Giuseppina P. P. Lima · Fabio Vianello
Editors

Food Quality, Safety and Technology

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Preface

The importance of safe and high-quality food products is doubtless and consumer demand for increased food quality and safety assurances moves down the chain with retailers and service providers asking suppliers and producers to provide verifiable proof that robust food quality and safety control systems have been effectively implemented. Furthermore, new analytical systems and process development are needed for a rigorous, credible food safety and quality management system in order to reduce assessment inconsistencies and production costs. The new global environment for food trade places considerable obligations on both importing and exporting countries to strengthen their food control systems and to implement and enforce risk-based food control strategies. Consumers are taking unprecedented interest in the way food is produced, processed, and marketed and are increasingly calling for their governments to accept greater responsibility for food safety and consumer protection.

This book collects selected contributions from several researchers, coming from Brazil, Italy, and Spain, working in the field of food science, and participating at the II spring school in "Food Quality, Safety and Technology," which was held in Botucatu (SP, Brazil), on September 24th–27th, 2012, at the Botucatu Campus of the Universidade Estadual Paulista "Julio Mesquita Filho" (UNESP). The goal of the conference was to provide a scientific forum covering large areas of agronomy, nutrition, food science and technology, veterinary, and related areas to food technology development, and it was addressed to educational, career advancement, and networking opportunities teachers, professionals, and graduate and postgraduate students in Food Science, Food and Agriculture Engineering, Veterinary, Science and Food Technology, and related areas by providing an exchange of knowledge and technologies. The initiative aimed at the delivery of consistent, globally recognized scientific principles on food safety and quality, which could be consistently applied to industry and production sectors and stakeholders, taking into account that effective food control systems are essential to protect the health and

safety of domestic consumers, enabling the assurance of safety and quality of foods entering in the international trade, and to ensure that imported foods conform to national requirements.

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Giuseppina Pace Pereira Lima
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Part I
Food Quality

Chapter 1
Antioxidants in Brazilian Plant Species

Rene A.S. Campos, Fabio Vianello, Luciana F. Fleuri, Valber A. Pedrosa, Paola Vanzani, and Giuseppina P.P. Lima

Abstract Brazil presents a huge variety and diversity of plant species and several studies showed the antioxidant potential of most of these Brazilian species. In general, antioxidants can be defined as a heterogeneous family of natural molecules, which are present in low concentrations, and can prevent or reduce the oxidative damage in organisms. Among the most studied antioxidants in plants, besides vitamins, polyphenols, such as flavonoids, carotenoids, and thiols, stand out. This chapter highlights the antioxidant properties of some Brazilian fruits, medicinal plants, herbs, and seasonings and proposes a review about the characteristics of Brazilian flora species, which were found to show antioxidant properties.

Keywords Phenolic compounds • Vitamins • Carotenoids • ROS

Abbreviations

ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
BHT	Butylhydroxytoluene
DPPH	1,1-Diphenyl-2-picryl-hydrazyl

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IC_{50}	The half maximal inhibitory concentration. The concentration of antioxidant needed to reduce the amount of radical by 50 %
ROS	Reactive oxygen species

1.1 Introduction

Brazil is a country of continental dimension, recognized for its great biodiversity. The richness of plant species is one of the highest in the world, with an estimated occurrence of almost 34,000 species, of which 54.2 % are endemic (Scariot 2010). This number may be even higher and some estimates indicate that there are over 56,000 species of plants, nearly 19 % of world flora (Giulietti et al. 2005).

However, despite the uncertainty about the composition and potential of the flora, the occupation of forest areas, whether for use of forest resources or their transformation into agricultural or urban areas, has caused the accelerated loss of natural resources and associated traditional knowledge (Brandão et al. 2013). Thus, new strategies must be implemented to promote the use and conservation of this heritage.

