (1966) Epidemiological studies related to coronary heart disease: characteristics of men aged 40–59 in seven countries. Acta Med Scand Suppl 460:1–392
Keys A, Menotti A, Karvonen MJ, Aravanis C, Blackburn H et al. (1986) The diet and 15-year death rate in the seven countries study. Am J Epidemiol 124:903–915
Kiritsakis A, Markakis P (1987) Effect of olive collection regime on olive oil quality. J Sci Food Agric 35:677–678
Knoops KT, de Groot LC, Kromhout D, Perrin AE, Moreiras-Varela O et al (2004) Mediterranean diet, lifestyle factors, and 10-year mortality in elderly European men and women: the HALE project. JAMA 292:1433–1439
Konstantinidou V, Covas MI, Muñoz-Aguayo D, Khymenets O, de la Torre R et al (2010) In vivo nutrigenomic effects of virgin olive oil polyphenols within the frame of the Mediterranean diet: a randomized controlled trial. FASEB J 24:2546–2557
Konturek JW, Hengst K, Konturek SJ, Sito E, Stachura J et al (1998) Physiological role of cholecystokinin in gastroprotection in humans. Am J Gastroenterol 93:2385–2390
Kopelman PG (2000) Obesity as a medical problem. Nature 404:635–643

Kouris-Blazos A, Gnardellis C, Wahlqvist ML, Trichopoulos D, Lukito W et al (1999) Are the advantages of the Mediterranean diet transferable to other populations? A cohort study in Melbourne, Australia. Br J Nutr 82:57–61

Kris-Etherton P (1999) Monounsaturated fatty acids and risk of cardiovascular disease. Circulation 100:1253–1258

Lairon D (2007) Intervention studies on Mediterranean diet and cardiovacular risk. Mol Nutr Food Res 51:1209–1214

Lanzon A, Albi T, Cert A, Gracian J (1994) The hydrocarbon fraction of virgin olive oil and changes resulting from refining. J Am Oil Chem Soc 71:285–291

Lasheras C, Fernandez S, Patterson AM (2000) Mediterranean diet and age with respect to overall survival in institutionalized, nonsmoking elderly people. Am J Clin Nutr 71:987–992

Lee C, Barnett J, Reavon PD (1998) Liposomes enriched in oleic acid are less susceptible to oxidation and have less proinflammatory activity when exposed to oxidizing conditions. J Lipid Res 39:1239–1247

Léger CL, Carbonneau MA, Michel F, Mas E, Monnier L et al (2005) A thromboxane effect of a hydroxytyrosol-rich olive oil wastewater extract in patients with uncomplicated type I diabetes. Eur J Clin Nutr 59:727–730

Lehner R, Kuksis A (1995) Triacylglycerol synthesis by purified triacylglycerol synthetase of rat intestinal mucosa. Role of acyl-CoA acyltransferase. J Biol Chem 270:13630–13636

Levi F, Pasche C, La Vecchia C, Lucchini F, Franceschi S (1999) Food groups and colorectal cancer risk. Br J Cancer 79:1283–1287

Li W, Sperry JB, Crowe A, Trojanowski JQ, Smith AB et al (2009) Inhibition of tau fibrillization by oleacanthal via reaction with the amino groups of tau. J Neurochem 110:1339–1351

Libby P (2002) Inflammation in atherosclerosis. Nature 420:868–874

Libby P, Ridker PM, Maseri A (2002) Inflammation in atherosclerosis. Circulation 105: 1135–1145

Liese AD, Schulz M, Moore CG, Mayer-Davis EJ (2004) Dietary patterns, insulin sensitivity and adiposity in the Multi-ethnic insulin resistance atherosclerosis study population. Br J Nutr 92:973–984

Linos A, Kaklamani VG, Kaklamani E, Koumantaki Y, Giziaki E et al (1999) Dietary factors in relation to rheumatoid arthritis: a role for olive oil and cooked vegetables? Am J Clin Nutr 70:1077–1082

Lipworth L, Martinez ME, Angell J, Hsieh CC, Trichopoulos D (1997) Olive oil and human cancer: an assessment of the evidence. Prev Med 26:181–190

Lloyd-Jones D, Adams R, Carnethon M, De Simone G, Ferguson TB et al (2009) Heart disease and stroke statistics-2009 update. A report from the American Heart Association statistics committee and stroke statistics subcommittee. Circulation 119:e21–e181

Logroscino G, Marder K, Cote L, Tang MX, Shea S et al (1996) Dietary lipids and antioxidants in Parkinson's disease: a population-based, case–control study. Ann Neurol 39:89–94

López-Miranda J, Delgado-Lista J, Pérez-Martínez P, Jiménez-Gómez Y et al (2007) Olive oil and the hemostatic system. Mol Nutr Food Res 51:1249–1259

López-Miranda J, Pérez-Jiménez F, Ros E, De Caterina R, Badimón L et al (2010) Olive oil and health: summary of the II international conference on olive oil and health consensus report. Nutr Metab Cardiovasc Dis 20:284–294

Lutsey PL, Steffen LM, Stevens J (2008) Dietary intake and the development of the metabolic syndrome: the atherosclerosis risk in communities study. Circulation 117:754–761

Martel J, Gracián J (1962) Minor components of olive oil and sulphur olive oil. I. Alcoholic compound isolated from the unsaponifiable fraction of sulphur olive oil. Grasas Aceites 13: 212–215

Martín R, Carvalho-Tavares J, Ibeas E, Hernández M, Ruiz-Gutierrez V et al (2007) Acidic triterpenes compromise growth and survival of astrocytoma cell lines by regulating reactive oxygen species accumulation. Cancer Res 67:3741–3751

Martín R, Ibeas E, Carvalho-Tavares J, Hernández M, Ruiz-Gutierrez V et al (2009) Natural triterpenic diols promote apoptosis in astrocytoma cells through ROS-mediated mitochondrial depolarization and JNK activation. PLoS One 4(6):e5975

Martin-Moreno JM, Willett WC, Gorgojo L, Banegas JR, Rodríguez-Artalejo F et al (1994) Dietary fat, olive oil intake and breast cancer risk. Int J Cancer 58:774–780

Masala G, Ceroti M, Pala V, Krogh V, Vineis P et al (2007) A dietary pattern rich in olive oil and raw vegetables is associated with lower mortality in Italian elderly subjects. Br J Nutr 98:406–415

Masala G, Bendinelli B, Versari D, Saieva C, Ceroti M et al (2008) Anthropometric and dietary determinants of blood pressure in over 7000 Mediterranean women: the European Prospective Investigation into Cancer and Nutrition-Florence cohort. J Hypertens 26:2112–2120

Materljan E, Materljan M, Materljan B, Vlacic H, Baricev-Novakovic Z et al (2009) Multiple sclerosis and cancers in croatia- a possible protective role of the "Mediterraean diet". Coll Antropol 33:539–545

Mattson FH, Grundy SM (1985) Comparison of effects of dietary saturated, monounsaturated, and polyunsaturated fatty acids on plasma lipids and lipoproteins in man. J Lipid Res 26:194–202

Medzhitov R (2008) Origin and physiological roles of inflammation. Nature 454:428–435

Mena MP, Sacanella E, Vázquez-Agell M, Morales M, Fitó M et al (2009) Inhibition of circulating immune cell activation: a molecular antiinflammatory effect of the Mediterranean diet. Am J Clin Nutr 89:248–256

Méndez MA, Popkin BM, Jakszyn P, Berenguer A, Tormo MJ et al (2006) Adherence to a Mediterranean diet is associated with reduced 3-year incidence of obesity. J Nutr 136: 2934–2938

Miller NE (1982) Coronary atherosclerosis and plasma lipoproteins: epidemiology and pathophysiologic considerations. J Cardiovasc Pharmacol 4(Suppl 2):S190–S195

Mitrou PN, Kipnis V, Thiébaut AC, Reedy J, Subar AF et al (2007) Mediterranean dietary pattern and prediction of all-cause mortality in a US population: results from the NIH-AARP Diet and Health Study. Arch Intern Med 167:2461–2468

Miyake Y, Sasaki S, Tanaka K, Fukushima W, Kiyohara C et al (2010) Dietary fat intake and risk of Parkinson's disease: a case–control study in Japan. J Neurol Sci 288:117–122

Moreno JA, López-Miranda J, Pérez-Martínez P, Marín C, Moreno R et al (2008) A monounsaturated fatty acid-rich diet reduces macrophage uptake of plasma oxidised low-density lipoprotein in healthy young men. Br J Nutr 100:569–575

National Cholesterol Education Program (NCEP) Adult Treatment Panel III (2001) Executive summary of the third report of The National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults. JAMA 285: 2486–2497

Neel JV (1999) The "thrifty genotype" in 1998. Nutr Rev 57:S2–S9

Orchard TJ, Temprosa M, Goldberg R, Haffner S, Ratner R et al (2005) The effect of metformin and intensive lifestyle intervention on the metabolic syndrome: the Diabetes Prevention Program randomized trial. Ann Intern Med 142:611–619

Osler M, Scroll M (1997) Diet and mortality in a cohort of elderly people in a North European community. Int J Epidemiol 26:155–159

Owen RW, Mier W, Giacosa A, Hull WE, Spiegelhalder B et al (2000a) Phenolic compounds and squalene in olive oils: the concentration and antioxidant potential of total phenols, simple phenols, secoroids, lignans and squalene. Food Chem Toxicol 38:647–659

Owen RW, Giacosa A, Hull WE, Haubner R, Wurtele G et al (2000b) Olive-oil consumption and health: the possible role of antioxidants. Lancet Oncol 1:107–112

Owen RW, Giacosa A, Hull WE, Haubner R, Spiegelhalder B et al (2000c) The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. Eur J Cancer 36: 1235–1247

Pacheco YM, Bermúdez B, López S, Abia R, Villar J et al (2007) Minor compounds of olive oil have postprandial anti-inflammatory effects. Br J Nutr 98:260–263

Pacheco YM, Lopez S, Bermúdez B, Abia R, Villar J et al (2008) A meal rich in oleic acid beneficially modulates postprandial sICAM- and sVCAM-1 in normotensive and hypertensive hypertriglyceridemic subjects. J Nutr Biochem 19:200–205

Paniagua JA, de la Sacristana AG, Sánchez E, Romero I, Vidal-Puig A et al (2007) A MUFA-rich diet improves posprandial glucose, lipid and GLP-1 responses in insulin-resistant subjects. J Am Coll Nutr 26:434–344

Papandreou C, Mourad TA, Jildeh C, Abdeen Z, Philalithis A et al (2008) Obesity in Mediterranean region (1997–2007): a systematic review. Obes Rev 9:389–399

Parillo M, Rivellese AA, Ciardullo AV, Capaldo B, Giacco A et al (1992) A high-monounsaturated-fat/low-carbohydrate diet improves peripheral insulin sensitivity in non-insulin- dependent diabetic patients. Metabolism 41:1373–1378

Parker DR, Weiss ST, Troisi R, Cassano PA, Vokonas PS et al (1993) Relationship of dietary saturated fatty acids and body habitus to serum insulin concentrations: the Normative Aging Study. Am J Clin Nutr 58:129–136

Pasinetti GM, Eberstein JA (2008) Metabolic syndrome and the role of dietary lifestyles in Alzheimer's disease. J Neurochem 106:1503–1514

Pereira MA, Kartashov AI, Ebbeling CB, Van Horn L, Slattery ML et al (2005) Fast- food habits, weight gain, and insulin resistance (the CARDIA Study): 15-year prospective analysis. Lancet 365:36–42

Perez-Camino MC, Cert A (1999) Quantitative determination of hydroxyl pentacyclic triterpene acids in vegetable oils. J Agric Food Chem 47:1558–1562

Pérez-Jiménez F (2005) International conference on the healthy effect of virgin olive oil. Eur J Clin Invest 35:421–424

Pérez-Jiménez F, Ruano J, Pérez-Martínez P, López-Segura F, López-Miranda J (2007) The influence of olive oil on human health: not a question of fat alone. Mol Nutr Food Res 51:1199–1208

Pérez-López FR, Chedraui P, Haya J, Cuadros JL (2009) Effects of the Mediterranean diet on longevity and age-related morbid conditions. Maturitas 64:67–79

Perona JS, Cabello-Moruno R, Ruiz-Gutiérrez V (2006) The role of virgin olive oil components in the modulation of endothelial function. J Nutr Biochem 17:429–445

Perona JS, Martínez-González J, Sanchez-Domínguez JM, Badimon L, Ruiz-Gutierrez V (2004) The unsaponifiable fraction of virgin olive oil in chylomicrons from men improves the balance between vasoprotective and prothrombotic factors released by endothelial cells. J Nutr 134:3284–3289

Pitt J, Roth W, Lacor P, Smith AB, Blankenship M et al (2009) Alzheiemer's-associated Abeta oligomers show altered structure, immunoreactivity andd synaptotoxicity with low doses of oleacanthal. Toxicol Appl Pharmacol 240:189–197

Psomiadou E, Tsimidou M (2001) Pigments in Greek virgin olive oils: occurrence and levels. J Sci Food Agric 81:640–647

Psaltopoulou T, Naska A, Orfanos P, Trichopoulos D, Mountokalakis T et al (2004) Olive oil, the Mediterranean diet, and arterial blood pressure: the Greek European Prospective Investigation into Cancer and Nutrition (EPIC) study. Am J Clin Nutr 80:1012–1018

Quiles JL, Ochoa JJ, Ramírez-Tortosa MC, Huertas JR, Mataix J (2006) Age-related mitochondrial DNA deletion in rat liver depends on dietary fat unsaturation. J Gerontol A Biol Sci Med Sci 61:107–114

Raederstorff D (2009) Antioxidant activity of olive polyphenols in humans: a review. Int J Vit Nutr Res 79:152–165

Ragione FD, Cucciolla V, Borriello A, Pietra VD, Pontoni G et al (2000) Hydroxytyrosol, a natural molecule occurring in olive oil, induces cytochrome c-dependent apoptosis. Biochem Biophys Res Commun 278(3):733–739

Ramesh BN, Rao TS, Prakasam A, Sambamutri K, Rao KS (2010) J Alzheimer's Dis 19:1123–1139

Ramirez M, Amate L, Gil A (2001) Absorption and distribution of dietary fatty acids from different sources. Early Hum Dev 65:S95–S101

Rastrelli L, Passi S, Ippolito F, Vacca G, De Simone F (2002) Rate of degradation of alpha-tocopherol, squalene, phenolics, and polyunsaturated fatty acids in olive oil during different storage conditions. J Agric Food Chem 50:5566–5570

Reaven GM (2005) The insulin resistance syndrome: definition and dietary approaches to treatment. Annu Rev Nutr 25:391–406
Reaven PD, Witztum JL (1996) Oxidized low density lipoproteins in atherogenesis: role of dietary modification. Annu Rev Nutr 16:51–71
Renaud SC, Ruf JC, Petithory D (1995) The positional distribution of fatty acids in palm oil and lard influences their biologic effects in rats. J Nutr 125:229–237
Reyes FJ, Centelles JJ, Lupianez JA, Cascante M (2006) (2Alpha,3beta)-2,3-dihydroxyolean-12-en-28-oicacid, a new natural triterpene from Olea europea, induces caspase dependent apoptosis selectively in colon adenocarcinoma cells. FEBS Lett 580:6302–6310
Ringbom T, Segura L, Noreen Y, Perera P, Bohlin L (1998) Ursolic acid from plantago major, a selective inhibitor of cyclooxygenase-2 catalyzed prostaglandin biosynthesis. J Nat Prod 61:1212–1215
Roberfroid MB (2002) Global view on functional foods: European perspectives. Br J Nutr 88 (suppl 2):S133–S138
Rojo-Martínez G, Esteva I, Ruiz de Adana MS, García-Almeida JM, Tinahones F et al (2006) Dietary fatty acids and insulin secretion: a population-based study. Eur J Clin Nutr 60:1195–1200
Ros E (2003) Dietary cis-monounsaturated fatty acids and metabolic control in type 2 diabetes. Am J Clin Nutr 78:617S–625S
Ruíz-Gutiérrez V, Muriana FJ, Guerrero A, Cert AM, Villar J (1996) Plasma lipids, erythrocyte membrane lipids and blood pressure of hypertensive women after ingestion of dietary oleic acid from two different sources. J Hypertens 14:1483–1490
Ryan M, McInerney D, Owens D, Collins P, Johnson A et al (2000) Diabetes and the Mediterranean diet: a beneficial effect of oleic acid on insulin sensitivity, adipocyte glucose transport and endothelium-dependent vasoreactivity. QJM 93:85–91
Salas J, López-Miranda J, Jansen S, Zambrana JL, Castro P et al (1999) The diet rich in monounsaturated fat modifies in a beneficial way carbohydrate metabolism and arterial pressure. Med Clin (Barc) 113:765–769
Salas-Salvado J, Fernández-Ballart J, Ros E, Martínez-González MA, Fito M et al (2008a) Effect of a Mediterranean diet supplemented with nuts on metabolic syndrome status: one-year results of the PREDIMED randomized trial. Arch Intern Med 168:2449–2458
Salas-Salvado J, García-Arellano A, Estruch R, Márquez-Sandoval F, Corella D et al (2008b) Components of the Mediterranean-type food patttern and serum inflammatory markers among patients at high risk for cardiovascular disease. Eur J Clin Nutr 62:651–659
Sales C, Oliviero F, Spinella P (2009) The Mediterranean diet model in inflammatory rheumatic diseases. Reumatismo 61:10–14
Sanders TA (2001) Olive oil and the Mediterranean diet. Int J Vitam Nutr Res 71:179–184
Scalbert A, Williamson G (2000) Dietary intake and bioavailability of polyphenols. J Nutr 130:2073S–2085S
Schroder H, Marrugat J, Vila J, Covas MI, Elosua R (2004) Adherence to the traditional Mediterranean diet is inversely associated with body mass index and obesity in a Spanish population. J Nutr 134:3355–3361
Schwartz GJ, Fu J, Astarita G, Li X, Gaetani S, Campolongo P, Cuomo V, Piomelli D (2008) The lipid messenger OEA links dietary fat intake to satiety. Cell Metab 8:281–288
Serra-Majem L, de la Cruz JN, Ribas L, Salleras L (2003) Mediterranean diet and health: is all the secret in olive oil? Pathophysiol Haemost Thromb 33:461–465
Serra-Majem L, de la Cruz JN, Ribas L, Salleras L (2004) Mediterranean diet and health: is all the secret in olive oil? Pathophysiol Haemost Thromb 33:461–465
Shai I, Schwarzfuchs D, Henkin Y, Shahar DR, Witkow S et al (2008) Weight loss with a low-carbohydrate, Mediterranean, or low-fat diet. N Engl J Med 359:229–241
Simon A, Najid A, Chulia AJ, Delage C, Rigaud M (1992) Inhibition of lipoxygenase activity and HL60 leukemic cell proliferation by ursolic acid isolated from heather flowers (Calluna vulgaris). Biochim Biophys Acta 1125:68–72
Sirtori CR, Tremoli E, Gatti E, Montanari G, Sirtori M et al (1986) Controlled evaluation of fat intake in the Mediterranean diet: comparative activities of olive oil and corn oil on plasma lipids and platelets in high-risk patients. Am J Clin Nutr 44:635–642

Sofi F, Cesari F, Abbate R, Gensini GF, Casini A (2008) Adherence to Mediterranean diet and health status: meta-analysis. BMJ 337:1344

Sofi F, Macchi C, Abbate R, Gensini GF, Casinin A (2010) Effectiveness of the Mediterranean diet: can it help delay or prevent Alzheimer's disease? J Alzheimer's Dis 20:795–801

Solfrizzi V, Panza F, Torres F, Mastroianni F, Del Parigi A et al (1999) High monounsaturated fatty acids intake protects against age-related cognitive decline. Neurology 52:1563–1569

Soriguer F, Esteva I, Rojo-Martínez G, Ruiz de Adana MS, Dobarganes MC et al (2004) Oleic acid from cooking oils is associated with lower insulin resistance in the general population (Pizarra study). Eur J Endocrinol 150:33–39

Stunkard AJ (1996) Current views on obesity. Am J Med 100:230–236

Su Q, Rowley KG, Itsiopoulus C, ODea K (2002) Identification and quantitation of major carotenoids in selected components of the Mediterranean diet: green leafy vegetables and olive oil. Eur J Clin Nutr 56:1149–1154

Tousoulis D, Papageorgiou N, Antoniades C, Giolis A, Bouras G et al (2010) Acute effects of different types of oil consumption on endothelial function, oxidative stress status and vascular inflammation in healthy volunteers. Br J Nutr 103:43–49

Trichopoulou A (1995) Olive oil and breast cancer. Cancer Causes Control 6:475–476

Trichopoulou A, Vasilopoulou E (2000) Mediterranena diet and longevity. Br J Nutr 84:S205–S209

Trichopoulou A, Kouris-Blazos A, Wahlqvist ML, Gnardellis C, Lagiou P et al (1995) Diet and overall survival in elderly people. Br Med J 311:1457–1460

Trichopoulou A, Lagiou P, Kuper H, Trichopoulos D (2000) Cancer and Mediterranean dietary traditions. Cancer epidemiology. Biomark Prev 9:869–873

Trichopoulou A, Orfanos P, Norat T, Bueno-de-Mesquita B, Ocké MC et al (2005) Modified Mediterranean diet and survival: EPIC-elderly prospective cohort study. BMJ 30:330, (7498):991

Trichopoulou A, Bamia C, Trichopoulos D (2009) Anatomy of health effects of Mediterranean diet: Greek EPIC prospective cohort study. BMJ 338:b2337

Tsimidou M, Papadopoulos G, Boskou D (1992) Phenolic-compounds and stability of virgin olive oil. Food Chem 45:141–144

Tsimikas S, Philis-Tsimikas A, Alexopoulos S, Sigari F, Lee C et al (1999) LDL isolated from Greek subjects on a typical diet or from American subjects on an oleate-supplemented diet induces less monocyte chemotaxis and adhesion when exposed to oxidative stress. Arterioscler Thromb Vasc Biol 19:122–130

Tuck KL, Hayball PJ (2002) Major phenolic compounds in olive oil: metabolism and health effects. J Nutr Biochem 13:636–644

Ukiya M, Akihisa T, Tokuda H, Suzuki H, Mukainaka T et al (2002) Constituents of Compositae plants III. Anti-tumor promoting effects and cytotoxic activity against human cancer cell lines of triterpene diols and triols from edible chrysanthemum flowers. Cancer Lett 177:7–12

Vassiliou EK, González A, García C, Tadros JH, Chakraborty G et al (2009) Oleic acid and peanut oil high in oleic acid reverse the inhibitory effect on insulin production of the inflammatory cytokine TNF-alpha both in vitro and in vivo systems. Lipids Health Dis 8:25, on line

Vessby B, Unsitupa M, Hermansen K, Riccardi G, Rivellese AA et al (2001) Substituting dietary saturated for monounsaturated fat impairs insulin sensitivity in healthy men and women: The KANWU study. Diabetologia 44:312–319

Waterman E, Lockwood B (2007) Active components and clinical applications of olive oil. Altern Med Rev 12:331–342

Weisburger JH (1997) Dietary fat and risk of chronic disease: mechanistic insights from experimental studies. Am Diet Assoc 97:S16–S23

Williams DE, Prevost AT, Whichelow MJ, Cox BD, Day NE et al (2000) A cross-sectional study of dietary patterns with glucose intolerance and other features of the metabolic syndrome. Br J Nutr 83:257–266

Wolf-Maier K, Cooper RS, Banegas JR, Giampaoli S, Hense HW et al (2003) Hypertension prevalence and blood pressure levels in 6 European countries, Canada, and the United States. JAMA 289:2363–2369

Wood LG, Hazlewood LC, Foster PS, Hansbro PM (2010) Lyprinol reduces inflammation and imporves lung function in a mouse model of allergic airway disease. Clin Exp Allergy 40:1785–1793

World Health Organization (WHO) (1999) Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus. Report No. 99.2. http://whqlibdoc.who.int/hq/1999/WHO_NCD_NCS_99.2.pdf

Yaqoob P, Calder P (1995) Effects of dietary lipid manipulation upon inflammatory mediator production by murine macrophages. Cell Immunol 163:120–128

Yaqoob P, Knapper JA, Webb DH, Williams CM, Newsholme EA et al (1998) Effect of olive oil on immune function in middle-aged men. Am J Clin Nutr 67:129–135

Yu SZ, Lu RF, Xu DD, Howe GR (1990) A case–control study of dietary and nondietary risk factors for breast cancer in Shanghai. Cancer Res 50:5017–5021

Zampelas A, Kafatos AG, Levin BE (2004) Olive oil intake in relation to cardiovascular diseases. Grasas Aceites 55:24–32

Zhang SM, Hernán MA, Chen H, Spiegelman D, Willett WC et al (2002) Intakes of vitamins E and C, carotenoids, vitamin supplements, and PD risk. Neurology 59:1161–1169

Zhang X, Cao J, Zhong L (2009) Hydroxytyrosol inhibits pro-inflammatory cytokines, iNOS, and COX-2 expression in human monocytic cells. Naunyn Schmiedebergs Arch Pharmacol 379:581–586

Chapter 19
Olive Oil Refining Process

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Contents

19.1 Introduction

Virgin olive oil (VOO) designations defined in Chap. 1 must be presented to consumers duly bottled and labeled, clearly indicating the category of oil contained within the bottle. However, not all olive oil production is classified as extra virgin and virgin, which can be consumed directly. Besides lampante virgin olive oil,

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties*,
DOI 10.1007/978-1-4614-7777-8_19, © Springer Science+Business Media New York 2013

which is not fit for human consumption because of its physicochemical and sensorial properties, there is olive-pomace oil, which is obtained from the solid residue resulting from the mechanical extraction of olive oil. Today, olive-pomace oil can be obtained from the three olive oil extraction systems (Chap. 3): pressing, three-phase decanter, and two-phase decanter. Olive-pomace oil represents approximately 8 % of olive oil production.