Since the seventeenth century, travelers and scientists from Europe had collected several Brazilian native plants species, used as foods or medicines, in the treatment of chronic and degenerative diseases. However, even today, most of these species have not undergone any evaluation to confirm their benefits and verify their potential use as a source of bioactive compounds. There is also the need for agronomical studies, as well as ecological and conservational ones (Oliveira et al. 2012).

Since it was proposed that aging is the result of cumulative damages caused by free radicals, in the mid-twentieth century (reviewed by Gutteridge and Halliwell 2010), researchers aimed to prove the benefits of a vegetal rich diet, source of natural antioxidants. It is believed that this may retard the aging process. Since then, several epidemiological studies have shown that the consumption of fruits and vegetables is associated with a lower incidence of chronic and degenerative diseases (Hartman et al. 2006; Zibadi et al. 2007).

The term "antioxidant" refers to a molecule that protects a biological target against an oxidative damage. In cells and tissues this damage is mainly caused by reactive oxygen species (ROS). By definition, ROS are oxygen radicals including hydroxyl, $OH^{\bullet}$ or superoxide, $O2^{\bullet -}$, some other reactive molecules, such as H_2O_2, and non-oxygen derived radicals, among which reactive species of nitrogen, chlorine, transition metal ions, and sulfur can be found (Valko et al. 2007; Halliwell 2011).

The body's antioxidant defenses against ROS produced during cellular aerobic respiration can be of endogenous, enzymatic, or nonenzymatic nature, or supplied by the diet. When natural defenses are overwhelmed by excessive production of pro-oxidants, oxidative stress occurs, which is a serious imbalance between the generation of reactive species and antioxidant protection, causing excessive

oxidative damage which can affect proteins, lipids, and nucleic acids, oxidizing both cellular and extracellular macromolecules, and causing injury to tissues and affecting the immune system (Limón-Pacheco and Gonsebatt 2009; Gutteridge and Halliwell 2010).

Various natural antioxidants are present in plant samples. Among them, carotenoids, such as carotenes and xanthophylls, polyphenols, such as flavonoids and phenolic acids, and vitamins C, E, and A are the most studied. Protective effects of nutritional antioxidants in health come from their ability to scavenge free radicals by acting as a hydrogen or electron donor or by directly reacting with them (Oliveira et al. 2009).

Polyphenols show a higher antioxidant capacity in vitro than ascorbic acid and tocopherols. These compounds are present in significant quantities in most vegetables and fruits, reinforcing the importance of polyphenols consumed from the diet, and emphasizing their availability and effects in vivo (Pulido et al. 2000). Hundreds of polyphenols, with a wide diversity of structures and molecular masses, exist and information about their consumption, bioavailability, and metabolism is currently partial and incomplete (Saura-Calixto 2011).

The diversity of antioxidant structure and properties makes difficult to separate and quantify these compounds in vegetable matrixes. Thus, in recent years, several methods have been developed to measure the total antioxidant activity, antioxidant capacity, or total antioxidant potential. Among them, the total phenols assay by Folin–Ciocalteu reagent, DPPH (1,1-diphenyl-2-picryl-hydrazyl) (Brand-Williams et al. 1995), trolox equivalent antioxidant capacity—TEAC (Van den Berg et al. 1999), total radical antioxidant potential—TRAP (Evelson et al. 2001), ferric reducing ability of plasma—FRAP (Benzie and Strain 1996), oxygen radical absorbance capacity—ORAC (Cao and Prior 1999), and lipid peroxidation assay (Zhang et al. 2006) are the most representative. These assays are based on hydrogen atom or electron transfer and measure the capacity of an antioxidant to reduce an oxidant, which changes its color when reduced. The degree of color change is correlated with the concentration of antioxidant in the sample. Synthetic antioxidants, such as BHT, trolox, gallic acid, rutin, and ascorbic acid, or plant extracts with recognized antioxidant power, such as *Gingko biloba*, are used as standards to calibrate the measurements in laboratory.