The refining process removes the undesirable flavor of those oils and chemical compounds that might be toxic or affect olive oil stability.

In Spain, the world's largest producer of olive oil with an average of around 1.1 million t/year, extra virgin olive oil (EVOO) represents only 35 % of production (MARM 2010). Thus, around 60 % of the production destined for human consumption should be refined before bottling. Therefore, the refining industry has a great economic importance in the world of olive oil.

19.2 Refining Process

Refining is the term applied to the process of neutralizing the free fatty acids (FFAs) present in oil by treatment with alkali and the centrifugal separation of the resulting insoluble material (e.g., phospholipids, color compounds, and soluble and insoluble impurities). The term refining is used with both physical and chemical processes (Anderson 2005). Table 19.1 displays the chemical or alkali refining process, which involves a caustic treatment step in order to neutralize the oil, while FFAs are removed by distillation during the deodorizing step in the physical refining process.

19.2.1 Settling of Olive Oil

Lampante VOO contains different types of undesirable substances ranging from 1 % to 10 % of FFAs, phospholipids, traces of metals, colored compounds, and water (Antonopoulos et al. 2006). The process of refining vegetable oils begins with a degumming step. In the olive oil industry, however, that step is replaced by the settling step because of the small amount of phospholipids in the oil, and also because settling removes moisture, impurities, and some waxes. There are three main factors

Table 19.1 Basic steps in refining process

Alkali or chemical refining	Main group of compounds removed	Physical refining
Washing/degumming	Phospholipids	Washing
Neutralization	Free fatty acids	–
Bleaching	Pigments/metals/soaps	Bleaching
Winterization	Waxes/saturated triacylglycerols	Winterization
Deodorization	Volatiles/free fatty acids	Deacidification/distillation

in effective settling: (1) time: a minimum of 4 weeks is required, depending on moisture, impurities, phospholipid content, and freshness of raw material; (2) temperature: a constant temperature between 20 °C and 25 °C yields the best results; (3) tank shape: an inverted cone is the most suitable shape for oil settling.

19.2.2 Chemical Refining Process

19.2.2.1 Degumming

The objective of this step is to eliminate phospholipids, also called *gums*. These compounds consist of a glycerol molecule esterified at the *sn*-1 and *sn*-2 positions with fatty acids (FAs) and at the *sn*-3 position with phosphoric acid normally linked to a polar group of varying nature. Phospholipids are strongly emulsifying compounds that are normally associated with prooxidative metals, which diminish stability and result in murky oil due to the appearance of precipitates. In addition, if phospholipids are present prior to deodorizing or physical refining, the oil produced will develop a darker color, showing poor organoleptic characteristics and stability (Dijkstra and Van Opstal 1989; Zamora et al. 2004).

These compounds are present in olive-pomace oil, but not in olive oil, due to the effect of the solvent on cellular membranes during the extraction process (Vioque and Maza 1973), phosphatidylcholine being the major phospholipid present in olive-pomace oil. These oils also contain a significant amount of nonhydratable phospholipids, normally in the form of calcium or magnesium salts. To achieve a complete elimination of these compounds, it is necessary to transform nonhydratable phospholipids into their hydratable forms by breaking metal/phospholipid complexes down with a strong acid, namely, phosphoric acid or citric acid (Thomopoulos and Tzía 1993).

The degumming step, which can be carried by continuous or discontinuous systems, precedes the neutralization step. In a continuous system, phospholipids are eliminated along with soaps in soapstocks (Dijkstra 1998).

19.2.2.2 Caustic Refining

This generally consists of three stages: neutralizing, washing, and drying.

Neutralizing

The neutralization step is aimed at eliminating FFAs (prooxidants) and adding an alkali, which results in the formation of soaps insoluble in the oil. It is considered costly since a small quantity of neutral oil remains lodged in what is called soapstocks (Prieto-Soler and Ramos-Ayerbe 1972).

An excess of 10–20 % of the calculated amount of NaOH is normally added to promote the saponifying reaction by which minor compounds (e.g., sterols and tocopherols) are removed (Lanzón et al. 1987; Pasqualone and Catalano 2000) together with other toxic compounds (Gozek et al. 1999).

The continuous process has been possible because of plate centrifuge separators. The first continuous process consists of four basic components: (1) a system that controls the rate of fluids to assure an exact proportion between alkali and oil in a continuous way, (2) a mechanical mixer, (3) a heat transfer system to break up the emulsion, and (4) a centrifuge separator (Hendrix 1990). Currently, the use of a self-cleaning centrifuge separator in the neutralization step is becoming the most widespread. It consists of a vertical centrifuge that allows the continuous discharge of soapstocks, which results in decreased losses in neutral oil and longer operating periods (De Greyt and Kellens 2000).

Washing and Drying

The oil separated by centrifugation contains residual soaps at 200–500 mg/kg. This amount is too high for the oil to be directly bleached because the soaps act as poisons for the bleaching earth and produce a strong adherence of the soapstocks to the filtering plates. To decrease the residual soap below 50 mg/kg, one or two washings are carried out using 8–10 % hot water (90–95 °C). The water added is then separated by a second centrifugation step. The washed oil contains between 0.3 % and 0.5 % water. If the oil needs to be stored after neutralization, then it must be dried until its water content is below 0.1 %. However, if it is going to be bleached immediately, then the oil can be dried in bleaching tanks, where the process is carried out under vacuum conditions. This process basically depends on the installation design.

19.2.2.3 Winterization

The crystallization step, which is carried out by cooling down the oil, is called winterization. The objective is the elimination of any compound that might cause the final product to appear cloudy or murky and, hence, unacceptable to consumers (De Greyt and Kellens 2000). It is an essential step in the refining of olive-pomace oil.

Winterization involves a cooling of the oil at around 5–8 °C that allows the margarine (solid portion, mainly constituted by waxes and saturated triglycerides) to crystallize in 24–48 h. The process is followed by separation into two phases through filtration or centrifugation.

Today, however, the process is carried out by means of an induced crystallization (Fig. 19.1), where those soaps resulting from the neutralization phase are used to improve the formation of microcrystals prior to maturation, or growth phase, where crystals reach macroscopic dimensions. The crystals are then separated by centrifugation, thereby minimizing losses in the process (Mastrobattista and Gabriele 1992).

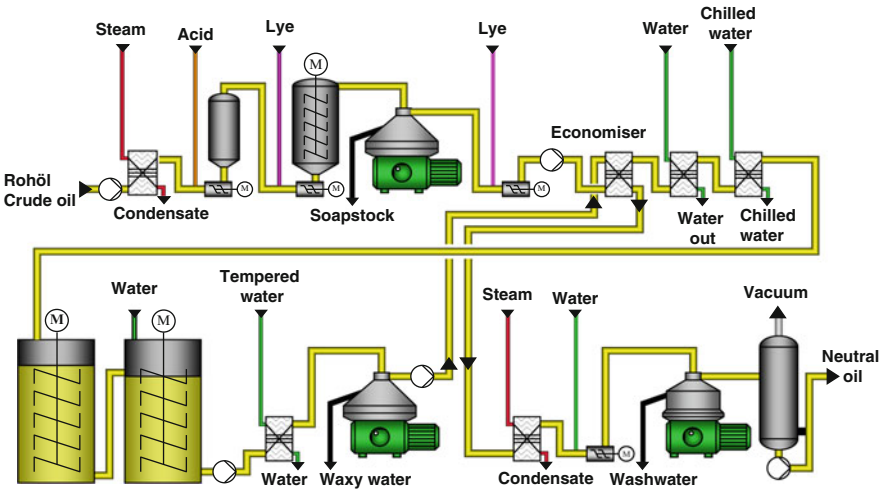

Fig. 19.1 Alkali refining: plant design complete three-stage plant (Courtesy of GEA Westfalia Separator Group)

One of the main problems associated with chemical refining is the production of residues. Soapstocks consist mainly of sodium salts produced from FFAs. They also contain entrained neutral oil along with gums. Moreover, in the case of olive-pomace oil, waxes, proteins, pigments, and other compounds are eliminated from the crude oil. For economic reasons, the soapstocks are acidified by the use of a strong acid treatment, yielding acid oil, which has some commercial value, and an aqueous phase, which requires its treatment before being discarded as wastewater (Cook 2000).

19.2.2.4 Bleaching

Bleaching is the step where the color of the oil is reduced due to the adherence of colored compounds onto bleaching earth. The pigments present in olive oil are carotenoids and chlorophylls, together with compounds derived from these compounds due to acidity (e.g., pheophytins and pheophorbides from chlorophylls and isomers of 5,8-furanoids from ring 5,6-epoxides from carotenes) (Gandul et al. 1999).

The elimination of chlorophylls from olive-pomace oil is especially important because they are not susceptible to removal in the deodorization step, in contrast to carotenoid pigments (Ouyan et al. 1980).

Bleaching clay also facilitates decomposition of oxidation products, and consequently, the peroxide value diminishes. If the raw material (olive-pomace oil) is not too much degraded, the anisidine value can be also reduced (Grompone 1991). Primary and secondary oxidation compounds are adsorbed during bleaching, along with metals, detergents, phospholipids, pesticides, and polyaromatic substances (Kock et al. 2005; Ruiz-Méndez et al. 2005).

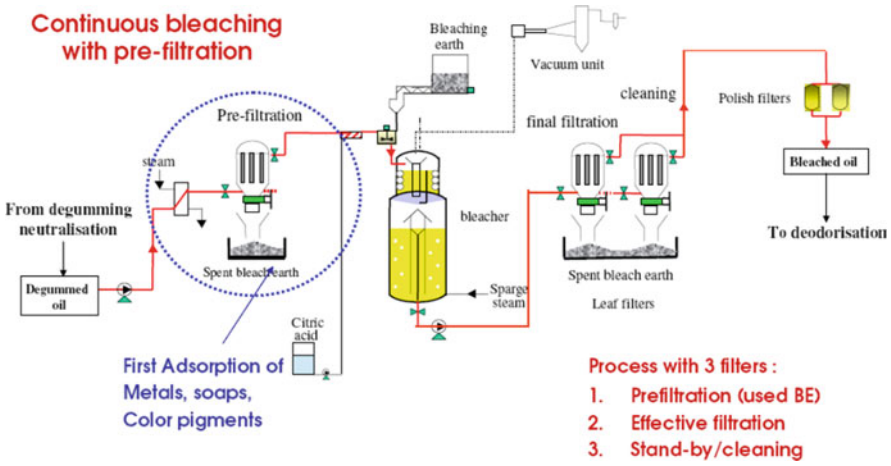

Fig. 19.2 Flowchart of continuous bleaching unit (Courtesy of Desmet Ballestra Group)

Bleaching earth, whether activated or not, consists of bentonites and montmorillonites (aluminum-magnesium silicate) with a particle size in the range of 20–80 μm (Gonzalez-Pradas et al. 1993). The most important properties of the bleaching earth, from the adsorption viewpoint, are physical (surface area, active points on the surface, and pore size) and chemical (acidity and humidity). On the other hand, both the filtration and retention of the oil are affected by particle size. Typical amounts of earth used range between 0.3 % and 1 % (Mag 1990).

Other commonly used adsorbents are active charcoal, which is being used more and more frequently in the elimination of polycyclic aromatic hydrocarbons and other toxic colored contaminants related to these compounds in olive-pomace oils (León-Camacho et al. 2003). Synthetic silica removes all the compounds that might interfere with pigment adsorption, such as soaps or phospholipids (Welsh et al. 1989), but it is, however, less efficient in retaining colored pigments, chlorophylls in particular (Lanzani et al. 1993).

Temperature selection is important because it affects equilibrium and because a decrease in temperature results in an increase in the viscosity of the oil.

The bleaching step is always carried out under vacuum conditions to reduce the possible effects of oxidation (Amati et al. 1969). It can be performed using a discontinuous or a continuous system. In the first case, the process involves cylinder containers equipped with a mixer and a heating system (Bockisch 1998). When bleaching is carried out in a continuous system, air is first removed from the oil to avoid oxidation. The oil is then passed through a filter with already used earth to reduce the total amount of bleaching earth that is required for the whole process. From there the oil is transferred to a mixer and then to a retention tank to allow for the necessary time of contact with the bleaching earth. Finally, the oil is passed through a filter to separate the bleaching earth (Bockisch 1998). When bleaching is carried out in a continuous system, it is necessary to have at least two filters. Figure 19.2 outlines the process.

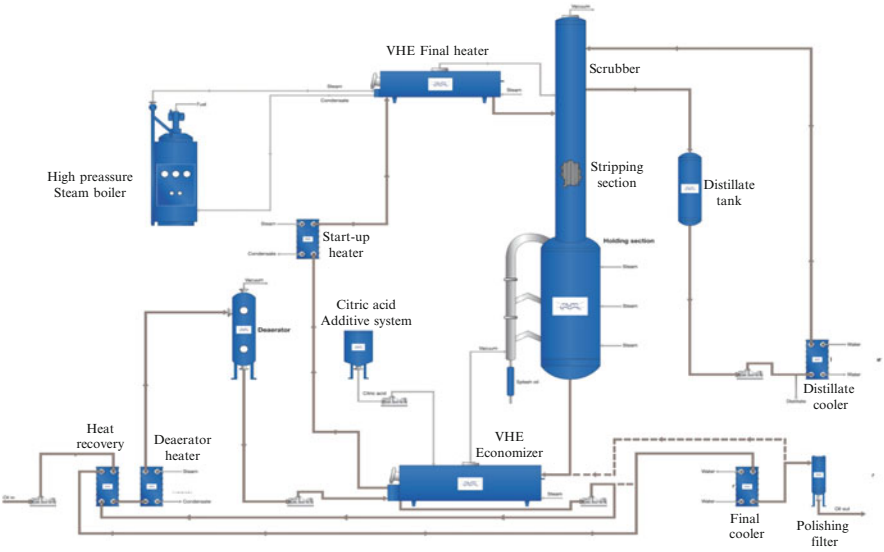

Fig. 19.3 Flowchart of continuous deodorization by "soft column" (Courtesy of Alfa Laval)

19.2.2.5 Distillation of Fatty Acids and Deodorization

In addition to triglycerides, all olive oils contain a large amount of minor compounds in much smaller quantities. Among them are volatiles that contribute not only to its appreciated sensory perceptions (e.g., fruity, green) but also to undesirable odors (e.g., rancid, fusty, vinegary) (Chap. 8). Deodorization, which allows removing those undesirable odors, consists of distillation under vacuum with stripping gas. In the physical refining process, as FFAs were not previously removed during neutralization with alkali, they are also eliminated during deodorization.

The deodorization and distillation of FFAs is made possible by the great differences in volatility between triglycerides and the other substances present. The injection of a stripping gas is required because, while maintaining a total constant low pressure, a reduction in the partial pressures of volatile compounds will be achieved, and the distillation will begin at a lower temperature.

This process consists of four steps. First, it is always necessary to prepare the oil by removing the air due to the fact that oxygen is highly reactive in the oil at deodorization temperatures and can drastically affect the stability of the resulting product. After that, the oil must be heated and the temperature maintained throughout the process, with either a high-pressure heating steam or thermal fluids. The carrier gas is injected into the bottom of the distiller; finally, the oil is cooled down under vacuum to prevent it from deterioration once it has been deodorized. This process may be carried out in a discontinuous, semicontinuous, or continuous system (Belchen 1999). Figure 19.3 shows a continuous system.

The optimal combination of time and temperature will vary according to the design characteristics of the operating plant, particularly concerning the efficiency of the distribution of the stripping gas in the oil, along with the elimination of residues and heat loss, which results in a significant decrease in stripping steam consumption (De Greyt and Kellens 2005).

The temperatures used for deodorization are between 180 °C and 270 °C. The upper limit was established as a result of the chemical decomposition and hydrolysis reactions that take place during this step (Sjöberg 1991). However, the use of temperatures above 200 °C is recommended when an additional bleaching effect is required because at higher temperatures a rapid decomposition of peroxides and pigments takes place (Ouyan et al. 1980).

Prieto-González et al. (2007) recommend operating at two different temperatures in the physical refining, first at a lower temperature to remove most of the fatty alcohols that produce waxes, and then at higher temperatures to adjust the required outlet acidity.

The minimum time required usually depends on the degree of thermal bleaching to be obtained. Operating time varies according to the type of oil and its initial quality (Sjöberg 1991), and it is directly related to the temperature used because the vapor pressure of the volatile compounds increases with temperature. On the other hand, the stability of the refined oil largely depends on the relationship between the time and temperature of deodorization, mainly due to degradation and, therefore, the loss of natural antioxidants (Verleyen et al. 2001).

The biggest improvements to the vacuum system allow for the substitution of steam with nitrogen in the deodorizing step of physical refining (Huesa and Dobarganes 1990). This technique (Decap et al. 2004) yielded significant savings, both in the consumption of water and energy and in the amount of gas required, resulting in the use of quantities that are five and ten times lower than those needed when steam is used. In addition, part of the contaminated wastewater is eliminated and the byproducts of deodorization are recovered with less degradation due to the inert nature of the gas (Ruiz-Méndez et al. 1996a, b).

Oxidation produces the most damaging effects on the quality of the resulting oil during storage, and the exclusion of oxygen is essential to preserve the quality of the finished product. The most usual procedure consists in introducing nitrogen when the oil leaves the deodorizer until it reaches the storage tank, maintaining a pressurized system by means of a regulator.

Soft Deodorization

Although virgin oils cannot undergo this treatment, some lampante oils (those showing only slight defects in chemical or sensory characteristics and which do not meet EVOO standards) are submitted to a “soft” deodorization to reduce or eliminate unpleasant odor compounds. After the treatment, these olive oils present chemical characteristics meeting chemical standards but without flavor.

Soft deodorization can be carried out by molecular distillation under vacuum or by a stream of nitrogen at low temperatures using adsorbent powders or filtering membranes (Cerretani et al. 2008; Pérez-Camino et al. 2008).

19.2.3 Physical Refining of Olive Oil

As stated previously, the main difference between olive oil and seed oils, from the refining viewpoint, is the content of phosphorus compounds that requires a degumming process. The maximum content of these compounds to carry out a physical refining is 5 mg/kg (Ong 1980). Phospholipids are not present in lampante VOOs because the oil is obtained by mechanical means from a mass of crushed olives malaxed with water (Chap. 3). The water addition during the malaxation process is enough to flocculate and separate these compounds in the aqueous phase, and furthermore, a water wash process is commonly used to remove mechanical impurities prior to the refining of the olive oil. Consequently, lampante olive oil is suitable for the physical refining.

Physical refining is more complicated than chemical refining from a technical viewpoint. However, it only requires three steps compared to more than six steps involved in conventional refining. Therefore, the main advantage of physical refining is the reduction in neutral oil losses and, at the same time, a partial reduction of the amount of pollution produced by washing water.

Other refining methods under investigation involve the elimination of FAs using molecular distillation (Lanzani et al. 1988) or supercritical fluids (Bondioli et al. 1992; Goncalves et al. 1991).

19.2.4 Environmental Impact

From what has been written so far, it has been established that the refining process is carried out with reactive chemicals that are toxic and dangerous. In addition, refining produces several types of residues and emissions that must be controlled, such as wastewater, solid waste (used bleaching earth), gas emissions (odors), and oil spills (Cook 2000).

Although regulations regarding environmental issues vary among countries, they are rigorous enough with respect to the control of residual waste, for example, the requirement to install purification systems for the wastewaters to be dumped offsite (Bozoglan and Hepbasli 2010).

19.3 Quality Control During Refining

The consumer of edible oils, according to market studies, pays special attention to appearance, flavor and scent, health effects, and the extent to which it fulfills its function. The International Olive Council (IOC) standards and the regulations of the Commission of the European Communities have defined the various categories of olive oil and olive-pomace oils (EC 2011). The quality control established by the refineries coincides with official regulations concerning the final product.

19.3.1 Quality Control of Virgin Lampante Olive Oil

Quality control begins with the evaluation of the crude oil, which is as complete (if not more) as the evaluation made on the resulting refined oil. The tests are applied not only to fulfill the marketing specifications but also to determine the most appropriate process for the raw material. The main factor to assure the quality of the refined oil stems is based on a high-quality crude oil, which must be treated and stored under the best conditions to minimize changes in the product. To obtain high-quality refined oil, the refineries must establish control points during the process with the aim of obtaining deodorized oils that fulfill the required specifications.

Due to the behavior of the solvent, crude olive-pomace oil contains more minor compounds than lampante VOO. Parameters that differentiate lampante virgin and olive-pomace oils are their contents in unsaponifiable matter (e.g., eritrodiol, uvaol, waxes) and their absorbency in ultraviolet light at K_{270}, which measure conjugated double bonds mostly caused by oxidation.

The ideal oil for physical refining is one containing no more than 2 % FFAs. This limitation avoids any increases in the percentage of saturated FAs in the beta position of triacylglycerols (TAGs) as a consequence of the interesterification with FFAs in random order at high temperatures during the deodorization step (Amelotti 1987; Gracian and Mancha 1971). This is the main reason for the selection of physical refining for oils with low acidity but not for olive-pomace oils.

19.3.2 Quality Control of Crude Olive-Pomace Oil

A large number of olive mills have been adapted to obtain VOO by two-phase decanter (Chap. 3) to optimize production costs and to avoid the production of wastewater, which is a severe environmental contaminant. The resulting byproduct, so-called *alperujo*, is a mixture of wastewater and olive-pomace oil that can be subjected to a second centrifugation in a decanter (Alba-Mendoza et al. 1996) to obtain what is known as two-phase olive-pomace oil (Sanchez-Moral and Ruiz-Méndez 2006); between 50 % and 70 % of the remaining oil can be extracted. To identify this type of olive oil, which fulfills the specifications for the olive-pomace oil designation, an analysis of aliphatic alcohols or a simple treatment with bleaching earth of the crude olive oil, due to the cloudy appearance in the bleached oils, is enough. The cloudiness results from the presence of high amounts of oleanolic acid (Pérez-Camino and Cert 1999).

The extraction of the olive-pomace oil remaining in the alperujo is extremely difficult when the oil proportion falls below 2 % because the oils coming from wet storage materials contain significant quantities of phospholipids, waxes, and other anomalous compounds. Furtheremore, the extracted oil has significantly inferior quality.

From the refining viewpoint, all these oils, although low in acidity, require a chemical refining process to eliminate fatty alcohols and triterpenic acids present in

Table 19.2 Characteristics of some crude pomace oil samples

	Crude oil				Degummed oil
Samples	Acidity (% oleic)	Phosphorus ($mg \cdot kg^{-1}$)	Soap (%)	Ca (%)	Acidity (% oleic)
A	7.30	479	4.15	1.15	14.75
B	2.39	357	0.05	0.60	4.37
C	7.45	446	1.95	0.60	11.5
D	3.36	332	0.05	0.50	4.09
E	11.20	432	2.71	0.39	13.48
F	8.81	432	2.41	0.35	10.52
G	14.47	454	4.03	0.49	17.22
H	12.43	443	0.22	0.30	14.73
I	18.57	486	0.37	0.43	21.73

the oil and to avoid the formation of waxes during the deodorization step (Servege 1983). Table 19.2 shows the acidity, together with the percentages of phosphorus, soaps, and calcium present in samples of crude olive-pomace oils; a substantial amount of calcium was also detected, possibly in the form of soaps and combined with phospholipids. The last column of the table shows the acidity determined in the same samples when the oil is subjected to a heat treatment in the presence of phosphoric acid, just as if it had suffered an exhaustive degumming process; there is an increment in FFAs, which in some cases reaches 50 %. The soaps could mask the actual free acidity in the oil, which has traditionally been the main parameter to evaluate the quality of crude oils.

Other components are formed during the storage of olive-pomace oil, such as FA ethyl esters and phenols. Substantial amounts of FA esters, formed by their reaction with short-chain alcohols resulting from the fermentation of organic materials in the presence of water, can be present with values ranging from 1 % to 3 %, and even 5 % in some cases (Ruiz-Méndez and Ramos-Hinojosa 2003). Although these compounds are removed during deodorization, the consequent losses must be considered.

On the other hand, although 4-ethylphenol is found in all olive oils intended for refining, its presence was particularly significant in the second centrifugation of olive oils, with its concentration increasing with time during olive paste storage. Similar trends were observed for hydroxytyrosol, hydroxytyrosol acetate, tyrosol, and catechol, with the concentration of these substances reaching values of up to 600 mg/kg oil (Brenes et al. 2004; García et al. 2008).

Finally, due to the drastic conditions associated with the drying process, usually high amounts of polycyclic aromatic hydrocarbons (PAHs) (400–200 ppb benzo-alfa-pirene) can be determined in crude olive-pomace oil, and they must be completely removed during the refining process (León-Camacho et al. 2003). These compounds, and those derived from other alterations because of wet storage, have had a great influence on the refining process and have led to modifications in the traditional refining processes.

Table 19.3 Routine analytical control at each stage of refining process of pomace olive oil

Oil type	Analysis
Crude oil	Free fatty acid (%) and after lab degumming (%), color, moisture and impurities, peroxide value, specific extinction and phosphorus, sterols, polycyclic aromatic hydrocarbons, and axes
Refined and winterized oil	Free fatty acids, soaps, peroxide value, phosphorus, and waxes
Bleached oil	Free fatty acids, soaps, color, peroxide value, and phosphorus
Deodorized oil	Organoleptic characteristics, free fatty acid, color, peroxide value

Table 19.3 shows the main analyses made throughout the refining process of olive-pomace oil. The control measures are numerous and necessary for establishing the proper amount of acids in the degumming step as the excess in the concentration of caustic soda in alkaline neutralization and the right proportion of active carbon in the bleaching phase.

The contents of phosphorus, soap, free acidity, and the color of the dried neutral oil are determined prior to bleaching. At this point of the refining process, the soap contents should be lower than 200 mg/kg, free acidity should not exceed 0.05 %, and the phosphorus content should be 30 mg/kg at most (Roden and Ullyot 1984; Podmore 1992). In addition, the oil should have a peroxide level equal to zero after bleaching and before deodorizing. The principal objectives of these control measurements are to verify whether the oil has received the appropriate treatment and to guarantee the effective action of the bleaching earth.

Finally, the deodorized product is analyzed before storage to ensure that the FFA level is lower than 0.1 %, the peroxide index is below 5 meqo^2/kg, and color and odor are detectable (Podmore 1992).

19.3.3 Quality Regulations for Refined Oils

It should be mentioned at this point that there is some lack of understanding surrounding an appropriate definition for the quality of refined oils. The analytical methodology used to evaluate quality is based on indexes indicating that the objectives established for each phase of the refining process have been reached. Thus, the determinations of free acidity, color, and odor characteristics are directly related to the effectiveness of the degumming, neutralization, bleaching, and deodorization steps of the refining process. Additional analyses to evaluate the oxidative level (e.g., λ_{270}) are difficult to interpret because they can vary according to the level of alteration as well as the presence of prooxidants and antioxidants in the oil. On the other hand, it is well known that the behavior of refined oil during storage and in different applications (e.g., frying) depends largely on the quality of the initial crude oil that is subjected to refining and on the quality of the final processed oil (Márquez-Ruiz et al. 2010).

Quality and authenticity specifications for refined olive oils are more rigorous than those established for other edible oils, even though legislation permits high acidity and makes no mention of parameters such as phosphorus or soap contents (EC 2011). Table 16.6 shows the main characteristics of refined olive oils. Regulations (EC 2011) and IOC trade standards permit high levels of compounds related to the refining process. These modifications are mainly due to the drying process of olive-pomace oil, where the value of oxidized triglycerides can increase by up to 35 % (Gomes and Capornio 1997).

There are exceptions to this general rule in specific cases where the required quality characteristic is stricter. That is the case in the canning industry, which requires a maximum percentage of FFA of 0.1 %. With respect to their appearance, the oils must be clear and bright and at a temperature of 20 °C, and they must be clear and free of sediment over a 24-h period. Therefore, any component that could produce cloudiness in the oil must be removed during the refining process. Clear examples are the winterization step to reduce waxes and long-chain saturated triglycerides, the vacuum drying step to eliminate water, and filtration to eliminate the remaining bleaching earth and other impurities. These last two requirements are specifically controlled since EU regulations and IOC trade standards establish that the contents of moisture and volatile compounds in a vacuum furnace should not exceed 0.1 % and the content of insoluble impurities in light petroleum must be below 0.05 %. Regarding color specifications, they may vary according to the marketing demands.

The specifications for refined oils are also related to sensory characteristics since the refined olive oil must be free from rancid odor, have no contamination by foreign substances, and not show deodorization defects. Nevertheless, no regulation has yet been established to monitor the sensory analyses of refined oils, and only the peroxide value (less than 10 meq O_2/kg) is a guarantee to limit the amount of modified compounds present in refined olive oil. In addition, refined olive oil must be produced to maintain acceptable sensory characteristics over its shelf life.

19.3.4 Contaminants

Persistent organic pollutants (POPs) (e.g., PAHs, pesticides, and PCBs) are well known because of their toxicity and their persistence in the environment. They must be removed during the refining process to achieve residual levels defined as “as low as reasonably achievable” (ALARA) (De Greyt and Kellens 2005).

The presence of pesticides in olive oil can elicit endocrine disorders (Skilourakis and Psillakis 2007). Amvrazi and Albanis (2009) determined that 10 % of the analyzed Greek samples from olive trees of conventional orchards contained no detectable residues, whereas other samples had residues of up to 20 insecticides. The highest detection rates were of fenthion, dimethoate, and endosulfan. Endosulfan, which is a pesticide in the same class as DDT, is highly persistent in the environment and has a high potential for bioaccumulation and biomagnification.

Past investigations suggested that the amount of most of the organochlorine and organophosphorus pesticides in edible oils can be reduced considerably by a chemical refining process or by adsorption on specific lipophilic adsorbents (e.g., activated carbon), by vacuum-steam distillation, or by extraction with supercritical CO_2 (de Diujn 2008; Kock et al. 2005; Kawashima et al. 2009). Bleaching seemed to be effective for the elimination of simazine only. To remove the other pesticides, the physical refining treatment requires a temperature of 240 °C at the deodorizing step, for time periods depending on the pesticide, for example,1 h for diflufenican, oxifluorfen, and alpha-endosulfan, 2 h for beta-endosulfan, and 3 h for endosulfan sulphate (Ruiz-Méndez et al. 2005).

Removal by photochemical degradation using ultraviolet light seemed to be an effective alternative for the reduction of these chemicals without harming the quality parameters of the lampante olive oil (Martínez Nieto et al. 2009).

19.4 Modifications in Oil Produced During Refining Process

From a general viewpoint, the main alterations to fats during processing and storage are due to the effects of humidity, temperature, and atmospheric oxygen. The effects of these variables involving steps prior to and during the refining process determine the initial quality of the refined oil, which may be further modified during storage and packaging as a result of oxidative alterations. The main difference among the three variables is that while humidity affects the ester bonds of glycerol leading to hydrolytic release of FFAs and partial glycerides, temperature and oxygen mainly affect the double bonds of the unsaturated acyl moieties that are constituents of the TAG molecule and, consequently, affect the formation of cyclic monomers, *trans* acid isomers, or dimers and polymers, series of compounds that are of great importance in the evaluation of thermal alteration in refined oils (Grandgirard et al. 1984). The following paragraphs describe the principal modifications to minor glyceridic compounds and the most relevant unsaponifiable compounds as a consequence of the refining process.

19.4.1 Minor Glyceridic Compounds in Oil

The quantification of total minor glyceridic compounds can be carried out with a previous separation by silica gel column chromatography and then the analysis by high-performance size-exclusion chromatography (HPSEC) (Ruiz-Méndez et al. 1997). Figure 19.4 shows a HPSEC chromatogram with four main peaks: triacylglycerol dimers (TAGDs), characteristic of thermal degradation; oxidized triacylglycerol monomers (oxTAGMs), related to oxidative alteration; diacylglycerols (DAGs); and FFAs, the last two mainly produced through hydrolytic reactions. The FA peak also includes a part of the polar unsaponifiable fraction and, consequently, it is not of interest from a quantitative viewpoint. Thus, the main groups of polar

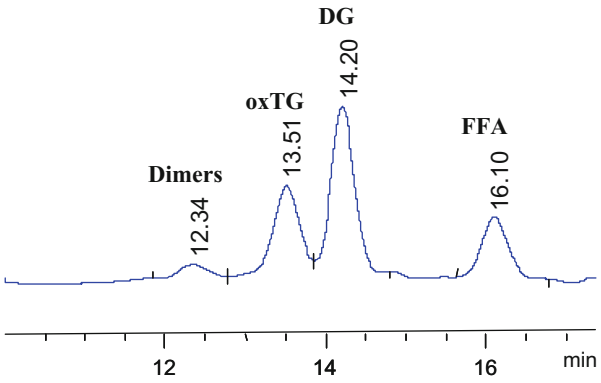

Fig. 19.4 Representative HPSEC chromatogram of refined oil polar compounds. Abbreviations: *oxTG* oxidized triglycerides, *DG* diglycerides, *FFA* free fatty acids

compounds, which elute in inverse order to their molecular weight, are the glyceridic compounds, which are associated with the main alterations in fats and oils.

In the case of lampante olive oil and olive-pomace oil, regardless of their percentages of polar compounds, the main minor lipids are DAGs and FFAs due to hydrolysis (Serani et al. 2001). With respect to oxidation, as stated earlier, most of the alterations are produced during the drying step of the olive-pomace oil before extraction of the oil by solvent.

Oxidized TAGMs and DAGs are difficult to remove during the refining process due to their polarity and volatility. An appreciable decrease in their quantity occurs in the neutralizing step, while no change is observed in the bleaching step. This is because the basic objective of the bleaching step is to remove compounds present in small quantities.

With regard to the deodorizing step, the significant increase in the amount of dimers is the consequence of the elevated temperature of this step. Most of this increase takes place immediately once the oil has been bleached. This indicates that the initial heating step, until the treatment temperature is reached, has an important effect on the formation of polymerized compounds. This could be due to the presence of large amounts of oxygen during this initial period (Ruiz-Méndez 2003). Once a dimer is formed, the existence of double bonds in other FAs of TAG molecules could lead to further reactions producing trimers and polymers.

The relative effect of both pathways depends on the temperature, the proportion of polyunsaturated fatty acids (PUFAs), and the presence of conjugated double bonds. The main implication is the formation of nonpolar dimers or acylic isomers that alter the natural structure of TAGs, which, at significant quantities, could modify the oil nutritional properties.

On the other hand, the formation of compounds related to polymerization does not seem to be related merely to the temperature and unsaturation degree of the oil, but also to the quality of the crude oil subjected to the process, a fact that has been corroborated with other edible oils (Ruiz-Méndez et al. 1997).

The changes that acylglycerol compounds undergo during the refining process lead to the following general conclusions:

1. There is a significant decrease in the amount of minor glyceride compounds in the oil during the neutralization phase due to the removal of FFAs.
2. The formation of compounds related to polymerization depends on the quality of the oil itself.
3. The oxidized TAG monomers and the DAGs undergo minor quantitative changes, which makes it easy to establish the relationship between the crude oil and the oil produced from any of the refining steps.

These conclusions lead to the proposal that the quantification of minor glyceridic compounds, in particular of polymers, and the relationship between TAGs and DAGs be used as parameters for the evaluation of the quality of refined lampante VOOs (Pérez-Camino et al. 1993) and olive-pomace oils (Gomes and Capornio 1998 and 2001).

19.4.1.1 Hydrolysis

The effect of humidity results in the formation of FFAs and partial acylglycerols, especially DAGs, as stated previously. FFA formation takes place during the neutralization and deodorization steps (Szabo 1959). Although FFAs are not easily formed during the storage and packaging of the refined oils, their elimination during the refining process is crucial, owing to the fact that FFAs and partial acylglycerols are more susceptible to oxidation than the TAGs from which they are formed, and they can therefore contribute to an acceleration of the autooxidation process (Tatum and Chow 2000).

19.4.1.2 Conjugated Double Bonds

An increase in the amount of bleaching earth with the aim of reducing the color can generate conjugated dienes and trienes (determined by K_{232} and K_{270}, respectively) derived from PUFAs (Sanelli 1979). High temperatures also have an adverse effect on the formation of conjugated double bonds (Passaloglou-Emmanouilidou 1990).

19.4.1.3 Geometric Isomerization

In the bleaching and deodorization steps of physical refining, *trans* isomers are formed from PUFAs. In the deodorizing step, the content of *trans* FAs depends on the temperature, the treatment time, the flow rate of the carrier, and the oil layer in the deodorizer (León-Camacho et al. 1999, 2001).

19.4.1.4 Transesterification of Fatty Acids in Triacylglycerol

A significant increase in the percentage of saturated FAs in the *sn*-2 results when the temperature in the deodorization step increases or oils with high acidity are deodorized (Amelotti 1987; Gracian and Mancha 1971). This is the main reason that lampante VOOs with low acidity are subjected to physical refining, in contrast to olive-pomace oils.

19.4.2 Other Compounds Affected by Refining Process

No relevant changes are observed in the total amount of the unsaponifiable fraction, with the exception of a decrease during the deodorization step. The compounds removed are important from the sensory viewpoint. They are the aldehydes and ketones that originate from hydroperoxide derivatives of FAs, as mentioned earlier (Chap. 8).

With respect to the amounts and compositions of sterols, tocopherols, and other minor compounds (Chap. 6), no significant differences were found in the amounts of the individual compounds in the refining process (Kocchar 1983; Pasqualone and Catalano 2000).

The hydrocarbon fraction suffers a considerable decrease during processing, more specifically during caustic refining and in deodorization (Lanzón et al. 1994). A considerable amount of squalene is collected from the distillates of the deodorization step and is considered a valuable byproduct (Bondioli et al. 1993).

At the same time, hydrocarbons of a diverse nature are formed, mainly in the bleaching step (Bortolomeazzi et al. 2000), such as alkadienes, products of the isomerization of squalene, and steroidal hydrocarbons with double bonds in the ring of the molecule. The content of stigmasta-3,5-diene, a product of the dehydration of β-sitosterol formed as result of the action of the bleaching earth (Lanzon et al. 1989; Cert et al. 1994), has been established in regulations to detect the addition of refined oils to VOOs.

Regarding waxes, there is a specific step to remove them in the refining of olive-pomace oil. The waxes are FA esters with long-chain alcohols, and their amounts in olive oils have been used for detecting the presence of olive-pomace oil (Chap. 16). Refined olive oil generally has wax contents close to the limit of 300 mg/kg used for detecting adulteration. Tubaileh et al. (2002) observed that the wax content increased during the heating period and decreased throughout the process upon reaching the operating temperature. Once a level has been reached, it does not vary during the storage period (Paganuzzi et al. 1997). At the same time, the total contents of fatty alcohols decrease during the deodorization step of physical refining.

The refining process also removes polyphenols. Diphenols (hydroxytyrosol, catechol, and hydroxytyrosol acetate) and flavonoids (luteolin and apigenin) are removed during the alkaline treatment (Nergiz 1993), whereas tyrosol and 4-ethylphenol remain until the deodorization step. A large amount of phenolic compounds has been found in refining byproducts, such as soapstocks and

deodorization distillates. In the latter streams, the concentrations of tyrosol and 4-ethylphenol have reached up to 149 mg/kg and 3,720 mg/kg, respectively. This high level of 4-ethylphenol and its well-known strong off-odor can interfere with the further processing of the deodorization distillates. This information opens up the possibility of recovering phenolic compounds from second-centrifugation olive oils by adding a new washing step prior to the refining process (García et al. 2006).

The concentration of triterpenic acids in olive oil depends on the oil quality; the higher the quality, the lower the concentration; average amounts are 200 mg/kg for EVOO, 300 mg/kg for VOO of 1 % acidity, and 10 g/kg for olive-pomace oil. In fact, enrichment in triterpenic acids occurs in the crude olive-pomace oil during the storage of the alperujo from which it is extracted (García et al. 2008), but a significant loss in these substances occurs during the refining process of the oil, particularly when the neutralization step of the chemical refining is used (Pérez-Camino and Cert 1999; Ruiz-Méndez et al. 2010).

19.5 Byproducts: Soapstocks and Deodorization Distillates

Distillates constitute good starting materials for obtaining compounds of high added value that have very different uses depending on their composition (Pickard et al. 1996). In the case of olive oil, this fraction, which can contain between 15 % and 30 % of unsaponifiable matter (Ruiz-Méndez et al. 1995), can have direct applications in the cosmetic and food industries (Abadlla 1999).

Phytosterols, for example, have been used in the pharmaceutical and cosmetic industries, although new applications are being discovered, such as precursors for synthetic prostaglandins and other compounds of particular interest in the food industry (Miettinen and Gylling 1997; Fernandes and Cabral 2007). The distillates from olive oil are also a good source of hydrocarbons since squalene comprises 15–30 % of such products and has widespread applications in cosmetics and pharmacology. Different solutions have been proposed for the separation of these hydrocarbons from deodorization distillates, such as the addition of detergents and further distillation (Serra-Masía and Martinez 1981) or fluid extraction in a supercritical state (Bondioli et al. 1993).

19.6 Challenges and Perspectives

Olive oil is valued for its content of minor compounds of nutritional interest, but the refining process removes a portion of these compounds. The olive oil industry intends to keep as much as possible of these compounds and simultaneously prevent the degradation of glyceridic components, as avoiding trans-FAs and polymeric TAGs formation with mild deodorizing conditions, and establishing more efficient processes in an attempt to reduce/valorize added-value byproducts, such as deodorizer distillates.

In this sense, green technologies, such as supercritical fluid extraction, membrane technology, and molecular distillation, have potential applications in the olive oil industry.

Deacidification using supercritical fluids has been performed at the laboratory scale (Bondioli et al. 1992) as has tocopherol concentration from deodorizer distillate (Ibañez et al. 2000).

However, after two decades of significant developments, the handling of large volumes of oil is still a major challenge. Even though equipment manufacturers have been working on a continuous system at the commercial scale for some time, it is not available yet (Temelli 2009).

Membrane technology can be used in many areas of the vegetable oil industry. A membrane-based process for deacidification of lampante oil was undertaken by Hafidi et al. (2005). It was reported that, while complete deacidification was achieved, some desirable components, mainly phenolics, were eliminated during the filtering process. Thus, it was suggested that focus be placed on reducing the elimination of phenolic compounds and improving the organoleptic characteristics of the filtered oils.

Some applications involving the extraction and refining of vegetable oils are now established – for example, degumming – while others are still under development. These include solvent recovery by reverse osmosis (RO) or nanofiltration (NF), deacidification by NF, dewaxing by microfiltration (MF), and wastewater treatment by MF, NF, and RO. The major limitation to date has been the lack of membranes that are stable in hexane and other solvents (Coutinho et al. 2009).

Molecular distillation, also called short path distillation, has become an important alternative for the separation of heat-sensitive compounds or substances with very high boiling points. Although this technique is a promising separation and purification method, it is not commonly applied in the olive oil industry. The cost of the system and possible alterations in the structure of the oil during the process seem to be serious disadvantages (Ciftci et al. 2012).

The production of refined olive oil is compulsory in a growing olive oil market, and the objectives of new developments in edible oil processing tend to enhance the nutritional quality of olive oils. Global trends show that green products and technologies are needed and all efforts should be made to supply them.

References

Abadlla AEM (1999) Antioxidative effect of olive oil deodorizer distillate on frying oil and quality of potato chips. Fett/Lipid 101:57–63

Alba-Mendoza J, Hidalgo Casado F, Ruíz Gómez Mª A, Martinez Román F, Moyano Pérez MJ, Cert-Ventulá A, Pérez-Camino MC, Ruíz-Méndez MV (1996) Características de los aceites de oliva de primera y segunda centrifugación. Grasas y Aceites 47:163–181

Amati A, Minguzzi A, Losi G (1969) Bleaching of olive oils I. Variations in some physico-chemical characteristics as a function of operation conditions. Riv Ital Sostanze Grasse 46:73–79

Amelotti G (1987) Effects of the interesterification for physical refining of some olive oils I. Riv Ital Sostanze Grasse 64:223–226
Amvrazi EG, Albanis TA (2009) Analytical methods: Pesticide residue assessment in different types of olive oil and preliminary exposure assessment of Greek consumers to the pesticide residues detected. Food Chem 113:253–261
Anderson D (2005) A primer on oil processing technology. In: Shahidi F (ed) Bailey's industrial oil and fat products, edible oil and fat products: processing technologies, vol 5, 5th edn. Wiley, Hoboken, pp 1–56
Antonopoulos K, Valet N, Spiratos D, Siragakis G (2006) Olive oil and pomace olive oil processing. Grasas Aceites 57:56–67
Belchen S (1999) Deodorization principles: Stripping efficiency in cross-flow and counter current operations. Inform 10:245–255
Bockisch M (1998) Oil purification. In: Bockisch M (ed) Fats and oils handbook. AOCS Press, Champaign (Ill), pp 613–626
Bondioli P, Mariani C, Lanzani A, Fedeli E, Mossa A, Muller A (1992) Lampante olive oil refining with supercritical carbon dioxide. J Am Oil Chem Soc 69:477–480
Bondioli P, Mariani C, Lanzani A, Fedeli E, Muller A (1993) Squalene recovery from olive oil deodorizer distillates. J Am Oil Chem Soc 70:763–766
Bortolomeazzi R, Zan M, Pizzale L, Conte LS (2000) Identification of new steroidal hydrocarbons in refined oils and the role of hydroxy sterols as possible precursors. J Agric Food Chem 48:1101–1105
Bozoglan E, Hepbasli A (2010) Performance improvements for olive oil refining plants. Int J Energy Res 34:476–493
Brenes M, Romero C, García A, Hidalgo FJ, Ruiz-Méndez MV (2004) Phenolic compounds in olive oils intended for refining: formation of 4-ethylphenol during olive paste storage. J Agric Food Chem 52:8177–8181
Cerretani L, Bendini A, Barbieri S, Lercker G (2008) Preliminary observations on the change of some chemical characteristics of virgin olive oils subjected to a soft deodorization process. Riv Ital Sostanze Grasse 85:75–82
Cert A, Lanzón A, Carelli A, Albi T, Amelotti G (1994) Formation of stigmasta-3,5-diene in vegetable oils. Food Chem 49:287–293
Ciftci ON, Ciftci D, Jenhab E (2012) Potential applications of green technologies. In: Boskou D (ed) Olive oil industry. Olive oil - constituents, quality, health properties and bioconversions. InTech - Open Access Company, Rijeka (Croatia), pp 293–307
Cook R (2000) Safety. In: Hamm W, Hamilton RJ (eds) Edible oil processing. Sheffield Academic Press, Sheffield, pp 219–243
Coutinho CM, Chiu MC, Basso RC, Ribeiro AP, Gonçalves L, Viotto LA (2009) State of art of the application of membrane technology to vegetable oils: a review. Food Res Int 42:536–550
De Duijn G (2008) Industrial experiences with pesticide removal during edible oil refining. Eur J Lipid Sci Technol 110:982–989
De Greyt W, Kellens M (2000) Refining practice. In: Hamm W, Hamilton RJ (eds) Edible oil processing. Sheffield Academic Press, Sheffield, pp 90–105
De Greyt W, Kellens M (2005) Deodorization. In: Shahidi F (ed) Bailey's industrial oil and fat products, edible oil and fat products: processing technologies, vol 5, 6th edn. Wiley, Hoboken, pp 352–357
Decap P, Braipson-Danthine S, Vanbrabant B, de Greyt W, Deroanne C (2004) Comparison of steam and nitrogen in the physical deacidification of soybean oil. J Am Oil Chem Soc 81:611–617
Dijkstra AJ (1998) Degumming revisited. Oléagineux, Corps Gras, Lipides 5:367–370
Dijkstra AJ, Van Opstal M (1989) The total degumming process. J Am Oil Chem Soc 66:1002–1009
European Communities (EC) (2011) Official J Comm European Communities. Regulation nº 61/2011, L 23, 27 Jan 2011
Fernandes P, Cabral JMS (2007) Phytosterols: applications and recovery methods. Bioresour Technol 98:2335–2350

Gandul B, Roca M, Mínguez M (1999) Chlorophyll and carotenoid pattern in virgin olive oil. Adulteration control. In: Minguez-Mosquera MI, Jaren-Galan M, Hornero-Mendez H (eds) Proceeding of the 1st international congress on pigments in food technology (PFT), Sevilla, PFT Press, pp 381–386. ISBN 8469901850

García A, Ruiz-Méndez MV, Romero Barranco C, Brenes M (2006) Effect of refining on the phenolic composition of crude olive oils. J Am Oil Chem Soc 83:159–164

García A, Brenes M, Dobarganes MC, Romero-Barranco C, Ruiz-Méndez MV (2008) Enrichment of pomace olive oil in triterpenic acids during storage of alpeorujo olive paste. Eur J Lipid Sci Technol 110:1136–1141

Gomes T, Capornio F (1997) Evaluation of the state of oxidation of crude olive-pomace oils. Influence of olive pomace drying and oil extraction with solvent. J Agric Food Chem 45:1381–1384

Gomes T, Caponio F (2001) Possibility of improving the quality characteristics of olive-pomace oil and enhancing its differentiation from refined olive-pomace oil. J Sci Food Agric 81:62–67

Gomes T, Capornio F (1998) Evaluation of the state of oxidation of crude olive-pomace oils. Influence of the refining process. J Agric Food Chem 46:1137–1142

Goncalves M, Vasconcelos AMP, Gomes-de-Azevedo EJS, Chaves-das-Neves HJ, Nunes-da-Ponte M (1991) On the application of supercritical fluid extraction to the deacidification of olive oils. J Am Oil Chem Soc 68:474–480

Gonzalez-Pradas E, Villafranca-Sánchez M, Gallego-Campo A (1993) Influence of the physical-chemistry properties of an acid activated bentonite in the bleaching of olive oil. J Chem Tech Biotechnol 57:213–216

Gozek K, Yucel U, Ilim M, Aysal P, Tuncbilek AS (1999) 14C-dimethoate residues in olive oil during oil processing. J Environ Sci Health B 34:413–429

Gracian J, Mancha M (1971) Fenómenos de transesterificación durante el calentamiento de aceites de oliva y de orujo y su influencia en la alteración de la estructura glicerídica. Grasas Aceites 22:30–39

Grandgirard A, Sebedio JL, Fleury J (1984) Geometrical isomerization of linolenic acid during heat treatment of vegetable oils. J Am Oil Chem Soc 61:1563–1568

Grompone MA (1991) El índice de anisidina como medida del deterioro latente de un material graso. Grasas y Aceites 42:8–13

Hafidi A, Pioch D, Ajana H (2005) Effects of a membrane-based soft purification process on olive oil quality. Food Chemistry 92(4):607–613

Hendrix B (1990) Neutralization. In: Erickson DR (ed) Edible fats and oils processing: basic principles and modern practices. AOCS Press, Champaign, pp 94–100

Huesa J, Dobarganes MC (1990) Patente ES 2.013.206. Chem Abstr 113:1744–1764

Ibañez E, Palacios J, Señorans FJ, Santa-Maria G, Tabera J, Reglero G (2000) Isolation and separation of tocopherols from olive by-products with supercritical fluids. J Am Oil Chem Soc 77:187–190

Kawashima A, Watanabe S, Iwakiri R, Honda K (2009) Removal of dioxins and dioxin-like PCBs from fish oil by countercurrent supercritical CO2 extraction and activated carbon treatment. Chemosphere 75:788–794

Kocchar SP (1983) Influence of processing on sterols of edible oils. Prog Lipid Res 22:161–188

Kock J, de Greyt W, Gibon V, Kellens M (2005) Développements récents en matières de raffinage et de modifications: élimination des contaminants dans les huiles alimentaires et réduction du taux d'acides gras trans. OCL 12:378–384

Lanzani A, Bondioli P, Allevi C, Baretteau P, Bertini CG (1988) Un impianto di distillazione con caratteristiche innovative nella tecnología di raffinazione di olio di sansa. Riv Ital Sostanze Grasse 65:439–443

Lanzani A, Bondioli P, Fedeli E (1993) Quality in fats and oils production. Riv Ital Sost Grasse 70:1–5

Lanzón A, Albi T, Gracián J (1987) Alteraciones regristradas en los componentes alcohólicos del insaponificable del aceite de oliva en el proceso de refinación. Primeros ensayos. Grasas y Aceites 38:203–209

Lanzon A, Cert A, Albi T (1989) Detección de la presencia de aceite de oliva refinado en el aceite de oliva virgen. Grasas Aceites 40:385–389

Lanzón A, Albi T, Cert A, Graciani J (1994) The hydrocarbon fraction of virgin olive oil and changes resulting from refining. J Am Oil Chem Soc 71:285–291

León-Camacho M, Ruiz-Méndez MV, Graciani-Constante E (1999) Isomerisation of fatty acids during deodorization/physical refining stripping with nitrogen. Fett/Lipid 101:290–296

León-Camacho M, Ruiz-Méndez MV, Graciani-Constante E (2001) Kinetics of the *cis-trans* isomerisationof linoleic acid in the deodorization and/or physical refining of edible fats. Eur J Lipid Sci Technol 102:85–92

León-Camacho M, Viera-Alcaide I, Ruiz-Méndez MV (2003) Elimination of polycyclic aromatic hydrocarbons by bleaching of olive pomace oil. Eur J Lipid Sci Technol 105:9–16

Mag TK (1990) Bleaching: theory and practice. In: Erickson DR (ed) Edible fats and oils processing: basic principles and modern practices. AOCS Press, Champaign, pp 107–116

MARM (2010) Ministerio de Agricultura Rural y Marino de España Observatorio del consumo y la distribución alimentaria. Informe anual. 2009

Márquez-Ruiz G, Ruiz-Méndez MV, Velasco J, Dobarganes MC (2010) Preventing oxidation during frying of foods. In: Decker E, Elias R, McClements DJ (eds) Oxidation in foods and beverages and antioxidant applications, vol 2. Woodhead Publishing, Cambridge, pp 239–273

Martínez Nieto L, Hodaifa G, Casanova MS (2009) Elimination of pesticide residues from virgin olive oil by ultraviolet light: preliminary results. J Hazard Mater 168:555–559

Mastrobattista G, Gabriele F (1992) Valutazione di una nuova tecnica di reffinazione su oli di sansa. Ind Aliment 31:447–449

Miettinen TA, Gylling H (1997) Sitostanol-ester margarines. In: Yalpani M (ed) New technologies for healthy foods and nutraceuticals. ATL Press Inc, Shrewsburry, pp 71–82

Nergiz C (1993) The effect of refining processes on the total polyphenol and 1 2-diphenol content of olive oil. Int J Food Sci Technol 28:461–464

Ong JTL (1980) Die Notwendigkeit neue Qualität-sanforderungen für rohes Sojaöl einzuführen. Fette Seifen Anstrichm 82:169–171

Ouyan H, Daun S, Chang SS, Ho CT (1980) Formation of carbonyl compounds from β-carotene during palm oil deodorization. J Food Sci 45:1214–1222

Paganuzzi V, Iorgi F, Malerba A (1997) Influence of ageing and temperature on some parameters provided by EEC regulation N. 2568/91 on olive oil. Riv Ital Sostanze Grasse 74:231–239

Pasqualone A, Catalano M (2000) Free and total sterols in olive oils. Effects of neutralization. Grasas Aceites 51:180–182

Passaloglou-Emmanouilidou S (1990) A comparative study of UV spectrophotometric methods for detection of olive oil adulteration by refined oils. Z Lebensm Unters Forsch 191:132–134

Pérez-Camino MC, Cert A (1999) Quantitative determination of hydroxy pentacyclic triterpenic acids in vegetable oils. J Agric Food Chem 47:1558–1562

Pérez-Camino MC, Ruiz-Méndez MV, Márquez-Ruiz G, Dobarganes MC (1993) Aceites de oliva vírgenes y refinados: diferencias en componentes menores glicerídicos. Grasas Aceites 44:91–96

Pérez-Camino MC, Cert A, Romero-Segura A, Cert-Trujillo R, Moreda W (2008) Alkyl esters of fatty acids a useful tool to detect soft deodorized olive oils. J Agric Food Chem 56:6740–6744

Pickard MD, Jones TJ, Tyler RT (1996) By-products utilization. In: Shahidi F (ed) Bailey's industrial oil and fat products, edible oil and fat products: processing technologies, vol 4. Wiley, New York, pp 603–630

Podmore J (1992) Controlling quality in an edible oil refinery. Inform 3:317–323

Prieto Gonzalez MM, Bada JC, Graciani E (2007) Temperature effects on the deacidification of mixtures of sunflower oil and oleic acid. J Am Oil Chem Soc 84:473–478

Prieto-Soler JM, Ramos-Ayerbe F (1972) Refinación de aceites. Estudio de las determinaciones de las pérdidas en neutralización y sus correspondencias con las pérdidas en la industria. Grasas Aceites 6:419–427

Roden A, Ullyot G (1984) Quality control in edible oil processing. J Am Oil Chem Soc 61:1109–1111
Ruiz-Méndez MV (2003) Refinación de aceite de oliva y de orujo de aceituna. In: Aparicio R, Harwood J (eds) Manual del Aceite de Oliva. Vicente Ediciones, Madrid, pp 563–595
Ruiz-Méndez MV, Ramos-Hinojosa AE (2003) Fatty acid esters with short-chain alcohols in two-phase olive pomace oils. Eur J Lipid Sci Technol 105:346–350
Ruiz-Méndez MV, Márquez-Ruiz G, Dobarganes MC (1995) Determinación cuantitativa de componentes mayoritarios presentes en los destilados obtenidos en la desodorización de aceites y grasas. Grasas Aceites 46:21–25
Ruiz-Méndez MV, Márquez-Ruiz G, Dobarganes MC (1996) Comparative performance of steam and nitrogen as stripping gas in physical refining of edible oils. J Am Oil Chem Soc 73:1641–1645
Ruiz-Mendez MV, Garrido-Fernández A, Rodríguez-Berbel FC, Graciani-Constante E (1996) Relationship among the variables involved in the physical refining of olive oil using nitrogen as stripping gas. Fett/Lipid 98:121–125
Ruiz-Méndez MV, Márquez-Ruiz G, Dobarganes MC (1997) Relationships between quality of crude and refined edible oils based on quantitation of minor glyceridic compounds. Food Chem 60:549–554
Ruiz-Méndez MV, Pérez I, Jiménez-Márquez A, Uceda-Ojeda M (2005) Elimination of pesticides in olive oil by refining using bleaching and deodorization. Food Addit Contam 22:23–30
Ruiz-Méndez MV, Dobarganes MC, Sanchez-Moral P (2010) Edible olive pomace oil concentrated in triterpenic acids, procedure of physical refining utilised for obtainment thereof and recovery of functional components present in the crude oil. Patent WO/2010/010219
Sanchez-Moral P, Ruiz-Méndez MV (2006) Production of pomace olive oil. Grasas Aceites 57:47–55
Sanelli B (1979) Variations of the UV spectrophotometric indices and removal of colour in refined olive oil. Freundlich equation of adsorption. III. Removal of coloured pigments. Riv Ital Sostanze Grasse 57:295–300
Serani A, Piacenti D, Staiano G (2001) Identification of deodorized oils in virgin olive oils. Note 2. Kinetics of diacylglycerols isomerization in virgin olive oils. Riv Ital Sostanze Grasse 78:525–528
Serra-Masía A, Martinez MJ (1981) Obtención de escualeno de los destilados de desodorización del aceite de oliva. Grasas Aceites 32:313–317
Servege A (1983) Difficulties in physical refining of olive oil due to presence of triterpene oleanolic acid. J Am Oil Chem Soc 60:584–587
Sjöberg P (1991) Deodorization technology. Lipid Technol 3:52–57
Skilourakis A, Psillakis E (2007) Endocrine disrupting compounds in olive oil. In: Ho P, Cortez Vieira MM (eds) Case studies in food safety and environmental health (Integrating safety and environmental knowledge into food studies towards european sustainable development) volume 6. Part I. Springer, Boston, pp 21–27
Szabo SD (1959) Hydrolysis during deodorization of fatty oils. Catalytic action of fatty acids. J Am Oil Chem Soc 36:143–145
Tatum J, Chow CK (2000) Effects of processing and storage on fatty acids in edible oils. In: Chow CK (ed) Fatty acids in foods and their health implications. Marcel Dekker Inc, New York, pp 411–426
Temelli F (2009) Perspectives on supercritical fluid processing of fats and oils. J Supercritical Fluids 47:583–590
Thomopoulos CD, Tzía C (1993) The effect of degumming by hydration on the purification of high acidity solvent extracted olive oil. Grasas Aceites 44:259–261
Tubaileh RM, Garrido-Fernández A, Ruiz Méndez MV, León-Camacho M, Graciani-Constante E (2002) Effects of deodorization/physical refining variables in wax and fatty alcohols content of refined olive oil. J Am Oil Chem Soc 79:971–976

Verleyen T, Verhe R, Huyghebaert A, Dewettinck K, Greyt W (2001) Identification of alpha-tocopherol oxidation products in triolein at elevated temperatures. J Agric Food Chem 49:1508–1511

Vioque E, Maza MP (1973) Glicolípidos de la aceituna. Grasas y Aceites 24:226–235

Welsh WA, Bogdanor JM, Toeneboehn G (1989) Silica refining of oils and fats. In: Erickson DR (ed) Edible fats and oils processing: basic principles and modern practices. AOCS Press, Champaign, pp 189–202

Zamora R, Olmo C, Navarro JR, Hidalgo FJ (2004) Contribution of phospholipid pyrrolization to the color reversion produced during deodorization of poorly degummed vegetable oils. J Agric Food Chem 52:4166–4171

Chapter 20
Tables of Olive Oil Chemical Data

Diego L. García-González, Carmen Infante-Domínguez, and Ramón Aparicio

Contents

20.1 Introduction

The nutritional assessment, authentication task, and traceability of any foodstuff require accurate compositional data validated by multilab comparisons. To do this with olive oil, it is necessary to carry out a prospective study of the existing databases and standardization to avoid errors in quantification. At present, available databases are scarce and incomplete and are thus difficult to cross tabulate. In recognition of these problems, the olive sector, in the research and industrial realms, demands a validated world database that would make possible a multicountry, multicultivar comparison and lead to better traceability and control of authenticity problems. The global olive oil trade, which has a high degree of competitiveness and rapid regulatory changes, needs a database on olive oil that is accepted

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties*,
DOI 10.1007/978-1-4614-7777-8_20, © Springer Science+Business Media New York 2013

worldwide to adapt the legal limits of some chemical parameters to a new world trade scenario free from adulteration and low-quality oils (García-González and Aparicio 2010). A standardized database that includes information from all producing countries would also allow regulatory bodies to reach a consensus on fixed legal limits, instead of proposing values based on partial studies, in order to avoid the continual readjustment of these values. Furthermore, the achievement of a world database on regular chemical compounds (e.g., fatty acids, sterols) would spur research institutions to supply information on other minor parameters (i.e., metals and n-alkanes) that could be applied to traceability and authentication control.

A profile of olive oil composition depends on several variables that can be clustered into four large groups: climate, soil, olive ripeness, and cultivar. These groups are intimately related to each other. The first two concern geographical provenance if we accept that other variables, such as the altitude and latitude of orchards, are ultimately related to climate and soil, which are the real actors directly affecting the concentration of chemical compounds. Thus, some previous studies proved that the temperature in certain time frames during olive ripening and the concentration of some metals in the soil significantly affect the activity of the enzymes responsible for olive oil chemical composition. Olive ripeness determines the profile of the chemical compounds responsible for olive oil quality (e.g. volatiles, pigments, and phenols). However, if olives are harvested at their optimum level of maturity, as often happens, then the influence of maturity on the other chemical compounds (e.g. fatty acids, sterols, alcohols) is almost negligible.

The fourth and last variable, cultivar, is the first source of information on olive oil characterization and traceability because it embodies the prevalent genetic diversity of all living organisms. Furthermore, as was just explained, the olive tree, being a living organism, is affected by the aforementioned external variables, which explains the interrelationship among those variables. Thus, all avenues of database standardization converge on the study of cultivars as the main parameter to explain the chemical composition of olive oils. This study may result in the recognition of a protected designation of origin (PDO) or protected geographical indication (PGI) when it is associated with a particular geographic location. Consequently, the cultivars (monovarietal olive oils) may serve as the backbone of any database on olive oil chemical composition. Ideally, this database should include those new varieties resulting from cross breedings since, in some cases, they have burst onto the agricultural scene and could be significant in the future. The current location of cultivars and the wide variety of synonyms in cultivar nomenclature also play an important role in making a database potentially useful for backward traceability. Furthermore, this information on cultivar locations and synonyms (Bartolini et al. 1998) gives the database a desirable flexibility to reshape the information in the case of a sudden alteration in the cultivar mapping of a particular region.

In this chapter a multicountry, multicultivar composition database for olive oil is presented by studying and analyzing information from different sources. The chemical parameters being studied are analyzed using standard or commonly accepted methods, from either the SEXIA project or from different literature sources (see references cited in the tables). Although the relevance of the information on tocopherols,

pigments, volatiles, and phenols is unquestionable, these compounds varied with time and with many variables other than cultivar and location. In addition to their labile nature, the lack of official methods for most of these compounds leads to a confusing collection of data with very different value ranges that cannot be compared. Consequently, the information presented in this section is primarily based on invariable compounds in order to obtain a long-lasting database. The methods used to analyze those compounds are described in official regulations (Chaps. 6 and 16). Thus, the following tables represent a first approach to reorganizing the complex information on olive oil composition and can be considered a starting point for a more ambitious project that would require a well-planned strategy of information gathering and normalization.

20.2 Chemical Composition of Olive Oils

As was stated elsewhere in this book, olive oil composition is strictly regulated by a series of limits established for analytical parameters that determine its purity and authenticity. The considerable efforts that have been devoted to improving the current analytical techniques have not been continued with a systematic characterization of the kinds of olive oil most frequently encountered in the market. Furthermore, in the current context, the existence of discrepancies in the authenticity of olive oils and errors between laboratories are mostly due to the scarce information on the samples analyzed (e.g., cultivar + geographical origin) and, obviously, to the application of different methods or quantification procedures. The first deficiency may be remedied by databases that store information for most of the chemical compounds of olive oils. With respect to the second problem, even though many methods are standard and are well defined in regulations, many laboratories still devise slight variations, skip steps, or present the data in different units. Those methods are even more prone to error when they are used to analyze minor components that are not usually determined by routine labs. For this last reason, we have included restricted information on chemical compounds that are not determined by official methods, although the analytical procedures are well described in the literature; they are hydrocarbons, 4-4′-dimethylsterols, and 4-monomethylsterols (Aparicio and Alonso 1994; Guinda et al. 1996; Azardmard-Damirchi and Dutta 2006; García-González et al. 2012).

To present results that can be compared with other sources, the information on chemical compounds is given in milligrams per kilogram, with the exception of fatty acids. Thus, 4-desmethylsterols, which are usually given in percentages for authenticity purposes, are displayed in milligrams per kilogram in the tables. This kind of quantification avoids the collinearity statistical phenomenon that affects calculations regarding predictors when applying statistical procedures and might prevent statistical software from performing the matrix inversion that is used in some nonsupervised statistical algorithms.

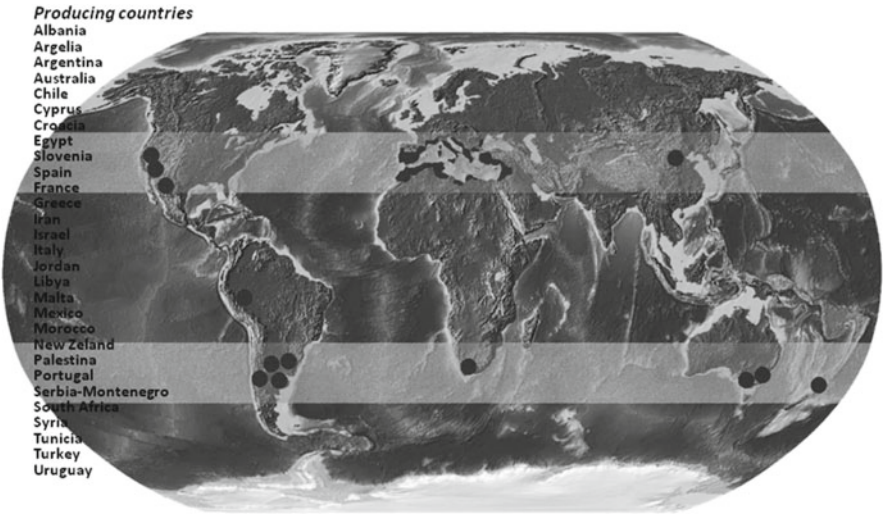

Fig. 20.1 Location of olive-oil-producing regions on a world map

The estimated seven million hectares planted with around 600 million productive olive trees of more than 1,275 cultivars (Bartolini et al 1998) are not exclusively limited to the Mediterranean basin but include countries as far flung as Australia and Chile (Fig. 20.1). To the diversity of the geographical locations of orchards we must add the enormous range of olive oils throughout Europe, which, having acquired a reputation for being genuine articles of high quality, are now protected under the umbrella of protected designation of origin (PDO) and protected geographical indication (PGI) labels (Table 20.1). In the first case, a PDO olive oil is one that is produced, processed, and prepared in a given geographical area using recognized techniques, and at least one of these stages in the second case (PGI). Databases have assisted researchers in their objective of characterizing PDOs (Fig. 20.2) by means of chemical compounds and so avoiding the presence of frauds in the market.

Therefore, future work on olive oil characterization and traceability should focus on building an olive oil map, where the most productive cultivars, the most prominent geographical zones, and all of the approved PDOs are characterized by chromatographic, spectroscopic, and isotopic information. It is hoped that the tables in this chapter will contribute to that aim.

Table 20.1 Names of European protected designations of origins (PDO) of virgin olive oils clustered by country

French PDO olive oil	*Portuguese PDO olive oil*
Huile d'olive d'Aix-en-Provence	
Huile d'olive De Corse	Azeite de Moura
Huile d'olive de Haute-Provence	Azeite de Tras-os-Montes
Huile d'olive de la Vallee des Baux-de-Provence	Azeite do Alentejo interior
Huile d'olive de Nice	Azeites do Norte Alentejano
Huile d'olive de Nimes	Azeite do Ribatejo
Huile d'olive de Nyons	Azeites da Beira interior
Greek PDO olive oil	*Italian PDO live oil*
Agios Mathaios Kerkyras	Alto Crotonese
Apokoronas Hanion Kritis	Aprutino Pescarese
Archanes Iraklio Kritis	Brisighella
Exeretiko partheno eleolado: "Thrapsano"	Bruzio
exeretiko partheno eleolado "Trizinia"	Canino
Finiki Lakonias	Cartoceto
Kalamata	Chianti Classico
Kolymvari Hanion Kritis	Cilento
Kranidi Argolidas	Collina di Brindisi
Krokees Lakonias	Colline di Romagna
Lygourgio Asklipiou	Colline Salernitane
Petrina Lakonias	Colline Teatine
Peza Iraklio Kritis	Dauno
Sitia Lasithi Kritis	Garda
Viannos Iraklio Kritis	Laghi Lombardi
Vorios Mylopotamos Rethymnis Kritis	Lametia
	Lucca
Spanish PDO olive oil	Molise
	Monte Etna
Aceite de La Rioja	Monti Iblei
Aceite de Mallorca/Aceite mallorquin/ Oli de Mallorca/Oli mallorqui	Penisola Sorrentina
	Pretuziano delle Colline Teramane
Aceite de Terra/Oli de Terra Alta	Riviera Ligure
Aceite del Baix Ebre-Montsia or Oli del Baix Ebre-Montsia	Sabina
	Sardegna
Aceite del Bajo Aragon	Tergeste
Aceite Monterrubio	Terra di Bari
Antequera	Terra d'Otranto
Baena	Terre di Siena
Gata-Hurdes	Terre Tarantine
Les Garrigues	Toscano
Montes de Granada	Tuscia
Poniente de Granada	Umbria
Priego de Cordoba	Valdemone
Sierra de Cadiz	Val di Mazara
Sierra de Cazorla	Valle del Belice
Sierra de Segura	Valli Trapanesi
Sierra Magina	Veneto, Veneto Euganei e Berici, Veneto del Grappa
Siurana	

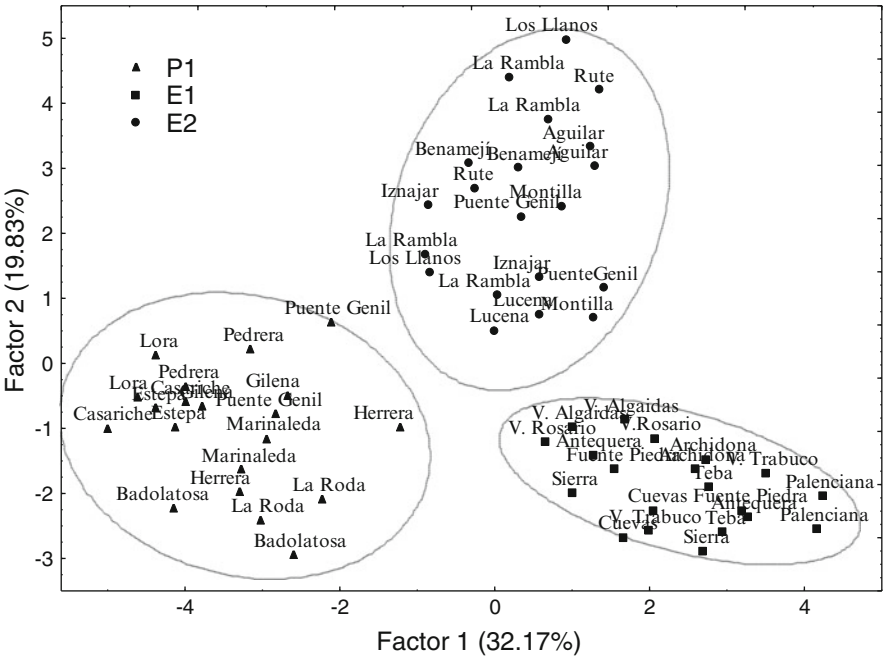

Fig. 20.2 Principal component analysis of VOOs produced in PDO "Estepa" and in some neighboring orchards in the Spanish provinces of Málaga (E1) and Córdoba (E2). Note: The names are of the villages where the olive mills are located (*Source*: García-González et al. (2012), with permission of Grasas y Aceites)

20.3 Tables with Information on Olive Oil Chemical Compounds

Tables 20.2–20.8 display information on the chemical composition of several varietals of olive oil. The number of characterized cultivars varies from 68, in the case of fatty acids, to 26, for the analysis of hydrocarbons. Some of these varietal olive oils have different geographical provenances (e.g., Picual from Spain, Chile, Argentina, and New Zealand), which makes it possible to analyze the influence of climate/latitude on the chemical composition of the same cultivar. The number of analyzed varietal oils is, obviously, high in the most common analytical methodology (fatty acids), which does not means that this information is more relevant than that from n-alkanes (hydrocarbons). Most of the information comes from the long-running large project funded by the Andalusian regional government to characterize Spanish production; many other foreign cultivars have been added to the database since it started in the last century. The frequent occurrence of "not reported" is an example of lack of complete information and dispersion of data published in scientific journals.

Table 20.2 Chemical composition of varietal olive oils: Fatty acids (%)

	C16:0		C16:1[a]		C17:0		C17:1n-8		C18:0		C18:1[b]		C18:2n-6		C18:3n-3		C20:0		C20:1n-9		C22:0			
Cultivar *(Country)*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	n	Reference
Arbequina (SP)	12.54	*3.76*	1.36	*0.3*	0.12	*0.01*	0.2	*0.04*	1.4	*0.36*	72.12	*3.44*	11.38	*0.17*	0.69	*0.11*	0.4	*0.07*	0.35	*0.05*	0.12	*0*	*17*	1
Carrasqueña (SP)	11.36	*0.26*	1.16	*0.07*	0.04	*0*	0.06	*0*	2.85	*0.1*	66.95	*1.12*	16.04	*2.11*	0.88	*0.04*	0.4	*0.01*	0.53	*0.01*	0.26	*0.03*	*7*	1
Cornicabra (SP)	8.62	*1.34*	0.70	*0.13*	0.06	*0.04*	0.09	*0.04*	2.5	*0.31*	78.71	*5.1*	7.53	*3.93*	0.66	*0.11*	0.53	*0.04*	0.36	*0.03*	0.11	*0.05*	*38*	1
Empeltre (SP)	9.59	*0.41*	0.79	*0.03*	0.1	*0*	0.18	*0.01*	1.23	*0.04*	77.51	*1.01*	9.08	*0.6*	0.6	*0.03*	0.36	*0.01*	0.38	*0.01*	0.15	*0.08*	*15*	1
Farga (SP)	10.43	*0.27*	0.70	*0.06*	0.16	*0.03*	0.27	*0.04*	2.16	*0.09*	72.33	*1.28*	11.93	*0.97*	0.63	*0.03*	0.42	*0.01*	0.4	*0.02*	0.15	*0*	*21*	1
Gordalilla (SP)	7.86	*0.65*	0.63	*0.03*	0.11	*0*	0.14	*0.01*	2.41	*0.11*	81.61	*0.82*	5.36	*0.13*	0.61	*0.01*	0.54	*0.01*	0.38	*0.01*	0.13	*0*	*7*	1
Hojiblanca (SP)	9.32	*1.17*	0.72	*0.22*	0.11	*0.04*	0.16	*0.06*	2.5	*0.62*	77.63	*3.04*	8.16	*1.81*	0.58	*0.17*	0.35	*0.11*	0.29	*0.1*	0.07	*0.05*	*44*	1
Imperial (SP)	9.67	*1.09*	0.88	*0.16*	0.08	*0.03*	0.08	*0.02*	2.27	*0.35*	80.09	*1.94*	4.63	*0.85*	0.91	*0.16*	0.41	*0.09*	0.28	*0.04*	0.08	*0.01*	*6*	1
Lechín Sevilla (SP)	11.05	*1.06*	1.08	*0.14*	0.12	*0.01*	0.19	*0*	2.06	*1.02*	74.48	*1.45*	9.43	*1.15*	0.74	*0.22*	0.32	*0.14*	0.29	*0.13*	0.06	*0.08*	*6*	1
Manzanilla[c] (SP)	9.30	*1.45*	0.75	*0.14*	0.07	*0.04*	0.09	*0.05*	2.46	*0.4*	75.56	*5.66*	9.8	*4.53*	0.73	*0.11*	0.53	*0.05*	0.35	*0.04*	0.11	*0.04*	*19*	1
Manzanillad[d] (SP)	10.59	*1.34*	0.99	*0.31*	0.14	*0.05*	0.17	*0.06*	2.52	*0.17*	75.57	*3.27*	8.08	*1.62*	0.72	*0.2*	0.52	*0.17*	0.4	*0.15*	0.1	*0.05*	*6*	1
Manzanilla[e] (SP)	12.46	*0.64*	1.08	*0.43*	0.07	*0.02*	0.07	*0.01*	1.68	*0.19*	68.28	*0.87*	14.06	*0.91*	1.08	*0.43*	0.4	*0.12*	0.28	*0.1*	0.1	*0.03*	*4*	1
Morrut[f] (SP)	9.40	*0.30*	0.53	*0.04*	0.12	*0.03*	0.12	*0.01*	3.18	*0.08*	73.98	*0.4*	10.72	*0.33*	0.57	*0.01*	0.53	*0.03*	0.38	*0.04*	0.15	*0.03*	*7*	1
Negral (SP)	12.55	*1.96*	0.72	*0.06*	0.1	*0*	0.19	*0.01*	1.53	*0.2*	75.22	*0.46*	10.03	*0.26*	0.61	*0.01*	0.38	*0.02*	0.32	*0.02*	0.09	*0.01*	*5*	1
Nevadillo Blanco[2] (SP)	10.50	*0.83*	0.96	*0.2*	0.09	*0.07*	0.13	*0.05*	2.54	*0.49*	77.97	*2.58*	6.46	*1.57*	0.63	*0.09*	0.36	*0.05*	0.25	*0.04*	0.07	*0.04*	*6*	1
Pico Limón (SP)	9.13	*0.21*	0.89	*0.08*	0.16	*0.01*	0.2	*0.03*	2.45	*0.09*	78.08	*2.12*	8.04	*1.13*	0.69	*0.02*	0.54	*0.02*	0.34	*0.01*	0.13	*0*	*5*	1
Picual (SP)	9.59	*1.02*	0.85	*0.22*	0.04	*0.04*	0.07	*0.03*	2.76	*0.65*	80.79	*3.12*	4.65	*1.31*	0.55	*0.14*	0.35	*0.09*	0.22	*0.07*	0.05	*0.05*	*226*	1
Picudo (SP)	10.26	*0.71*	0.94	*0.16*	0.06	*0.05*	0.1	*0.05*	2.68	*0.42*	76.61	*3.22*	7.97	*2.18*	0.55	*0.22*	0.37	*0.09*	0.26	*0.12*	0.07	*0.05*	*18*	1
Serrana (SP)	10.66	*0.18*	0.66	*0.04*	0.18	*0.03*	0.3	*0.04*	2.12	*0.1*	71.67	*1.28*	12.37	*1.02*	0.65	*0.02*	0.41	*0.01*	0.4	*0.01*	0.15	*0*	*6*	1
Sevillenca[3] (SP)	11.13	*0.65*	0.69	*0.09*	0.17	*0.07*	0.26	*0.06*	1.65	*0.89*	66.49	*1.99*	17.06	*1.86*	1.04	*0.11*	0.48	*0.09*	0.41	*0.09*	0.17	*0.05*	*6*	1
Verdial de Huevar (SP)	10.23	*0.54*	0.7	*0.09*	0.17	*0.02*	0.27	*0.03*	2.55	*0.17*	74.68	*1.84*	8.97	*1.06*	0.83	*0.1*	0.51	*0.05*	0.53	*0.13*	0.16	*0.03*	*8*	1
Verdial de Vélez (SP)	9.59	*0.23*	0.64	*0.08*	0.15	*0.02*	0.22	*0.02*	2.08	*0.22*	73.45	*1.94*	10.9	*1.19*	1.24	*0.18*	0.52	*0.04*	0.4	*0.11*	0.13	*0.03*	*4*	1
Biancolilla (IT)	14.01	*1.78*	0.89	*0.2*	0.19	*0.06*	0.33	*0.08*	2.31	*0.49*	71.71	*3.72*	9.17	*2.01*	0.49	*0.19*	0.36	*0.09*	0.15	*0.11*	0.05	*0.04*	*3*	1
Bossana (IT)	12.41		0.96		0.04		0.09		2.01		73.32		10.49		0.83		0.36		0.33		0.12		*9*	5
Carolea (IT)	16.02		1.18		0.25		0.53		2.75		68.87		7.76		0.43		0.58		0.28		0.11		*9*	5
Cerasuola (IT)	10.00		0.3		0.1		0.1		2.6		76.3		9.2		0.5		0.4		0.3		0.1		*3*	5

(continued)

Table 20.2 (continued)

Cultivar *(Country)*	C16:0		C16:1[a]		C17:0		C17:1n-8		C18:0		C18:1[b]		C18:2n-6		C18:3n-3		C20:0		C20:1n-9		C22:0		n	Reference
	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*		
Coratina (IT)	9.13	*0.28*	0.44	*0.06*	0.06	*0.01*	0.11	*0.02*	2.36	*0.14*	80.8	*1*	6.07	*0.66*	0.68	*0.02*	0.37	*0.01*	0.42	*0.02*	0.12	*0*	*7*	1
Castiglionese (IT)	15		1.3		0.1		0.1		2.5		72		8		0.7		0.3		0.3		0.1		*4*	5
Dritta (IT)	12.17		0.95		0.06		0.09		2.58		77.52		4.99		0.39		0.46		0.39		0.15		*11*	5
Frantoio (IT)	10.2	*1.2*	0.6	*0.15*	nr		0.1	*0.01*	1.6	*0.19*	80.6	*3.3*	5.4	*1.1*	0.6	*0.16*	0.3	*0.11*	0.4	*0.08*	0.1	*0.03*	*13*	5
Leccino (IT)	12.69	*1.12*	1.18	*0.03*	0.04	*0*	0.1	*0.01*	1.67	*0.07*	75.98	*3.12*	5.77	*1.54*	0.65	*0.06*	0.3	*0.01*	0.25	*0.05*	0.1	*0.01*	*7*	1
Maiatica (IT)	11.2	*0.94*	1.07	*0.15*	0.03	*0.02*	0.09	*0.02*	3.1	*0.17*	75.42	*1.57*	7.49	*0.49*	0.51	*0.05*	0.41	*0.04*	0.27	*0.03*	0.1	*0*	*3*	1
Mix. Lucania[4] (IT)	*12.53*	*0.38*	*1.16*	*0.07*	*0.03*	*0.01*	*0.11*	*0.01*	*2.12*	*0.18*	*75.03*	*0.48*	*7.57*	*0.48*	*0.62*	*0.02*	*0.36*	*0.02*	*0.31*	*0.03*	*0.11*	*0.04*	*5*	1
Mix. Tuscany[5] (IT)	*11.82*	*0.38*	*0.91*	*0.08*	*0.04*	*0.01*	*0.08*	*0.01*	*2.03*	*0.09*	*75.33*	*0.69*	*7.84*	*0.36*	*0.6*	*0.02*	*0.39*	*0.01*	*0.36*	*0.02*	*0.12*	*0*	*6*	1
Moraiolo (IT)	10.99	*0.45*	0.61	*0.1*	0.04	*0.01*	0.08	*0.02*	1.84	*0.26*	78.48	*1.67*	6.15	*0.63*	0.62	*0.12*	0.29	*0.03*	0.32	*0.09*	0.09	*0.02*	*6*	1
Noccelara[f] (IT)	13.5		1		0.1		0.1		2.8		73.1		8.1		0.7		0.4		0.2		0.1		*3*	5
Noccelara[g] (IT)	14.94		1.35		0.12		0.31		1.58		64.64		15.71		0.29		0.73		0.26		0.08		*7*	5
Ogliarola (IT)	12.18	*0.53*	1.02	*0.18*	0.03	*0.01*	0.08	*0.01*	1.96	*0.14*	74.15	*1.12*	8.65	*0.69*	0.64	*0.02*	0.39	*0.02*	0.4	*0.04*	0.11	*0*	*7*	1
Ottobratica[h] (IT)	14.36		0.99		0.21		0.33		2.43		71.47		8.59		0.44		0.6		0.31		0.15		*18*	5
Ottobratica[i] (IT)	15.33	*0.47*	1.79	*0.3*	0.17	*0.03*	0.25	*0.06*	1.87	*0.26*	69.25	*0.57*	9.84	*0.77*	0.38	*0.04*	0.63	*0.05*	0.3	*0.01*	0.13	*0.01*	*3*	1
Sinopolese (IT)	13.24		0.92		0.15		0.24		2.42		76.09		5.2		0.45		0.78		0.35		0.16		*18*	5
Taggiasca (IT)	8.37	*0.24*	0.51	*0.03*	0.01	*0.01*	0.06	*0.01*	1.87	*0.06*	82.21	*0.58*	5.72	*0.23*	0.42	*0.03*	0.3	*0.01*	0.24	*0.01*	0.08	*0.02*	*8*	1
Toccolana (IT)	12.65		1		0		0.1		2.2		74.35		8.35		0.4		0.6		0.35		0		nr	5
Tonda Iblea (IT)	16.63	*0.04*	2.28	*0.07*	0.06	*0.01*	0.1	*0.01*	1.51	*0.07*	66.55	*0.13*	11.46	*0.07*	0.26	*0.03*	0.84	*0.04*	0.2	*0.03*	0.06	*0.01*	*16*	6
Koroneiki (GR)	11.21	*0.57*	0.95	*0.07*	0.25	*0.1*	0.15	*0.05*	2.59	*0.24*	77.75	*1.76*	5.63	*1.1*	0.55	*0.13*	0.5	*0.09*	0.55	*0.07*	0.27	*0.1*	*9*	1
Media Oblonga (GR)	11.79	*0.14*	0.57	*0.02*	0.12	*0.01*	0.19	*0*	2.15	*0*	61.92	*0.27*	19.53	*1.08*	0.67	*0.01*	0.34	*0.04*	0.32	*0.04*	0.09	*0.01*	*3*	1
Aglandau (FR)	11.94		1.01		0.19		0.38		2.43		75.5		7.06		0.63		0.4		0.28		nr		*8*	8
Cailletier (FR)	10.42		0.66		0.05		0.11		2.1		78.4		6.75		0.64		0.38		0.33		nr		*8*	8
Picholine (FR)	10.74		0.73		0.06		0.1		2.31		74.96		9.31		0.85		0.36		0.33		nr		*8*	8
Oblica (HR)	12.63	*0.97*	0.91	*0.08*	nd		0.12	*0*	2.29	*0.17*	70.21	*2.3*	12.07	*0.97*	0.74	*0.05*	0.42	*0.04*	0.34	*0.09*	nd		*13*	4
Lavtoska (HR)	12.3	*1*	0.95	*0.15*	nd		0.15	*0.05*	2.3	*0*	72.15	*1.45*	10.45	*0.35*	0.7	*0*	0.45	*0.05*	0.3	*0*	0.1	*0*	*2*	4
Levantinka (HR)	12.65	*1.28*	0.88	*0.08*	nd		0.1	*0*	3.18	*0.45*	72.97	*3.13*	8.23	*1.09*	0.78	*0.08*	0.57	*0.08*	0.35	*0.05*	0.18	*0.05*	*5*	4
Nabali Baladi (JO)	12.4	*0.05*	1.06	*0.02*	0.32	*0*	0.32	*0.01*	3	*0.2*	67.1	*0.3*	13.8	*0.2*	0.74	*0.03*	0.6	*0.01*	0.33	*0*	0.16	*0*	*4*	7

Nabali Muhasan (JO)	12.5	*0.2*	1.08	*0.02*	0.29	*0.03*	0.31	*0.01*	3.1	*0.05*	67	*0.4*	13.9	*0.2*	0.74	*0.05*	0.6	*0.05*	0.32	*0.01*	0.16	*0.01*	*4*	7
Shami (JO)	16	*0.3*	1.2	*0.2*	0.12	*0.02*	0.25	*0.03*	2.17	*0.1*	67.1	*0.5*	11.4	*0.3*	0.75	*0.1*	0.4	*0.03*	0.28	*0.02*	0.11	*0.02*	*4*	7
Aloui (TN)	11	*2.2*	0.62	*0.38*	nr		nr		2.96	*1*	67.2	*5.4*	17.2	*3.9*	0.67	*0.01*	0.44	*0.01*	nr		nr		*3*	3
Chetoui (TN)	13.11	*2.38*	0.28	*0.11*	0.05	*0.02*	0.09	*0.04*	3.2	*0.17*	70.2	*4.26*	12.3	*3.54*	0.77	*0.15*	0.5	*0.04*	0.23	*0.02*	0.1	*0.02*	*8*	1
Chladmi (TN)	14.9	*2.1*	1.72	*0.51*	nr		nr		2.85	*0.12*	69.8	*0.9*	9.77	*3.59*	0.62	*0.11*	0.4	*0.01*	nr		nr		*3*	3
Jarboui (TN)	14.53	*1.82*	0.75	*0.2*	0.04	*0.01*	0.04	*0.01*	2.12	*0.32*	62.77	*3.29*	18.38	*1.28*	0.79	*0.16*	0.41	*0.05*	0.23	*0.04*	0.1	*0.03*	*6*	1
Neb Jmel (TN)	16.47		1.27		0.04		0.04		2.68		70.98		10.87		0.72		0.51		0.23		0.1		*18*	2
Regregui (TN)	15.36	*1.05*	0.8	*0.23*	0.04	*0*	0.04	*0*	1.94	*0.12*	62.37	*1.29*	18.34	*0.55*	0.68	*0.18*	0.45	*0.04*	0.23	*0.15*	0.1	*0.01*	*18*	2
Rekhami (TN)	14.86	*2.18*	0.64	*0.32*	0.04	*0*	0.04	*0*	2.38	*0.59*	64.57	*5.58*	16.09	*5.13*	0.76	*0.11*	0.4	*0.14*	0.2	*0.08*	0.1	*0.01*	*18*	2
Sayali (TN)	11		0.2		nr		nr		2.7		77.4		5.9		1.7		0.2		0.6		nr		*nr*	10
Sredki (TN)	10.54	*0.7*	0.53	*0.06*	nr		nr		2.38	*0.19*	74	*0.8*	11.6	*1.3*	0.59	*0.03*	0.39	*0.12*	nr		nr		*3*	3
Arbequina (CL)	14.16	*0.29*	1.38	*0.16*	0.1	*0.02*	0.23	*0.08*	1.68	*0.26*	72.78	*0.64*	8.43	*0.74*	0.35	*0.05*	0.5	*0.07*	0.27	*0.04*	0.11	*0.04*	*4*	1
Barnea (CL)	11.99	*0.18*	0.75	*0.09*	0.05	*0.01*	0.08	*0.01*	2.12	*0.27*	77.3	*0.45*	6.45	*0.91*	0.35	*0.03*	0.57	*0.03*	0.24	*0.04*	0.1	*0.02*	*3*	1
Frantoio (CL)	12.59	*0.22*	0.98	*0.04*	0.04	*0.01*	0.08	*0.01*	2.01	*0.3*	74.11	*0.48*	8.91	*1.12*	0.31	*0.09*	0.61	*0.08*	0.27	*0.04*	0.09	*0.02*	*3*	1
Leccino (CL)	13.27	*0.31*	0.87	*0.1*	0.04	*0.01*	0.07	*0.03*	1.72	*0.12*	78.19	*0.95*	4.64	*0.62*	0.29	*0.07*	0.57	*0.04*	0.26	*0.04*	0.08	*0.02*	*3*	1
Picual (CL)	12.45	*0.25*	0.8	*0.07*	0.06	*0.01*	0.12	*0.04*	1.88	*0.2*	79.64	*1.01*	3.81	*0.84*	0.32	*0.06*	0.59	*0.06*	0.25	*0.03*	0.09	*0.01*	*4*	1
Arbequina (AR)	19.78	*0.26*	3.16	*0.15*	0.09	*0.01*	0.25	*0.06*	1.57	*0.02*	54.19	*0.77*	19.36	*0.41*	0.89	*0.01*	0.4	*0.01*	0.16	*0.01*	0.09	*0.01*	*20*	1,14
Arauco (AR)	16.82	*3.08*	1.77	*0.74*	0.08	*0.03*	0.11	*0.01*	2.53	*0.38*	61.83	*11.49*	15.12	*6.9*	0.7	*0.43*	0.6	*0.14*	0.23	*0.04*	0.16	*0.06*	*4*	1,14
Barnea (AR)	14.87	*0.92*	1.51	*0.32*	0.08	*0.04*	0.09	*0*	2.16	*0.16*	60.94	*3.33*	19.7	*2.25*	0.85	*0.04*	0.38	*0.04*	0.24	*0.05*	0.1	*0.01*	*6*	1,14
Coratina (AR)	12.21	*0.84*	0.55	*0.08*	0.02	*0.02*	0.08	*0.04*	1.94	*0.08*	70.66	*2.77*	13.03	*1.8*	0.54	*0.23*	0.5	*0.28*	0.41	*0.01*	0.1	*0.01*	*4*	1,14
Empeltre (AR)	12.9	*1.4*	1.19	*0.35*	0.11	*0.01*	0.32	*0.02*	1.73	*0.14*	72.94	*3.25*	9.16	*0.85*	0.77	*0.05*	0.25	*0.08*	0.31	*0.02*	0.05	*0.04*	*2*	14
Frantoio (AR)	16.18	*0.4*	1.71	*0.2*	0.07	*0.06*	0.11	*0.01*	1.99	*0.21*	64.03	*0.17*	14.47	*0.39*	0.81	*0.17*	0.44	*0.06*	0.26	*0.01*	0.09	*0.01*	*4*	1,14
Koroneiki (AR)	13.73	*0.29*	1.35	*0.07*	0.01	*0*	0.01	*0*	2.36	*0.21*	71.64	*1.28*	9.09	*0.95*	0.76	*0.08*	0.47	*0.02*	0.35	*0.03*	0.1	*0.01*	*3*	1
Manzanilla (AR)	15.77	*0.56*	1.98	*0.17*	0.1	*0*	0.3	*0.09*	2.54	*1.14*	67.18	*7.34*	10.65	*5.75*	0.99	*0.09*	0.4	*0.16*	0.36	*0.06*	0.1	*0.08*	*6*	1,14
Picual (AR)	11.55	*1.61*	1.68	*0.5*	0.04	*0.02*	0.07	*0.02*	3.1	*0.85*	72.15	*5.56*	8.59	*3.79*	0.9	*0.17*	0.37	*0.08*	0.21	*0.08*	0.08	*0.04*	*7*	1,14
Arbequina (AU)	15.53	*2.3*	2.05	*0.6*	nr		nr		1.52	*0.4*	67.15	*5.8*	12.18	*3.3*	0.63	*0.11*	nr		nr		nr		*8*	9
Barnea (AU)	11.4	*1.8*	0.85	*0.2*	nr		nr		1.98	*0.2*	71.65	*3.5*	12.68	*2.1*	0.63	*0.1*	nr		nr		nr		*8*	9
Coratina (AU)	11.05	*2*	0.4	*0.1*	nr		nr		1.78	*0.3*	77.53	*2.4*	7.53	*1.1*	0.75	*0.1*	nr		nr		nr		*8*	9
Frantoio (AU)	12.85	*1.33*	1.05	*0.27*	nr		nr		1.7	*0.11*	70.08	*3.13*	10.8	*1.49*	0.63	*0.14*	nr		nr		nr		*8*	9

(continued)

Table 20.2 (continued)

Cultivar *(Country)*	C16:0		C16:1[a]		C17:0		C17:1n-8		C18:0		C18:1[b]		C18:2n-6		C18:3n-3		C20:0		C20:1n-9		C22:0		n	Reference
	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*		
Koroneiki (AU)	11.87	*1.5*	0.9	*0.2*	nr		nr		2.23	*0.3*	77.27	*0.6*	6.2	*1.4*	0.57	*0.1*	nr		nr		nr		*8*	9
Leccino (AU)	13.5	*1.5*	1.1	*0.3*	nr		nr		1.75	*0.4*	75.88	*1.2*	6.78	*1.1*	0.6	*0.1*	nr		nr		nr		*8*	9
Manzanilla (AU)	13.6	*1.4*	1.47	*0.2*	nr		nr		3.1	*0.4*	70.3	*3.48*	9.67	*2.16*	0.67	*0.1*	nr		nr		nr		*8*	9
Picual (AU)	12.1	*1.7*	1.2	*0.5*	nr		nr		2.25	*0.7*	78.78	*2.28*	4.18	*0.9*	0.7	*0.2*	nr		nr		nr		*8*	9
Barnea (NZ)	8.34		0.51		0.04		0.09		1.94		80.38		7.41		0.33		0.6		0.26		0.09		*1*	1
Frantoio (NZ)	9.68	*0.17*	0.57	*0.03*	0.04	*0.01*	0.09	*0.01*	1.46	*0.03*	81.44	*0.43*	5.42	*0.31*	0.29	*0.02*	0.62	*0.03*	0.34	*0.03*	0.09	*0.02*	*3*	1
Koroneiki (NZ)	8.13		0.56		0.04		0.07		1.79		83.71		4.25		0.34		0.66		0.33		0.13		*1*	1
Leccino (NZ)	10.54		0.76		0.04		0.09		1.67		80.68		4.99		0.28		0.59		0.29		0.07		*1*	1
Picual (NZ)	8.81		0.51		0.04		0.08		2.11		84.11		3.17		0.28		0.58		0.25		0.07		*1*	1
Arbequina[5] (US)	14.06	*2.14*	1.44	*0.54*	0.12	*0.04*	0.26	*0.05*	2.02	*0.36*	71.32	*4.13*	9.16	*1.92*	0.73	*0.1*	0.39	*0.05*	0.32	*0.06*	0.11	*0.02*	*18*	17
Coratina[5] (US)	12.93	*5.53*	1,17	*1*	0.13	*0.06*	0.2	*0.1*	2.3	*0.35*	72	*11.47*	8.93	*4.48*	1.27	*0.64*	0.47	*0.06*	0.33	*0.06*	0.13	*0.06*	*3*	17
Frantoio[5] (US)	12.43	*0.83*	0.88	*0.16*	0.1	*0*	0.1	*0*	2.52	*0.31*	73.37	*1.8*	8.9	*0.98*	0.87	*0.18*	0.4	*0*	0.3	*0*	0.1	*0*	*6*	17
Koroneiki[5] (US)	12.36	*1.1*	0.94	*0.25*	0.1	*0*	*0.14*	0.09	2.34	*0.33*	76.14	*1.97*	6.24	*0.76*	0.74	*0.11*	0.42	*0.04*	0.32	*0.04*	0.16	*0.05*	*5*	17
Mission[5] (US)	10.16	*1.56*	0.81	*0.28*	0.14	*0.08*	*0.16*	0.1	2.58	*0.45*	76.63	*2.94*	7.84	*1.54*	0.92	*0.12*	0.36	*0.07*	0.31	*0.04*	0.11	*0.03*	*14*	17
Range for EVOO (IOC)	7.5 20		0.3 3.5						0.5 5		55 83		3.5 21		≤1.0		≤0.6		≤0.4		≤0.3		-	11

Note: [a], C16:1 is the sum of C16:1n-9 and C16:1n-7; [b], C18:1 is the sum of C18:1n-9 and C18:1n-7; [c], Manzanilla Cacereña; [d], Manzanillad de Huelva; [e], Manzanilla de Sevilla; [f], Noccelara del Belice; [g], Noccelara Messinese; [h], Ottobratica Rotondella; [i], Ottobratica PerciaSacchi; n, number of samples; ref, reference number; Std, standard deviation, only when reported by authors; nr, not reported; AR, Argentina; AU, Australia; CL, Chile; FR, France; HR, Croatia; IT, Italy; JO, Jordan; NZ, New Zealand; SP, Spain; TN, Tunisia; EVOO, Extra virgin olive oil; IOC, international Olive Council; [1], it might include a low percentage of Sevillenca; [2], it might include a low percentage of Nevadillo Negro; [3], it might include a very low percentage of Morrut; [4], it is a mixture of Ogliarola del Brandano (50%), Maiatica di Ferrandina (20%) and Coratina (30%); [5], it is a mixture of Frantoio (60%), Leccino (10-20%), Moraiolo (15-20%) and Seggianese (5%).

References: 1, Aparicio (2013); 2, Haddada et al. (2007); 3, ManaI-Djebali et al. (2012); 4, Zanetic et al. (2010); 5, Angerosa (2011); 6, Giuffré (2005); 7, Al-Ismail et al. (2011); 8, Ollivier et al. (2003); 9, Mailer et al. (2010); 11, EC, (2003); 14, Ceci and Carrelli (2007); 16, Stefanoudaki et al. (2000); 17, Wong and Flynn (2013).

Table 20.3 Chemical composition of varietal olive oil: Major 4-desmethylsterols and diols (mg/kg)

	S1		S2		S3		S4		A1			
Cultivar (*Country*)	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	n	Reference
Arbequina (SP)	61.01	*8.89*	14.85	*6.94*	1269.1	*195.73*	289.02	*60.25*	17.92	*4.78*	17	1
Carrasqueña (SP)	39		15.87		1312.02		85.7		39.45		9	13
Cornezuelo (SP)	38.81		10		1167.3		183.47		35.58		9	13
Cornicabra (SP)	52.7	*11.4*	18.8	*11.7*	1365.3	*212*	114.3	*52.7*	47.53	*15.25*	38	1
Corniche (SP)	75.99		12.7		1654.34		79.97		62.16		9	13
Empeltre (SP)	51.8	*17.7*	13	*7*	1526.5	*577.8*	128	*51.8*	17.59	*4.8*	15	1
Farga (SP)	58.5	*6.4*	37.5	*14.1*	1352.5	*150.9*	86.5	*58.5*	39.84	*15.8*	21	1
Gordalilla (SP)	39.63	*3.16*	38.89	*0.91*	1278.85	*77.01*	166.8	*12.2*	43.59	*0.43*	7	1
Hojiblanca (SP)	55.1	*13.7*	18	*5.7*	1594.2	*352.9*	119	*54.84*	30.3	*7.19*	44	1
Imperial (SP)	50.79	*9.15*	24.61	*3.76*	1389.65	*184.32*	52.43	*1.12*	29.75	*3.15*	6	1
Lechín de Sevilla (SP)	80.39	*5.72*	31.17	*6.24*	2427.11	*89.57*	115.81	*67.26*	52.17	*20.53*	6	1
Manzanilla Cacereña (SP)	43.5	*7.6*	26.5	*12*	1355.6	*281.8*	146.6	*43.5*	48.39	*16.82*	6	1
Manzanilla de Huelva (SP)	39.96	*2.89*	28.17	*3.07*	1306.79	*80.31*	122.69	*14.81*	50.62	*5.37*	6	1
Manzanilla de Sevilla (SP)	55.08	*3.94*	20.27	*2.16*	2035.42	*40.95*	119.06	*12.11*	37.18	*4.17*	4	1
Morrut[1] (SP)	60.71	*1.54*	33.89	*3.82*	1397.27	*39.69*	84.73	*3.98*	43.88	*1.86*	7	1
Negral (SP)	41.61	*1.8*	10.31	*0.48*	1239.15	*87.09*	98.08	*14.6*	22.2	*4.39*	5	1
Nevadillo Blanco[2] (SP)	49.4	*8.1*	21.8	*7.5*	1366.2	*214.9*	77.9	*49.4*	33.46	*10.01*	6	1
Pico Limón (SP)	34.54	*5.27*	31.12	*2.83*	1277.99	*100.29*	107.96	*17.91*	56.67	*1.34*	5	1
Picual (SP)	47.1	*7*	12.4	*4.3*	1220.3	*182*	82.8	*47.1*	20.87	*7.19*	226	1
Picudo (SP)	45.2	*4*	14.5	*3.8*	1242.8	*162.1*	99.1	*45.2*	23.4	*5.93*	18	1
Real Sevillana (SP)	40.26		12.03		1403.12		214.91		42.01		9	13
Royal (SP)	38.77	*0.29*	11.11	*3.43*	1105.36	*37.15*	155.13	*53.05*	27.91	*7.97*	3	1
Serrrana (SP)	56	*1.81*	13.91	*4.45*	1280.23	*46.76*	97.94	*8.26*	24.14	*2.48*	6	1
Sevillenca[3] (SP)	90.9		19.32		1813.52		101.35		32.08		6	1
Verdial de Badajoz (SP)	48.91		14.61		1305.76		114.27		46.6		9	13

(continued)

Table 20.3 (continued)

Cultivar (*Country*)	S1		S2		S3		S4		A1		n	Reference
	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*		
Verdial de Huevar (SP)	42.8	*4.1*	26.7	*7.4*	1275.8	*164.4*	105.7	*42.8*	112.51	*24.45*	8	1
Verdial de Vélez (SP)	56.09	*1.93*	15.33	*2.67*	1419.89	*157.94*	137.69	*10.11*	67.38	*3.18*	4	1
Carolea (IT)	46.93		9.04		1881.83		268.15		24.09		9	5
Castiglionese (IT)	39.63		8.59		1165.55		160.67		nr		4	5
Cerasuola (IT)	40.99		13.66		1050.88		104.06		17.87		3	5
Coratina (IT)	46.76	*0.68*	10.99	*0.96*	1157.6	*5.86*	98.2	*5.27*	26.8	*5.4*	7	1
Frantoio (IT)	83.01		41.3		2430.16		337.64		26.65		8	5
Grossa di Cassano (IT)	51.64		14.21		1399.99		67.11		nr		nr	15
Dritta (IT)	39.64		15.76		990.99		198.43		40.99		11	5
Itrana (IT)	39.75		11.25		1238.44		161.26		nr		nr	15
Leccino (IT)	46.19	*3.72*	20.47	*0.45*	1366.41	*103.38*	164.44	*4.78*	19.42	*5.29*	7	1
Maiatica (IT)	48.15	*2.86*	18.81	*7.41*	1201.74	*101.61*	503.01	*64.01*	32.36	*3.5*	3	1
Mix. Lucania[4] (IT)	47.39	*1.95*	14.85	*1.15*	1259.41	*43.5*	293.3	*86.39*	23.73	*1.82*	5	1
Mix. Tuscany[5] (IT)	44.63	*3.75*	13.27	*2.17*	1288.01	*91.19*	154.4	*10.95*	28.07	*2.99*	6	1
Moraiolo (IT)	82.02	*4.94*	27.19	*6.28*	2872.06	*135.96*	575.04	*44.29*	59.16	*4.15*	6	1
Noccelara del Belice (IT)	33.06		13.56		850.36		57.53		18.68		3	5
Noccelara Messinese (IT)	43.98		10.66		1172.72		80.87		9.9		7	5
Nociara (IT)	49.09		15.57		1382.91		194.31		nr		nr	15
Ogliarola (IT)	42.29	*2.53*	17.44	*1.82*	1108.07	*102.65*	181.61	*17.6*	37.29	*5.58*	7	1
Ottobratica Rotondella (IT)	38.9		14.71		1086.61		78.11		11.01		18	5
Ottobratica PerciaSacchi (IT)	35.2	*4.8*	12.1	*3.2*	980.1	*108.3*	141.21	*12.7*	12.8	*1.82*	3	1
Pendolino (IT)	39.37		14.96		1152.21		141.12		nr		nr	15
Picholine (IT)	66.39		16.07		1671.33		102.54		nr		nr	15
Sinopolese (IT)	56.78		28.7		1572.3		123.18		21.34		18	5

Taggiasca (IT)	32.33	*2.44*	6.94	*0.67*	996.99	*73.98*	129.62	*7.39*	15.44	*1.82*	8	1
Toccolana (IT)	31.1		19.28		988.09		164.58		25.25		nr	5
Koroneiki (GR)	43.25	*0.68*	6.95	*0.79*	831.1	*6.11*	187.1	*8.74*	27.67	*3.29*	7	1
Lianolia (GR)	46.15		9.63		1218.64		108.16		nr		10	12
Lavtoska (HR)	46.37		25.43		1287.9		168.13		25.43		2	4
Levantinka (HR)	35.89		8.28		1189.06		115.55		9.66		5	4
Oblica (HR)	47.25		21		1495.45		236.26		17.15		13	4
Nabali Baladi (JO)	35	*2*	10	*7*	1147	*30*	76	*5*	nr		4	7
Nabali Muhasan (JO)	75	*4*	42	*3*	2243	*50*	233	*10*	nr		4	7
Shami (JO)	49	*3*	18	*2*	1746	*50*	102	*4*	nr		4	7
Aloui (TN)	36.87	*1.29*	12.02	*0.56*	1015.96	*13.62*	150.61	*3.87*	27.6	*0.44*	3	3
Chemlali (TN)	72.7		7.45		1725.78		144.18		28.39		3	3
Chetoui (TN)	33.81	*0.4*	25.16	*10.71*	1022.22	*15.78*	168.54	*30.36*	25.63	*2.93*	8	1
Chladmi (TN)	54.22	*1.11*	5.43	*0.13*	1222.1	*17.04*	211.23	*4.03*	23.9	*0.63*	3	3
Jarboui (TN)	57.16	*0.22*	11.08	*0.19*	1685.9	*15.72*	161.19	*3.93*	27.29	*1.96*	18	2
Neb Jmel (TN)	58.69	*0.57*	11.21	*0.59*	1739.4	*28.53*	165.89	*2.85*	28.94	*2.84*	18	2
Rekhami (TN)	44.5	*0.33*	10.87	*0.66*	1411.09	*68.64*	179.19	*6.86*	42.63	*1.5*	18	2
Regregui (TN)	62.03	*0.94*	16.62	*2,82*	2112.33	*23.55*	88.72	*13.19*	42.37	*9.42*	18	2
Sayali (TN)	37.03	*1.72*	14.01	*0.94*	1475.02	*124.03*	183	*37.04*	nr		18	2
Sredki (TN)	51.8	*2*	15.5	*1.3*	1126.32	*51.01*	137.24	*16.03*	32.4	*1.04*	3	3
Arbequina (CL)	33.27	*0.45*	7.44	*0.85*	684.54	*5.12*	261.7	*3.17*	14.99	*2.72*	4	1
Barnea (CL)	40.2	*0.72*	4.98	*0.15*	753.29	*4.17*	67.91	*1.11*	18.93	*3.19*	3	1
Frantoio (CL)	44.76	*0.77*	7.31	*0.11*	1202.55	*8.39*	163.66	*6.23*	8.07	*1.15*	3	1
Leccino (CL)	37.5	*0.52*	10.67	*0.49*	892.12	*6.17*	109.18	*2.24*	24.66	*2.21*	3	1
Picual (CL)	37.12	*0.57*	5.64	*0.09*	863.32	*5.82*	88.28	*1.79*	26.16	*2.05*	4	1
Arauco (AR)	58.71	*10.18*	11.01	*2.89*	1113.8	*143.84*	108.21	*6.121*	78.88	*32.53*	4	1,14
Arbequina (AR)	96.17	*21.79*	24.29	*8.52*	1864.3	*349.95*	111.85	*32.77*	37.06	*5.29*	20	1,14

(continued)

Table 20.3 (continued)

Cultivar (*Country*)	S1		S2		S3		S4		A1		n	Reference
	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*		
Barnea (AR)	108.37	*2.1*	17.06	*3.7*	2043.51	*35.63*	104.36	*8.72*	26.45	*2.61*	6	1.14
Coratina (AR)	32.69	*0.9*	9.29	*0.96*	847.03	*57.49*	62.59	*0.84*	19.39	*2.37*	4	1,14
Empeltre (AR)	54.16	*0.34*	17.73	*93.04*	1394.06	*311.89*	147.07	*2.56*	12.37	*5.69*	2	14
Frantoio (AR)	63.86	*12.62*	14.19	*5.01*	1627.67	297.77	105.89	*20.16*	19	*5.05*	4	1,14
Manzanilla (AR)	73.63	*9.66*	34.33	*12.36*	2032.7	*249.69*	103.76	*47.21*	24.07	*8.26*	6	1,14
Picual (AR)	74.37	*5.78*	21.37	*2.63*	1936.3	*97*	122.71	*16.47*	22.47	*3.55*	7	1,14
Barnea (NZ)	61.87		8.72		1101.83		116.83		10.66		1	1
Frantoio (NZ)	40.92	*0.62*	10.18	*0.45*	910.11	*44.72*	117.98	*8.37*	8.63	*0.63*	3	1
Koroneiki (NZ)	42.91	*0.68*	11.67	*0.77*	681.41	*4.69*	142.08	*4.22*	20.98	*4.32*	1	1
Leccino (NZ)	50.73		17.92		989.37		169.33		12.12		1	1
Picual (NZ)	40.15		4.24		894.87		102.71		12.83		1	1
Values for EVOO (IOC)	≤4%[5]		S3>S5		≥93%[5]				≤4.5%[5]		–	11

Note: S1, campesterol; S2, stigmasterol; S3, β-sitosterol; S4, Δ^5-avenasterol; A1, Erythrodiol + uvaol; n, number of samples; ref, reference number; Std, Standard deviation, only when reported by authors; nr, not reported; EVOO, Extra Virgin Olive Oil; IOC, International Olive Oil; AR, Argentina; CL, Chile; GR, Greece; HR, Croatia; IT, Italy; JO, Jordan; NZ, New Zealand; SP, Spain; TN, Tunisia; [1], it might include a low percentage of Sevillenca; [2], it might include a low percentage of Nevadillo Negro; [3], it might include a very low percentage of Morrut; [4], it is a mixture of Ogliarola del Brandano (50%), Maiatica di Ferrandina (20%) and Coratina (30%); [5], it is a mixture of Frantoio (60%), Leccino (10-20%), Moraiolo (15-20%) and Seggianese (5%).

References: 1, Aparicio (2013); 2, Haddada et al. (2007); 3, ManaI-Djebali et al. (2012); 4, Zanetic et al. (2010); 5, Angerosa (2011); 7, Al-Ismail et al. (2011); 11, EC, (2003); 12, Longobardi et al. (2012); 13, Sánchez-Casas et al. (2004); 14, Ceci and Carrelli (2007); 15, Giuffrè et al. (2012).

Table 20.4 Chemical composition of varietal olive oil: Minor 4-desmethylsterols (mg/kg)

	S1		S2		S4		S6		S7		S9		S11		S12		S13			
Cultivars (*Country*)	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	n	Reference
Arbequina (SP)	2.17	*0.54*	6.84	*0.73*	7.24	*0.28*	1.99	*0.35*	1.99	*0.19*	18.29	*0.83*	12.13	*0.19*	2.72	*0.23*	6.88	*0.47*	17	1
Empeltre (SP)	1.82		2.99		7.96		1.99		2.32		13.6		10.78		10.28		20.57		15	1
Carrasqueña (SP)	5.9		1.97		3.48		1.66		16.78		15.27		10.13		1.97		2.27		9	13
Cornezuelo (SP)	7.5		4.56		2.65		3.23		18.52		12.06		10.29		4.7		6.91		9	13
Cornicabra (SP)	4.98	*1.27*	3.76	*1.27*	5.18	*0.91*	2.12	*1.67*	14.58	*1.15*	11.24	*4.12*	7.29	*2.02*	3.06	*0.56*	5.14	*0.98*	38	1
Corniche (SP)	9.48		3.03		3.22		3.03		21.22		13.45		10.42		3.79		4.36		9	13
Manzanilla Cacereña (SP)	5.18	*0.76*	2.29	*0.15*	1.81	*0.09*	0.36	*0.02*	12.17	*2.15*	5.67	*0.96*	6.63	*0.89*	3.13	*0.99*	8.08	*1.17*	6	1
Morisca (SP)	6.1		4.71		4.18		2.27		17.43		14.82		12.9		3.66		6.62		9	13
Verdial de Badajoz (SP)	7.84		3.08		2.77		0.31		14.92		15.07		10.15		2.92		7.38		9	13
Carolea (IT)	3.67		2.09		3.29		1.14		21.39		16.04		9.73		7.36		10.42		9	5
Castiglionese (IT)	2.42		0.73		1.32		1.9		14.73		16.51		12.64		3.46		8.61		4	5
Cerasuola (IT)	1.51		1.14		6.1		2.02		11.77		10.09		7.36		3.57		6.52		3	5
Coratina (IT)	3.6	*0.8*	4.05	*1.2*	5.89	*0.42*	4.73	*0.57*	14.06	*1.04*	18.87	*5.25*	6.79	*1.26*	3.18	*0.32*	4.26	*0.55*	7	1
Dritta (IT)	1.17		2.17		1.57		nr		12.05		12.27		10.48		1.53		7.85		11	5
Frantoio (IT)	2.3		4.31		0.91		2.62		32.44		15.64		30.6		15.71		26.93		8	5
Grossa di Cassano[1] (IT)	3.79		0.63		1.58		0.32		15.79		15.79		2.53		2.84		3.79		nr	15
Itrana (IT)	3.6		3.9		0.9		0.15		15.6		12.6		4.95		3.9		5.7		nr	15
Leccino (IT)	3.48	*0.92*	1.36	*0.27*	2.6	*1.19*	1.75	*0.7*	16.83	*1.54*	10.12	*0.42*	8.52	*2.75*	5.64	*2.91*	8.78	*3.38*	7	1
Moraiolo (IT)	5.16	*0.76*	7.01	*0.84*	1.05	*0.54*	nr		9.18	*0.29*	21.23	*6.97*	27.05	*8.95*	12	*4.99*	18.11	*8.34*	6	1
Noccelara di Belice (IT)	0.79		0.6		7.44		2.48		9.26		4.63		57.53		4.79		2.81		3	5
Noccelara Messinese (IT)	2.55		2.04		3.62		7.22		12.47		7.49		5.43		6.55		7.97		7	5
Nociara (IT)	3.42		5.82		3.76		0.34		16.25		14.71		12.66		5.47		7.7		nr	15
Ottobratica Perciasacchi (IT)	4.11	*1.32*	2.09	*0.83*	7.6	*2.23*	0.89	*0.64*	14.81	*2.52*	7.16	*5.7*	10.93	*2.78*	2.65	*0.63*	2.99	*0.38*	3	1
Ottobratica Rotondella (IT)	2.9		0.58		2.22		1.59		11.31		16.09		17.88		2.73		3.36		18	5
Pendolino (IT)	4.8		3.39		3.25		0.28		16.09		23.14		4.66		3.95		4.8		nr	15

(continued)

Table 20.4 (continued)

Cultivars (*Country*)	S1		S2		S4		S6		S7		S9		S11		S12		S13		n	Reference
	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*		
Picholine (IT)	6.5		2.87		1.72		1.72		16.84		10.33		4.97		4.21		6.5		nr	15
Sinopolese (IT)	3.5		1.09		3.11		2.38		18.16		15.09		5.92		4.96		5.23		18	5
Toccolana (IT)	0		2.49		2.49		8.09		9.95		12.44		11.2		1.24		6.97		nr	5
Koroneiki (GR)	3.59	*0.16*	4.71	*0.22*	5.49	*1.23*	1.1	*0.05*	12.77	*0.94*	8.63	*1.89*	6.39	*0.48*	2.91	*0.29*	4.37	*0.31*	7	1
Lianolia (GR)	1.42		0.99		2.83		2.69		8.78		1.42		7.93		4.39		7.93		10	12
Lavtoska (HR)	1.79		2.24		1.5		0.15		13.46		12.71		7.48		2.99		7.48		2	4
Levantinka (HR)	1.38		2.21		2.07		0.14		12.7		16.15		55.22		1.38		6.9		5	4
Oblica (HR)	1.75		1.75		2.98		0.18		14.88		17.5		5.25		1.75		7		13	4
Aloui (TN)	3.14	*0.37*	2.93	*0.19*	2.1	*0.29*	nd		15.52	*1.68*	9.38	*0.21*	11.05	*0.55*	2.65	*0.17*	5.76	*0.48*	3	3
Chemlali (TN)	2.22		4.03		1.01		0		19.94		7.45		11.68		3.83		13.69		3	3
Chetoui (TN)	1.74	*0.06*	3.24	*0.63*	1.11	*0.08*	1.42	*0.13*	13	*0.23*	4.55	*1.07*	8.87	*0.03*	1.98	*0.03*	4.33	*0.33*	8	1
Chladmi (TN)	3.31	*0.68*	3.62	*0.21*	2.96	*0.29*	4.45	*2.11*	16.61	*0.81*	14.57	*0.54*	14.1	*0.1*	3.64	*0.07*	10.92	*0.1*	3	3
Jarboui (TN)	1.58	*0.22*	2.77	*0.23*	0.79	*0.09*	1.98	*0.21*	19.98	*1.96*	4.35	*0.2*	13.65	*0.08*	4.75	*0.19*	14.83	*0.24*	18	2
Neb Jmel (TN)	1.43	*0.04*	2.65	*0.54*	1.02	*0.05*	2.04	*0.02*	19.36	*0.55*	4.48	*0.04*	13.86	*0.03*	4.89	*0.11*	15.08	*0.04*	18	2
Rekhami (TN)	2.38	*0.82*	4.76	*0.81*	1.7	*0.8*	1.7	*0.2*	18	*8.16*	6.45	*1.64*	8.15	*0.79*	5.77	*1.64*	5.44	*0.78*	18	2
Regregui (TN)	2.34	*0.94*	1.17	*0.93*	1.4	*0.94*	2.34	*0.2*	22.47	*1.88*	11.24	*3.01*	5.38	*1.88*	6.55	*2.83*	12.41	*5.65*	18	2
Sayali (TN)	18.02	*11.03*	5.04	*2.03*	2	*4*	3.01	*1.02*	9.03	*6.02*	7.03	*5.02*	3.04	*1.05*	2.01	*1.02*	3.04	*1.03*	18	2
Sredki (TN)	1.94	*0.27*	2.71	*0.2*	1.7	*0.08*	0.85	*0.75*	13.4	*0.9*	11.2	*1.4*	9.4	*2.22*	2.02	*0.66*	8.5	*1.84*	3	3
Arbequina (CL)	1.06	*0.11*	6.59	*0.34*	1.28	*0.11*	1.06	*0.05*	11.27	*0.84*	34.23	*1.06*	13.5	*0.54*	5.74	*0.18*	3.4	*0.34*	4	1
Barnea (CL)	0.98	*0.09*	3.81	*0.21*	2.73	*0.22*	0.98	*0.04*	55.72	*1.1*	37.66	*1.41*	3.51	*0.16*	2.15	*0.1*	3.81	*0.25*	3	1

Frantoio (CL)	1.52	*0.12*	2.44	*0.23*	1.83	*0.25*	1.52	*0.07*	17.96	*0.19*	60.14	*1.17*	9.9	*0.23*	2.28	*0.16*	9.59	*0.47*	3	1
Leccino (CL)	1.15	*0.14*	4.82	*0.2*	3.9	*0.16*	1.15	*0.05*	24.43	*0.75*	44.61	*0.98*	7.91	*0.19*	3.9	*0.15*	7.8	*0.39*	3	1
Picual (CL)	1.06	*0.1*	3.4	*0.21*	0.96	*0.34*	1.06	*0.06*	11.59	*0.84*	43.18	*1.23*	4.04	*0.09*	2.02	*0.11*	4.04	*0.16*	4	1
Arauco (AR)	11.65	*11.53*	2.82	*0.27*	2.15	*0.15*	4.64	*1.89*	14.39	*1.06*	28.11	*20.39*	9.51	*2.85*	2.94	*0.15*	6.36	*0.18*	4	1,14
Arbequina (AR)	6.76	*4.5*	2.49	*1.97*	8.84	*3.56*	5.14	*1.91*	21.34	*5.47*	21.21	*9.76*	12.45	*2.67*	4.57	*1.56*	5.04	*0.86*	20	1,14
Barnea (AR)	11.65	*11.53*	2.82	*0.27*	2.94	*0.15*	2.32	*0.7*	23.16	*2.13*	14.61	*1.74*	10.87	*1.28*	2.81	*0.42*	4.27	*0.93*	6	1,14
Coratina (AR)	3.64	*2.67*	1.69	*0.9*	3.8	*1.47*	3.12	*2.15*	10.84	*1.04*	29.64	*10.68*	5.16	*0.11*	3.74	*2.58*	2.45	*0.71*	4	1,14
Empeltre (AR)	1.67	*0.34*	1.68	*0.33*	4.35	*1.67*	3.44	*3.24*	15.04	*3.02*	10.36	*3.68*	8.36	*1.68*	2.34	*0.33*	6.35	*0.34*	2	14
Frantoio (AR)	18.08	*16.11*	1.52	*1.12*	3.26	*0.95*	4.34	*1.69*	16.76	*1.78*	25.44	*15.28*	17.57	*2.35*	6.51	*1.63*	8.58	*2.58*	4	1,14
Manzanilla (AR)	3.27	*1.1*	2.03	*1.71*	6.06	*1.72*	5.96	*1.85*	22.79	*3.9*	14.04	*4.24*	9.74	*1.02*	3.98	*1.5*	9.21	*2.3*	5	1,14
Picual (AR)	4.81	*0.69*	2.24	*0.12*	5.87	*0.72*	4.89	*0.66*	19.4	*1.9*	16.23	*5.28*	11.02	*1.56*	4.79	*0.25*	7.11	*0.86*	7	1,14
Barnea (NZ)	1.38		2.91		1.11		1.38		24.92		55.09		4.57		1.66		4.84		1	1
Frantoio (NZ)	1.15	*0.05*	2.58	*0.05*	2.44	*0.52*	1.15	*0.05*	11.77	*1.03*	42.48	*0.72*	5.93	*1.77*	2.04	*0.62*	5.72	*2.83*	3	1
Koroneiki (NZ)	0.94		4.52		2.07		0.94		11.2		34.06		4.8		2.07		4.14		1	1
Leccino (NZ)	1.32		6.46		2.77		1.32		13.97		49.42		7.77		3.69		6.33		1	1
Picual (NZ)	1.12		1.9		1.12		1.12		15.95		44.72		3.57		1.78		4.01		1	1
Values for EVOO (IOC)	≤0.5%[5]														≤0.5%[5]				-	11

Note: S1, cholesterol; S2, 24-methylen cholesterol; S4, campestanol; S6, Δ^7-campesterol; S7, Chlerosterol; S9, sitostanol; S11, $\Delta^{5,24}$-stigmastadienol; S12, Δ^7-stigmastenol; S13, Δ^7-avenasterol; n, number of samples; ref, reference number; EVOO, Extra Virgin Olive Oil; IOC, international Olive Council; Std, standard deviation, only when reported by authors; nr, not reported; [5], referred to the sum of all the sterols (from S1 to S13); AR, Argentina; CL, Chile; GR, Greece; HR, Croatia; IT, Italy; JO, Jordan; NZ, New Zealand; SP, Spain; TN, Tunisia.

References: 1, Aparicio (2013); 2, Haddada et al. (2007); 3, ManaI-Djebali et al. (2012); 4, Zanetic et al. (2010); 5, Angerosa (2011); 11, EC, (2003); 12, Longobardi et al. (2012); 13, Sánchez-Casas et al. (2004); 14, Ceci and Carrelli (2007); 15, Giuffrè et al. (2012).

Table 20.5 Chemical composition of varietal olive oils: Phytol and aliphatic alcohols (mg/kg)

	Ph		C22		C24		C26		C28		ST		
Cultivars (*Country*)	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	n	Reference
Arbequina (SP)	364.58	*60.17*	11.36	*2.23*	17.79	*1.85*	40.6	*4.19*	30.67	*1.98*	102.16	17	1
Carrasqueña (SP)	35.97	*7.25*	56.27	*7.16*	75.66	*10.11*	46.78	*7.64*	22.54	*1.99*	201.25	7	1
Cornicabra (SP)	185.43	*90*	41.85	*20.69*	60.63	*31.96*	58.3	*21.85*	25.7	*8.06*	186.48	38	1
Empeltre (SP)	141.33	*21.22*	25.08	*3.76*	45.36	*6.54*	49.08	*4.77*	24.07	*2.27*	143.59	15	1
Farga (SP)	82.06	*9.83*	45.82	*7.38*	71.89	*14.05*	59.13	*10.38*	28.63	*2.89*	205.47	21	1
Gordalilla (SP)	81.32	*3.11*	23.26	*4.97*	23.15	*2.53*	50.4	*1.99*	19.7	*1.64*	116.51	7	1
Hojiblanca (SP)	53.58	*15.01*	22.19	*10.52*	44.57	*21.64*	58.09	*22.97*	25.9	*10.08*	150.75	44	1
Imperial (SP)	90.68	*14.35*	28.56	*4.27*	44.68	*9.78*	85.54	*10.99*	29.38	*4.27*	193.36	6	1
Lechín de Sevilla(SP)	62.94	*7.26*	30.12	*9.89*	59.88	*16.43*	81.7	*31.37*	44.94	*14.73*	216.64	6	1
Manzanilla Cacereña (SP)	132.98	*72.74*	39.84	*20.05*	58.27	*33.43*	66	*26.84*	28.71	*10.09*	192.82	19	1
Manzanilla de Huelva (SP)	67.72	*10.37*	45.54	*14.21*	75.77	*40.7*	86.8	*33.23*	31.23	*10.91*	239.34	6	1
Manzanilla de Sevilla (SP)	118.62	*5.98*	19.62	*4.21*	19.29	*2.17*	28.22	*3.78*	22.2	*2.17*	88.95	4	1
Morrut[1] (SP)	111.38	*6.74*	42.04	*3.71*	69.18	*5.6*	86.53	*9.66*	33.74	*5.59*	231.48	7	1
Negral (SP)	219.92	*2.2*	46.49	*9.69*	64.36	*9.73*	71.7	*6.79*	31.23	*1.24*	213.78	5	1
Nevadillo Blanco[2] (SP)	63.12	*27.62*	34.12	*17.08*	69.27	*36.72*	93.81	*42.43*	28.54	*11.99*	225.75	6	1
Pico Limón (SP)	79.51	*8.12*	37.85	*6.26*	62.45	*9.73*	74.28	*12.03*	27.84	*2.37*	202.42	5	1
Picual (SP)	30.32	*9.81*	36.98	*16.04*	67.09	*29.21*	89.48	*39.24*	28.3	*12.32*	221.85	226	1
Picudo (SP)	41.9	*15.8*	20.5	*7.1*	37	*14.4*	64.3	*29.2*	23	*10.8*	144.8	18	1
Serrana (SP)	82.45	*11.62*	41.34	*6.94*	63.52	*13.36*	51.88	*8.79*	26.78	*2.63*	183.52	6	1
Sevillenca[3] (SP)	214.96	*23.36*	34.02	*4.17*	56.05	*4.75*	65.4	*2.84*	49.45	*4.17*	208.73	6	1
Verdial de Huevar (SP)	199.83	*27.06*	26.78	*14.63*	52.9	*31.42*	37.2	*14.63*	13.36	*4.68*	130.24	8	1
Verdial de Vélez (SP)	180.78	*21.17*	17.22	*9.89*	21.52	*8.98*	31.55	*10.74*	16.16	*7.23*	89.96	4	1
Coratina (IT)	71.81	*8.59*	23.52	*3.05*	33.17	*4.7*	52.52	*9.54*	26.77	*4.55*	135.98	7	1
Carolea (IT)	nr		9.39		17.81		15.14		10.25		57.45	9	5

Castiglione (IT)	216.4		25		52		28.8		19.7		145.1	4	5
Dritta (IT)	143.32		15.41		33.09		39.74		17.72		115.28	11	5
Frantoio (IT)	235.4		6.95	*2.91*	19.59	*7.43*	43.94	*12.39*	22.63	*6.34*	100.31	13	5
Gentile (IT)	281.5		25.7		28.9		39.7		17.7		112	16	5
Leccino (IT)	260.7		28.9		60		66.9		30.9		199	6	5
Maiatica (IT)	156.33	*7.31*	22.84	*3.06*	28.03	*4.74*	58.45	*13.96*	29.45	*7.21*	138.77	3	1
Mix Lucania[4] (IT)	114.86	*30.72*	23.46	*3.74*	35.94	*6.85*	45.78	*5.17*	24.2	*3.79*	129.38	5	1
Mix Tuscany[5] (IT)	129	*17.82*	34.58	*4.61*	56.21	*7.96*	63.58	*7.62*	32.97	*5.14*	188.04.	6	1
Moraiolo (IT)	81.4		5.9		16.4		37.7		5.9		79	nr	5
Noccellara Messinese (IT)	nr		4.77		6.81		13.36		10.53		39	3	5
Ogliarola (IT)	120.74	*28.39*	31.96	*13.91*	46.03	*18.57*	55.15	*11.77*	28	*5.02*	161.14	7	1
Ottobratica PerciaSacchi (IT)	nr		14.42	*4.85*	28.67	*4.93*	26.88	*8.52*	23.31	*2.91*	98.28	3	1
Ottobratica Rotondella (IT)	nr		19.83		32.17		34.28		29.83		126.33	18	5
Sinopolese (IT)	nr		13.5		24.44		45.28		25.11		120.95	18	5
Taggiasca (IT)	106.12	*14.98*	37.44	*2.36*	40.04	*3.85*	37.01	*3.36*	16.26	*1.88*	130.75	8	1
Toccolana (IT)	122.4		22.6		41.7		51.6		24.4		152	nr	5
Tonda Iblea (IT)	nr		11.66		18.7		9.02		9.6		54.03	16	6
Koroneiki (GR)	nr		13.16	*3.98*	35.69	*6.23*	73.48	*9.96*	26,71	*5.28*	162.87	21	1,16
Sayali (TN)	nr		20.1	*2.8*	36.5	*3.2*	49.3	*4.2*	28.9	*2.9*	177.8	nr	10
Values for EVOO (IOC)											≤350.00	–	11

Note: Ph, Phytol; Er, Erythrodiol; C22, Docosanol; C24, Tetracosanol; C26, Hexacosanol; C28, Octacosanol; ST, Total alcohols; n, number of samples; Std, standard deviation, only when reported by authors; nr, not reported; GR, Greece; IT, Italy; SP, Spain; TN, Tunisia; EVOO, Extra virgin olive oil; EVOO, Extra virgin olive oil; IOC, international Olive Council; [1], it might include a low percentage of Sevillenca; [2], it might include a low percentage of Nevadillo Negro; [3], it might include a very low percentage of Morrut; [4], it is a mixture of Ogliarola del Brandano (50%), Maiatica di Ferrandina (20%) and Coratina (30%); [5], it is a mixture of Frantoio (60%), Leccino (10-20%), Moraiolo (15-20%) and Seggianese (5%).

References: 1, Aparicio (2013); 5, Angerosa (2011); 6, Giuffrè (2005); 10, Sakouhi et al. (2010); 11, EC, (2003); 16, Stefanoudaki et al. (2000).

Table 20.6 Chemical composition of varietal olive oils: 4,4'-dimethylsterols (mg/kg)

	T1		T2		T3		T4		T5		T6		T7		T8			
Cultivars (*Country*)	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	n	Reference
Arbequina (SP)	9.66	*0.82*	23.59	*1.99*	22.94	*6.17*	64.82	*3.94*	6.33	*0.76*	163.69	*12.04*	144.67	*30.17*	2.79	*0.58*	17	1
Carrasqueña (SP)	4.14	*0.63*	12.6	*1.72*	14.9	*8.16*	33.33	*3.92*	7.93	*1.29*	94.63	*12.16*	605.99	*100.31*	4.37	*1.75*	7	1
Cornicabra (SP)	5.91	*1.25*	12.05	*4.86*	29.49	*14.2*	30.87	*10.84*	6.64	*2.13*	198.01	*36.43*	326.41	*169.66*	6.72	*5.77*	38	1
Empeltre (SP)	4.33	*0.38*	12.49	*2.06*	10.78	*0.7*	38.07	*5.06*	6.41	*0.98*	150.55	*28.93*	466.54	*46.27*	4.61	*1.1*	15	1
Farga (SP)	11.82	*2.08*	15.78	*1.92*	29.48	*3.64*	40.49	*4.48*	13	*1.98*	170.87	*16.78*	587.11	*91.89*	19.35	*9.4*	21	1
Gordalilla (SP)	7.65	*0.68*	11.68	*2.58*	70.56	*2.25*	36.84	*7.09*	7.43	*0.92*	150.21	*22.25*	709.9	*105.53*	11.29	*2.98*	7	1
Hojiblanca (SP)	10.29	*2.9*	17.81	*9.24*	26.25	*10.07*	51.69	*22.2*	8.85	*3.05*	166.2	*55.05*	922.46	*326.17*	8.72	*6.49*	44	1
Imperial (SP)	6.46	*0.62*	38.2	*1.97*	31.96	*4.78*	98.42	*8.94*	7.81	*1.75*	214.22	*23.96*	540.36	*80.75*	3.65	*1.17*	6	1
Lechin de Sevillla (SP)	5.72	*2.84*	5.14	*3.44*	17.25	*11.09*	25.57	*15.76*	8.01	*3.41*	83.49	*8.61*	749.35	*96.06*	8.66	*0.53*	6	1
Manzanilla Cacereña (SP)	6.14	*1.54*	12.02	*5.93*	32.8	*18.53*	32.12	*13.78*	7.19	*2.45*	112.08	*43.81*	539.21	*123.81*	6.86	*4.36*	19	1
Manzanilla de Huelva (SP)	6.9	*2.9*	12.18	*2.83*	35.2	*8.55*	35.12	*5.24*	9.56	*3.51*	143.72	*21.26*	723.52	*111.01*	8.38	*3.24*	6	1
Manzanilla de Sevilla (SP)	7.38	*1.1*	31.75	*4.17*	18.18	*3.62*	80.91	*7.38*	11.3	*2.84*	208.09	*39.56*	636.37	*94.13*	8	*1.23*	4	1
Morrut[1] (SP)	11.58	*1.38*	17.86	*3.74*	64.42	*4.7*	46.56	*6.99*	10.9	*1.28*	215.9	*24.61*	599.98	*69.71*	8.96	*3.07*	7	1
Negral (SP)	5.24	*1.14*	12.48	*2.4*	26.09	*10.3*	40.82	*4.8*	12.36	*6.09*	258.34	*47.21*	647.76	*68.95*	13.12	*6.16*	5	1
Nevadillo Blanco[2] (SP)	9.35	*2.11*	13.6	*6.47*	27.58	*7.63*	38.72	*17.22*	8.32	*2.25*	125.65	*43.65*	742.97	*160.05*	8.45	*7.55*	6	1
Pico Limón (SP)	6.09	*0.94*	19.31	*2.34*	37.68	*3.21*	46.19	*5.01*	7.87	*2.11*	150.52	*14.03*	597.28	*78.81*	3.88	*1.62*	5	1
Picual (SP)	7.04	*2.59*	13.47	*6.59*	20.39	*12.67*	42.18	*16.54*	8.11	*2.5*	137.79	*50.12*	847.96	*284.29*	8.57	*6.02*	226	1
Picudo (SP)	6.9	*1.9*	13.7	*5.9*	25.3	*13.3*	40.2	*14.5*	8.7	*2.7*	166.4	*48.6*	1002.2	*188.6*	9.1	*7.2*	18	1
Serrana (SP)	12.94	*2.08*	16.37	*2.16*	30.51	*4.13*	41.98	*5*	14.12	*1.94*	180.9	*15.92*	637.23	*91.13*	22.21	*10.6*	6	1
Sevillenca[3] (SP)	17.24	*5.16*	34.94	*12.03*	28.81	*6.21*	70.91	*9.16*	10.28	*2.17*	327.14	*16.21*	751.76	*40.23*	10.12	*3.11*	6	1
Verdial de Huevar (SP)	12.1	*1.62*	10.79	*4.26*	64.69	*13.07*	30.11	*9.01*	15.2	*1.79*	101.7	*20.8*	626.77	*119.04*	12.42	*4.24*	8	1
Verdial de Vélez (SP)	11.04	*1.79*	19.73	*3.23*	114.06	*2.16*	55.59	*4.23*	15.53	*1.87*	166.73	*23.91*	667.87	*80.84*	4.58	*1.11*	4	1

Coratina (IT)	14.02	*0.82*	15.27	*1.82*	47.74	*5.44*	34.45	*5*	17.72	*1.2*	388.69	*56.91*	679.83	*69.21*	17.32	*4.45*	7	1
Carolea (IT)	nr		24.91		34.63		71.27		nr		208		405		nr		9	5
Castiglione (IT)	nr		29.9		23.2		92.3		nr		652.8		349.5		nr		4	5
Dritta (IT)	nr		19.46		27.23		61.4		nr		275.68		539.92		nr		11	5
Frantoio (IT)	nr		44.9		121		156.7		nr		483.3		548.2		nr		13	5
Gentile (IT)	nr		nr		46.1		17.7		nr		323.8		395.9		nr		16	5
Leccino (IT)	nr		19.8		44.3		68.7		nr		288.7		435.3		nr		6	5
Leccino Calabria (IT)	nr		14.9		20.8		44.3		nr		165.6		286.9		nr		5	1
Mix Lucania[4] (IT)	12.96	*1.26*	15.28	*2.75*	47.55	*16.71*	37.57	*6.23*	20.46	*4.88*	367.48	*77.29*	657.82	*40.78*	11.39	*1.88*	5	1
Mix Tuscany[5] (IT)	11.84	*0.76*	13.03	*1.17*	38.18	*4.29*	30.86	*2.14*	17.68	*2.73*	261.9	*31.96*	595.5	*62.74*	nr		6	1
Maiatica (IT)	11.34	*2.49*	17.14	*3.33*	38.88	*10.78*	43.39	*12.5*	13.17	*7.33*	484.54	*113.44*	664.88	*100.53*	13.72	*1.49*	3	1
Moraiolo (IT)	nr		24.8		35.5		84.4		nr		312.19		250.7		nr		3	5
Noccellara Messinese (IT)	nr		20.6		33.8		65.8		nr		236.1		527.9		nr		7	1
Ogliarola (IT)	10.39	*1.21*	15.72	*2.09*	41.84	*9.82*	29.76	*3.91*	18.47	*6.12*	334.01	*66.16*	757.38	*67.04*	11.72	*2.8*	18	5
Ottobratica (IT)	nr		25.06		15		69.83		nr		178.44		173.44		nr		18	5
Sinopolese (IT)	nr		35.28		24.06		85.78		nr		268.78		233.83		nr		nr	5
Taggiasca (IT)	11.64	*0.87*	16.45	*2.52*	27.52	*2.8*	43.85	*5.93*	18.79	*1.57*	217.91	*31.98*	1261.14	*128.95*	13.24	*1.95*	8	1
Toccolana (IT)	nr		25.21		24.89		85.53		nr		366.4		508.1		nr		nr	5
Tonda Iblea (IT)	nr		5.1		18.38		37.95		nr		130.02		446.12		nr		16	6
Sayali (TN)	26	*11*	nr		101	*22*	nr		nr		523	*45*	1464	*126*	nr		nr	10

Note: T1, Taraxerol;T2, Dammaradienol; T3, β-amyrin; T4, Butyrospermol; T5, 24-methylene-lanost-8-en-3-β-ol; T6, Cycloartenol; T7, 24-Methylene-cycloartanol; T8, Lanosterol isomer; n, number of samples; Std, standard deviation, only when reported by authors; nr, not reported; IT, Italy; SP, Spain; TN, Tunisia; [1], it might include a low percentage of Sevillenca; [2], it might include a low percentage of Nevadillo Negro; [3], it might include a very low percentage of Morrut; [4], it is a mixture of Ogliarola del Brandano (50%), Maiatica di Ferrandina (20%) and Coratina (30%); [5], it is a mixture of Frantoio (60%), Leccino (10-20%), Moraiolo (15-20%) and Seggianese (5%).
References: 1, Aparicio (2013); 5, Angerosa (2011); 6, Giuffrè (2005); 10, Sakouhi et al. (2010).

Table 20.7 Chemical composition of varietal olive oils: 4-monomethylsterols (mg/kg)

	M1		M2		M3		M4		M5		OA			
Cultivars (*Country*)	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	n	Reference
Arbequina (SP)	19	*10.17*	8.87	*3.16*	10.01	*5.99*	12.85	*4.37*	50.27	*16.24*	3.17	*1.89*	17	1
Carrasqueña (SP)	24.85	*2.3*	14.76	*2.24*	56.4	*4.71*	8.9	*2.63*	110.18	*27.95*	9.12	*4.67*	7	1
Cornicabra (SP)	15.68	*6*	7.06	*2.19*	45.9	*20.61*	5.84	*1.99*	62.83	*24.7*	8.99	*3.55*	38	1
Empeltre (SP)	15.39	*0.93*	5.86	*0.56*	22.41	*2.26*	10.07	*0.65*	111.5	*13.44*	4.42	*1.03*	15	1
Farga (SP)	13.7	*2.65*	7.68	*1.02*	31.95	*4.76*	7.5	*0.88*	89.93	*13.2*	3.55	*1.07*	21	1
Gordalilla (SP)	15.19	*0.58*	6.95	*0.15*	34.25	*4.43*	8.84	*0.72*	89.97	*10.33*	14.73	*0.27*	7	1
Hojiblanca (SP)	26.81	*10.05*	9.1	*2.76*	81.45	*47.05*	11.06	*5.05*	111.79	*34.3*	8.86	*4.86*	44	1
Imperial (SP)	29.29	*8.89*	15.45	*2.76*	68.4	*11.94*	18.86	*6.73*	228.19	*40.85*	2.04	*0.89*	6	1
Lechin de Sevilla (SP)	24.51	*11.84*	14.86	*15.22*	66.81	*48.25*	18.77	*0.4*	168.27	*71.6*	9.23	*6.46*	6	1
Manzanilla Cacereña (SP)	16.23	*6.57*	7.97	*2.42*	33.77	*13.99*	6.52	*2.39*	79.29	*23.34*	10.06	*3.88*	19	1
Manzanilla de Huelva (SP)	12.44	*4.28*	9.1	*3.45*	26.91	*11.1*	7.14	*2.03*	103.94	*36.61*	10.63	*2.34*	6	1
Manzanilla de Sevilla (SP)	23.16	*14.15*	11.48	*4.16*	40.46	*18.95*	15.53	*2.78*	206.35	*40.16*	4.17	*1.34*	4	1
Morrut[1] (SP)	12.7	*2.77*	9.15	*1.16*	29.03	*7.07*	6.85	*1.33*	90.38	*11.26*	9.26	*1.61*	7	1
Negral (SP)	15.15	*2.05*	6.43	*0.8*	18.94	*2.88*	9.28	*1.01*	126.38	*19.23*	7.75	*1.22*	5	1
Nevadillo Blanco[2] (SP)	15.71	*5.5*	6.82	*2.59*	43.47	*19.11*	8.43	*2.42*	70.87	*23.08*	6.84	*4*	6	1
Pico Limón (SP)	21.35	*2.11*	10.05	*1.37*	38.17	*4.95*	7.76	*1.75*	109.14	*21.37*	11.27	*3.16*	5	1
Picual (SP)	14.75	*4.92*	7.09	*2.35*	42.24	*20.31*	7.27	*3.33*	67.17	*22.49*	4.8	*2.75*	226	1
Picudo (SP)	16.2	*6.2*	8.1	*2.7*	52.9	*19.8*	9	*2.6*	83	*25*	5.4	*3.3*	18	1

Serrana (SP)	12.66	*2.88*	7.16	*1.04*	29.51	*4.84*	7.37	*1.03*	86.98	*15.23*	2.92	*1.03*	6	1
Sevillenca[3] (SP)	18.51	*9.23*	20.71	*3.12*	49.35	*17.42*	9.48	*1.89*	154.03	*30.94*	5.3	*1.72*	6	1
Verdial de Huevar (SP)	19.85	*2.8*	12.87	*2.07*	23.09	*5.22*	13.22	*1.68*	169.97	*12.6*	17.36	*4.85*	8	1
Verdial de Vélez (SP)	24.8	*12.17*	13.35	*1.79*	36.55	*14.39*	13.05	*1.45*	142.56	*23.75*	8.9	*1.79*	4	1
Coratina (IT)	17.32	*1.82*	8.81	*1.2*	22.25	*3.58*	11.79	*1.44*	144.42	*14.79*	10.93	*1.43*	7	1
Mix Lucania[4] (IT)	15.9	*4.99*	9.4	*1.03*	19.28	*6.68*	13.74	*3.13*	175.17	*29.83*	6.31	*1.35*	7	1
Mix Tuscany[5] (IT)	11.61	*1.51*	6.76	*1.01*	13.36	*2.05*	8.6	*0.85*	138.11	*15.14*	6.1	*0.64*	3	1
Maiatica (IT)	23.93	*2.49*	11.31	*2.11*	36.02	*0.78*	12.92	*2.62*	211.38	*38.16*	6.55	*2.68*	3	1
Ogliarola (IT)	8.29	*2.57*	6.54	*2.26*	9.43	*3.47*	6.04	*1*	124.11	*26.06*	5.24	*0.36*	18	5
Taggiasca (IT)	15.25	*0.93*	5.84	*1*	26.77	*3.85*	7.25	*0.8*	101.4	*11.3*	3.65	*0.29*	8	1
Sayali (TN)	77	*19*	42	*14*	162	*38*	nr		421	*41*	nr		nr	10

Note: M1, Obtusifoliol; M2, Grammisterol; M3, Cycloeucalenol; M4, 24-Etillophenol; M5, Citrostadienol; OA, Oleanolic aldehyde; n, number of samples, ref, reference number; Std, Standard deviation, only when reported by authors; nr, not reported; IT, Italy; SP, Spain; TN, Tunisia; [1], it might include a low percentage of Sevillenca; [2], it might include a low percentage of Nevadillo Negro; [3], it might include a very low percentage of Morrut; [4], it is a mixture of Ogliarola del Brandano (50%), Maiatica di Ferrandina (20%) and Coratina (30%); [5], it is a mixture of Frantoio (60%), Leccino (10-20%), Moraiolo (15-20%) and Seggianese (5%).
References: 1, Aparicio (2013); 5, Angerosa (2011); 10, Sakouhi et al. (2010).

Table 20.8 Chemical composition of varietal olive oils: Hydrocarbons (mg/kg)

	C13:1		α-Copaene		Eremophylene		α-Muurolene		C17		C21			
Cultivar (*Country*)	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	n	Reference
Arbequina (SP)	0.43	*0.17*	1.42	*0.47*	0.54	*0.36*	0.89	*0.09*	0.19	*0.02*	0.93	*0.14*	12	1
Carrasqueña (SP)	1.55	*0.34*	0.74	*0.05*	0.47	*0.35*	0.19	*0.13*	0.8	*0.74*	0.6	*0.35*	7	1
Cornezuelo (SP)	0.42	*0.11*	2.82	*0.94*	1.01	*0.33*	0.06	*0*	0.07	*0.01*	0.36	*0.04*	10	1
Cornicabra (SP)	1.61	*1.12*	1.62	*1.83*	3.13	*2.92*	0.83	*1.04*	1.24	*2.01*	1.45	*2.43*	38	1
Empeltre (SP)	0.34	*0.09*	0.57	*0.08*	1.03	*0.14*	1.16	*1.23*	0.15	*0.04*	0.34	*0.05*	15	1
Farga (SP)	1.07	*0.56*	1.06	*0.65*	2.13	*0.95*	0.76	*0.35*	0.49	*0.22*	1.02	*0.35*	21	1
Gordalilla (SP)	2.11	*0.17*	1.4	*0.04*	7.46	*0.66*	0.89	*0.09*	0.18	*0.02*	0.26	*0.02*	7	1
Hojiblanca (SP)	2.51	*1.82*	1.84	*1.11*	1.42	*0.73*	0.83	*0.53*	0.23	*0.11*	0.32	*0.12*	44	1
Lechin de Sevilla (SP)	7.12	*2.97*	1.09	*0.61*	1.5	*0.78*	0.59	*0.31*	0.37	*0.3*	0.34	*0.11*	6	1
Manzanilla Cacereña (SP)	1.67	*1.19*	1.69	*1.48*	3.17	*2.9*	0.88	*0.86*	1.25	*1.2*	1.44	*1.24*	19	1
Manzanilla de Huelva (SP)	6.27	*1.51*	2.72	*0.92*	2.64	*0.81*	1.53	*0.53*	0.38	*0.24*	0.95	*0.92*	6	1
Morrut[1](SP)	5.77	*1.84*	0.34	*0.17*	2.69	*0.95*	0.42	*0.23*	1.53	*1.13*	2.59	*1.13*	7	1
Nevadillo Blanco[2] (SP)	3.74	*1.41*	0.55	*0.34*	2.13	*1.72*	0.29	*0.25*	0.38	*0.12*	0.47	*0.13*	26	1
Pico Limon (SP)	2.61	*1.52*	0.79	*0.23*	0.42	*0.11*	1.01	*0.78*	1.15	*0.67*	0.58	*0.23*	5	1
Picual (SP)	4.87	*1.53*	0.38	*0.24*	0.66	*0.62*	0.14	*0.13*	0.26	*0.16*	0.4	*0.28*	226	1
Picudo (SP)	5.81	*1.52*	1.33	*0.71*	0.42	*0.33*	0.71	*0.44*	0.23	*0.13*	0.31	*0.02*	18	1
Real Sevillana (SP)	1	*0.24*	1.59	*0.76*	0.68	*0.12*	0.05	*0*	0.1	*0.03*	0.4	*0.05*	9	1
Serrrana (SP)	0.19	*0.1*	0.47	*0.38*	1.16	*0.77*	0.35	*0.41*	0.27	*0.16*	0.44	*0.16*	6	1
Verdial de Badajoz (SP)	0.22	*0.09*	1.47	*0.69*	0.21	*0.09*	0.06	*0*	0.25	*0.02*	1.66	*0.76*	7	1
Verdial de Huevar (SP)	7.86	*1.14*	3.63	*1.22*	1.95	*0.53*	2.08	*0.77*	0.43	*0.34*	0.51	*0.26*	8	1
Taggiasca (IT)	0.67	*0.33*	0.61	*0.12*	0.23	*0.03*	0.27	*0.03*	0.33	*0.27*	0.67	*0.35*	5	1
Arbequina (CL)	9.46	*2.03*	6.56	*2.94*	3.21	*2.13*	0.24	*0.15*	0.11	*0.06*	0.21	*0.08*	4	1
Barnea (CL)	16.93	*3.12*	11.37	*1.04*	6.13	*2.19*	0.6	*0.52*	0.13	*0.05*	0.26	*0.13*	3	1
Frantoio (CL)	9.26	*1.46*	5.98	*0.97*	3.19	*1.15*	0.29	*0.12*	0.12	*0.09*	0.42	*0.11*	3	1
Leccino (CL)	8.07	*1.85*	5.18	*2.14*	2.44	*0.98*	0.66	*0.27*	0.23	*0.11*	0.44	*0.14*	3	1
Picual (CL)	10.86	*2.94*	7.52	*1.83*	4.34	*0.87*	0.74	*0.43*	0.15	*0.08*	0.3	*0.16*	4	1
Arbequina (AR)	6.54		4.83		2.45		0.19		0.15		0.42		1	1

Arauco (AR)	6.44		4.63		2.47		0.22		0.15		0.43		1	1
Coratina (AR)	6.24		4.23		2.09		0.16		0.23		0.87		1	1
Frantoio (AR)	4.05		2.69		1.39		0.14		0.11		0.34		1	1
Picual (AR)	4.36		2.83		1.32		0.1		0.09		0.19		1	1
Barnea (NZ)	3.29		2.06		0.89		0.15		0.33		0.77		1	1
Frantoio (NZ)	6.09	*4.2*	3.94	*2.75*	1.92	*1.3*	0.16	*0.02*	0.24	*0.05*	0.26	*0.05*	3	1
Koroneiki (NZ)	6.63		4.08		1.95		0.11		0.18		0.29		1	1
Leccino (NZ)	7.03		4.43		2.12		0.16		0.19		0.3		1	1
Picual (NZ)	10.63		6.92		3.26		0.59		0.69		0.74		1	1
	C23		C24		C25		C26		C27		C28			
Cultivar (*Country*)	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	n	Reference
Arbequina (SP)	9.46	*1.12*	6.15	*0.78*	17.23	*2.12*	3.47	*0.34*	21.76	*5.16*	4.12	*0.86*	12	1
Carrasqueña (SP)	1.92	*0.86*	0.74	*0.05*	3.55	*0.57*	0.74	*0.02*	3.99	*1.6*	0.74	*0.26*	7	1
Cornezuelo (SP)	1	*0.24*	0.59	*0.15*	2.34	*0.63*	0.85	*0.43*	2.56	*0.39*	0.59	*0.11*	10	1
Cornicabra (SP)	3.44	*4.81*	1.42	*0.43*	6.23	*6.62*	0.14	*1.51*	8.82	*7.93*	1.46	*1.41*	38	1
Empeltre (SP)	7.34	*0.34*	3.42	*4.54*	10.2	*9.66*	6.66	*2.66*	6.12	*1.43*	4.25	*1.81*	15	1
Farga (SP)	10.9	*2.95*	6.84	*1.83*	17.82	*3.76*	2.74	*0.61*	14.76	*3.65*	2.33	*0.62*	21	1
Gordalilla (SP)	1.04	*0.02*	0.18	*0.02*	3.1	*0.07*	0.26	*0.02*	3.98	*0.24*	0.73	*0.03*	7	1
Hojiblanca (SP)	1.21	*0.31*	0.82	*0.23*	3.83	*1.02*	1.11	*0.34*	6.63	*2.43*	1.21	*0.42*	44	1
Lechin de Sevilla (SP)	1.37	*0.17*	1.01	*0.23*	1.57	*0.57*	4.53	*0.86*	11.21	*3.54*	1.76	*0.03*	6	1
Manzanilla Cacereña (SP)	3.47	*2.89*	1.48	*1.41*	6.28	*6.06*	1.48	*1.35*	8.89	*7.9*	1.45	*1.4*	19	1
Manzanilla de Huelva (SP)	1.54	*0.21*	0.92	*0.4*	3.93	*0.52*	0.97	*0.13*	3.84	*0.35*	1.13	*0.19*	6	1
Morrut[1] (SP)	0.36	*0.11*	10.56	*4.69*	19.89	*8.73*	2.41	*0.57*	8.71	*6.19*	1.22	*0.45*	7	1
Nevadillo Blanco[2] (SP)	1.62	*0.34*	1	*0.32*	4.01	*1.37*	1.03	*0.36*	6.62	*2.32*	1.06	*0.2*	26	1
Pico Limon (SP)	1.61	*0.84*	1.01	*0.21*	3.86	*1.13*	1.06	*0.35*	6.72	*3.12*	1.24	*0.16*	5	1

(continued)

Table 20.8 (continued)

Cultivar (*Country*)	C23		C24		C25		C26		C27		C28		n	Reference
	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*		
Picual (SP)	1.64	*1.13*	0.71	*0.18*	3.72	*0.77*	0.99	*0.77*	5.06	*1.04*	1.1	*0.35*	226	1
Picudo (SP)	1.22	*0.11*	0.72	*0.14*	3.12	*0.63*	0.94	*0.15*	4.53	*1.15*	1.12	*0.54*	18	1
Real Sevillana (SP)	1.12	*0.31*	0.61	*0.2*	2.37	*0.7*	0.65	*0.16*	2.24	*0.65*	0.53	*0.07*	9	1
Serrrana (SP)	1.87	*0.68*	1.06	*0.42*	4.47	*0.82*	1.29	*0.2*	5.23	*0.84*	1.25	*0.18*	6	1
Verdial de Badajoz (SP)	2.24	*0.47*	0.66	*0.11*	2.89	*1.01*	0.73	*0.2*	4.02	*1.03*	0.71	*0.12*	7	1
Verdial de Huevar (SP)	1.68	*0.32*	1.87	*1.01*	5.12	*1.16*	1.84	*1.11*	5.07	*1.43*	1.98	*1.02*	8	1
Taggiasca (IT)	1.9	*0.15*	0.77	*0.07*	4.63	*0.28*	0.83	*0.49*	10.83	*0.92*	0,93	*0.09*	5	1
Arbequina (CL)	5.92	*1.39*	4.5	*2.15*	14.3	*5.75*	2.01	*0.24*	12.5	*2.94*	1.57	*0.45*	4	1
Barnea (CL)	7.53	*4.57*	5.19	*1.03*	13.42	*5.01*	1.9	*0.32*	12.48	*1.93*	1.72	*0.11*	3	1
Frantoio (CL)	4.52	*2.14*	3.14	*0.82*	11.37	*6.98*	2.1	*0.49*	15.62	*1.15*	6.27	*2.12*	3	1
Leccino (CL)	16.93	*6.91*	16.61	*6.23*	22.84	*8.93*	3.29	*0.38*	9.78	*2.01*	7.39	*0.37*	3	1
Picual (CL)	2.43	*1.1*	2.15	*0.47*	6.23	*4.16*	1.54	*0.45*	7.27	*1.17*	1.84	*0.34*	4	1
Arbequina (AR)	18.53		14.07		30.65		4.06		39.74		3.62		1	1
Arauco (AR)	6.17		4.72		15.14		2.14		15.52		4.38		1	1
Coratina (AR)	20.12		13.73		25.14		3.42		11.62		1.31		1	1
Frantoio (AR)	6.52		5.8		16.43		2.65		15.33		1.61		1	1
Picual (AR)	1.72		0.91		6		1.33		11.58		15.06		1	1
Barnea (NZ)	9.54		5.13		18.01		2.27		22.41		4.4		1	1
Koroneiki (NZ)	3.81	*0.91*	3.43	*0.82*	10.55	*1.94*	2.25		10.82		3.04		1	1
Frantoio (NZ)	6.54		6.56		14.31		2.06	*0.34*	13.23	*1.89*	4.6	*1.32*	2	1
Leccino (NZ)	6.55		6.56		14.22		2.22		10.9		9.43		1	1
Picual (NZ)	3.46		2.48		13.39		4.45		25.36		8.49		1	1

	C29		C30		C31		C32		C33		C35			
Cultivar (*Country*)	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	n	Reference
Arbequina (SP)	14.17	*1.03*	1.65	*0.3*	7.96	*0.64*	0.72	*0.11*	2.92	*0.61*	0.35	*0.23*	12	1
Carrasqueña (SP)	4.32	*2.15*	0.64	*0.29*	4.64	*3.18*	0.72	*0.51*	3.4	*2.64*	0.87	*0.66*	7	1
Cornezuelo (SP)	2.7	*0.74*	0.44	*0.1*	1.65	*0.93*	0.24	*0.1*	0.82	*0.11*	0.24	*0.08*	10	1
Cornicabra (SP)	8.95	*8.42*	1.11	*1.03*	7.06	*5.44*	0.95	*0.71*	3.96	*2.84*	1.07	*0.75*	38	1
Empeltre (SP)	4.03	*3.25*	0.64	*0.08*	2.21	*0.34*	0.22	*0.12*	0.09	*0.12*	0.23	*0.01*	15	1
Farga (SP)	11.04	*2.74*	0.92	*0.21*	4.34	*1.92*	0.47	*0.26*	1.71	*1.25*	0.49	*0.45*	21	1
Gordalilla (SP)	0.37	*0.04*	0.57	*0.03*	3.29	*0.16*	0.33	*0.02*	1.62	*0.11*	0.37	*0.04*	7	1
Hojiblanca (SP)	5.02	*1.94*	0.72	*0.25*	3.1	*1.41*	0.35	*0.23*	1.35	*0.84*	0.33	*0.24*	44	1
Lechin de Sevilla (SP)	7.03	*1.02*	0.8	*0.19*	4.18	*1.44*	0.41	*0.16*	1.9	*0.97*	0.32	*0.1*	6	1
Manzanilla Cacereña (SP)	8.9	*8.44*	1.17	*1.01*	7.06	*5.43*	0.98	*0.71*	3.95	*2.83*	1.05	*0.71*	19	1
Manzanilla de Huelva (SP)	4.99	*0.46*	0.62	*0.14*	4.68	*0.84*	0.63	*0.18*	2.82	*0.64*	0.77	*0.24*	6	1
Morrut[1] (SP)	6.32	*1.17*	0.42	*0.23*	2.43	*1.09*	0.22	*0.08*	0.77	*0.13*	0.16	*0.06*	7	1
Nevadillo Blanco[2] (SP)	4.43	*0.93*	0.64	*0.29*	2.81	*0.86*	0.38	*0.12*	1.34	*0.49*	0.38	*0.17*	26	1
Pico Limon (SP)	10.2	*6.94*	1.41	*0.23*	10.85	*4.26*	1.49	*0.62*	7.54	*2.48*	1.78	*0.25*	5	1
Picual (SP)	4.67	*3.91*	0.65	*0.16*	3.89	*1.4*	0.46	*0.41*	1.99	*1.17*	0.48	*0.46*	226	1
Picudo (SP)	4.04	*1.03*	0.6	*0.13*	3.11	*0.82*	0.33	*0.15*	1.54	*0.43*	0.43	*0.12*	18	1
Real Sevillana (SP)	2.34	*0.83*	0.39	*0.14*	1.52	*0.89*	0.21	*0.11*	0.86	*0.14*	0.27	*0.06*	9	1
Serrrana (SP)	5.55	*0.93*	0.73	*0.44*	2.88	*0.61*	0.68	*0.62*	1.33	*0.37*	0.29	*0.09*	6	1
Verdial de Badajoz (SP)	3.29	*1.12*	0.45	*0.16*	1.54	*0.97*	0.22	*0.08*	0.56	*0.18*	0.15	*0.03*	7	1
Verdial de Huevar (SP)	6.56	*2.57*	1.33	*1.1*	6.24	*3.88*	1.03	*0.83*	3.95	*2.69*	1.01	*0.78*	8	1
Taggiasca (IT)	6.8	*0.93*	0.62	*0.12*	3.63	*0.67*	0.3	*0.06*	1.53	*0.42*	0.37	*0.09*	5	1
Arbequina (CL)	10.18	*2.17*	0.94	*0.23*	6.05	*0.56*	0.47	*0.17*	2.32	*1.12*	0.39	*0.21*	4	1
Barnea (CL)	13.1	*2.05*	1.31	*0.35*	12.46	*9.87*	1.41	*0.94*	6.68	*2.87*	1.43	*0.25*	3	1

(continued)

Table 20.8 (continued)

Cultivar (*Country*)	C29		C30		C31		C32		C33		C35		n	Reference
	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*	Mean	*Std*		
Frantoio (CL)	9.36	*1.15*	0.86	*0.15*	4.83	*0.78*	0.37	*0.14*	1.48	*0.73*	0.32	*0.13*	3	1
Leccino (CL)	16.79	*1.99*	2.28	*0.34*	25.69	*14.16*	3.14	*1.91*	16.08	*6.71*	5.81	*3.94*	3	1
Picual (CL)	13.89	*3.26*	1.82	*0.17*	16.82	*10.11*	2.1	*1.79*	9.06	*4.93*	2.19	*1.83*	4	1
Arbequina (AR)	17.45		1.41		10.93		1.02		5.27		1.21		1	1
Arauco (AR)	15.61		1.68		15.74		2.29		11.75		3.66		1	1
Coratina (AR)	8.24		0.94		6.33		0.54		2.29		0.43		1	1
Frantoio (AR)	12.06		0.97		5.38		0.42		1.73		0.33		1	1
Picual (AR)	7.37		0.82		6.28		0.67		3.48		0.85		1	1
Barnea (NZ)	14.58		0.87		5.96		0.59		3.48		1		1	1
Koroneiki (NZ)	9.92		0.87		4.02		0.32		1.61		0.53		1	1
Frantoio (NZ)	16.26	*2.46*	1.48	*0.22*	6.99	*0.43*	0.4	*0.04*	1.71	*0.4*	0.41	*0.15*	2	1
Leccino (NZ)	10.04		0.93		4.16		0.35		1.71		0.59		1	1
Picual (NZ)	25.49		4.09		13.81		2.68		6.74		2.38		1	1

Note: C13:1, tridecene; C17: heptadecene; C21, heneicosane; C23, tricosane; C24, tetracosane; C25, pentacosane; C26, hexacosane; C27, heptacosane; C28, octacosane;C29, nonacosane; C30, triacontane; C31; hentriacontane; C32, dotriacontane; C33, tritriacontane; C35, pentatriacontane; n, number of samples; ref, reference number; Std, Standard deviation, only when reported by authors; nr, not reported; AR, Argentina; AU, Australia; CL, Chile; IT, Italy; NZ, New Zealand; SP, Spain; [1], it might include a low percentage of Sevillenca; [2], it might include a low percentage of Nevadillo Negro.
References: 1, Aparicio (2013).

Acknowledgments The authors wish to thank the Spanish cooperative societies, Italian and Greek producers of olive oil, Drs. Selina Wang, Marie Wong, Franca Angerosa, Nalda Romero, Ariel Bueno, and Dan Flynn, among many others, for supplying SEXIA Group with virgin olive oil samples.

References

Al-Ismail KM, Ahmad R, Al-Dabbas M, Ajo RY, Rababah T (2011) Some physicochemical properties of olive and olive oil of three Jordanian olive varieties. Riv Ital Sostanze Grasse 88:191–198

Aparicio R, Alonso V (1994) Characterization of virgin olive oils by SEXIA Expert System. Prog Lipid Res 33:29–38

Aparicio R (2013) SEXIA© (expert system for olive oil characterization) databases. Instituto de la Grasa-CSIC, Seville

Azadmard_Damirchi S, Dutta PC (2006) Novel solid-phase extraction method to separate 4-desmethyl- 4-monomethyl-, and 4-4′-dimetylsterols in vegetable oils. J Chromatogr A 1108:183–187

Bartolini G, Prevost G, Messeri C, Carignani G, Menini UG (1998) Olive germplasm: cultivars and world-wide collections. FAO, Roma

Ceci LN, Carelli AA (2007) Characterization of monovarietal Argentinian olive oils from new productive zones. J Am Oil Chem Soc 84:1125–1136

European Communities (EC) (2003) Official Journal of the Commission of the European Communities. Regulation nº 1989/2003, L298, pp 57–66

García-González DL, Aparicio R (2010) Research in olive oil: challenges for the near future. J Agric Food Chem 58:12569–12577

García-González DL, Tena N, Aparicio R (2012) Describing chemical singularity of Spanish protected designations of origin of virgin olive oil. Grasas Aceites 63:26–34

Giuffré AM (2005) Caratterizzazione dell'olio di olive della *cv*. Tonda Iblea coltivata in provincia di Reggio Calabria, in funzione di due tecnologie di estrazione. Riv Ital Sostanze Grasse 82:135–141

Giuffrè AM, Louadj L, Poiana M, Macario A (2012) Composition en sterols des huiles extradites d'olives de cultivars de la province de Reggio Calabria (Sud d'Italie). Riv Ital Sostanze Grasse 98:177–183

Griggs WH, Hartmann MV, Bradley BT, Iwakiri BT, Whisler JE (1975) Olive pollination in California. California Agr Expt Sta Bul 869

Guinda A, Lanzón A, Albi T (1996) Differences in hydrocarbons of virgin olive oils obtained from several olive varieties. J Agric Food Chem 44:1723–1726

Haddada FM, Manai H, Oueslati I, Sánchez J, Osorio E et al (2007) Fatty acid, triacylglycerol, and phytosterol composition in six Tunisian olive varieties. J Agric Food Chem 55:10941–10946

Longobardi F, Ventrella A, Casiello G, Sacco D, Catucci L et al (2012) Classification of olive oils according to geographical origin by using 1H NMR fingerprinting combined with multivariate analysis. Food Chem 133:169–175

Mailer RJ, Ayton J, Graham K (2010) The influence of growing region, cultivar and harvest timing on the diversity of Australian olive oil. J Am Oil Chem Soc 87:877–884

Manai-Djebali H, Krichène D, Ouni Y, Gallardo L, Sánchez J et al (2012) Chemical profiles of five minor olive oil varieties grown in central Tunisia. J Food Composition Analysis 27:109–119

Ollivier D, Artaud J, Pinatel C, Durbec JP, Guérère M (2003) Triacylglycerol and fatty acid compositions of French virgin olive oils. Characterization by chemometrics. J Agric Food Chem 51:5723–5731

Sakouhi F, Absalon C, Flamini G, Cioni PL, Kallel H et al (2010) Lipid components of olive oil from Tunisian Cv. Sayali characterization and authenticity. *C.R.* Biologies 333:642–648

Sánchez-Casas J, Osorio-Bueno E, Montaño-García AM, Martínez-Cano M (2004) Sterol and erythrodiol + uvaol content of virgin olive oils from cultivars of Extremadura (Spain). Food Chem 87:225–230

Stefanoudaki E, Kotsifaki F, Koutsaftakis A (2000) Sensory and chemical profiles of three European olive varieties (Olea europea L); an approach for the characterisation and authentication of the extracted oils. J Sci Food Agric 80:381–389

Zanetic M, Struceli D, Perica S, Rade D, Skevin D et al (2010) Chemical composition of Dalmatian virgin olive oils from autochtonous olive cultivars Oblica, Lastovka and Levantinka. Riv Ital Sostanze Grasse 87:24–33

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R. Aparicio and J. Harwood (eds.), *Handbook of Olive Oil: Analysis and Properties,*
DOI 10.1007/978-1-4614-7777-8, © Springer Science+Business Media New York 2013