The research area about the properties of natural antioxidants has grown in recent years, due to the increasing restrictions about the use of synthetic antioxidants and public awareness about health issues. Hence, various researches are focused on the identification and characterization of novel antioxidants from natural sources (Shahidi 2000).

The research for new plant products, as a source of antioxidants, has received great attention in Brazil. This can be verified from the literature recently produced in the country, such as "screening studies." The most studied plant materials are represented by fruits, conventional or "exotic" vegetables, teas and spices, medicinal plants, plant products, such "cachaças" (spirits), essential oils, and industrial wastes.

1.2 Fruits

The Amazon region presents a great variety of fruit species, characterized by different aromas and flavors, which may represent potential alternatives, economically attractive sources of antioxidants. A study on Brazilian fruits highlighted acerola (*Malpighia glabra*), cashew (*Anacardium occidentale*), mangaba (*Hancornia speciosa*), umbu (*Spondia tuberosa*), açaí (*Euterpe oleracea*), uvaia (*Eugenia pyriformis*), and murici (*Byrsonima crassifolia*) as a good source of antioxidants, when measured by the DPPH antioxidant capacity and compared with BHT. In this study, the antioxidant activity was related to the high levels of vitamin C and phenolic compounds present in these fruits. Acerola presented 1,360 mg/100 g of vitamin C and 1,060 mg/100 g of polyphenols (Rufino et al. 2009).

Another study on economically important fruits, camu-camu (*Myrciaria dubia*) and acerola (*Malpighia emarginata*), displayed a considerable amount of vitamin C: 1,882 and 1,357 mg/100 g, respectively. Açaí (*Euterpe oleracea*) and jussara (*Euterpe edulis*) showed large amount of different antioxidants: 111 and 192 mg/100 g anthocyanins, 91.3 and 375 mg/100 g flavonoids, and 20.8 and 21.5 mg/100 g chlorophyll, respectively. Puça-preto (*Mouriri pusa*) was also considered an excellent source of anthocyanins showing 103 mg/100 g, as well as murta (*Blepharocalyx salicifolius*) with 143 mg/100 g, jambolão (*Syzygium cumini*) with 93.3 mg/100 g, jabuticaba (*Myrciaria cauliflora*) with 58.1 mg/100 g, and camu-camu with 42.2 mg/100 g. The fruits of gurguri (*Mouriri guianensis*) are a rich source of carotenoids with 4.7 mg/100 g. The richest fruits in polyphenols were camu-camu with 1,176 mg/100 g, acerola with 1,063 mg/100 g, and puçá-preto with 868 mg/100 g, indicating that these fruits are excellent sources of bioactive compounds (Rufino et al. 2010).

Camu-camu has demonstrated an antioxidant activity, determined by the DPPH method, of 119 % higher than pure α-tocopherol. Researchers also studied murta, guriri, carnauba, jabuticaba, and puçá-preto (Rufino et al. 2011a). In a recent in vivo study, the camu-camu juice also showed antigenotoxic activity, being able to reduce DNA damage caused by H_2O_2 (Da Silva et al. 2012a, b). Ascorbic acid is the main component responsible for the antioxidant capacity; however, this fruit contains also phenolic compounds, ellagic acid derivatives, anthocyanins, flavonoids (rutin and its derivatives) and flavones (derivatives of naringenin and eriodictyol), and hydrolyzed tannins, providing additional evidence about the importance of this fruit as a source of bioactive compounds (Chirinos et al. 2010).

In açaí fruits (*Euterpe oleracea*), the highest antioxidant capacity was observed (1.82 mmol BHT equivalent/100 g fresh weight) among frozen fruit pulps sold in Brazil, followed by cashew (*Anacardium occidentale*), apple (*Malus domestica*), and blackberry (*Morus nigra*) (Hassimotto et al. 2009). In a variety of açaí, the "BRS Pará," a high fiber (71 %) and oil (20.8 %) content and a high antioxidant capacity were found, being higher than the virgin olive oil, demonstrating considerable potential for nutritional applications (Rufino et al. 2011b). The antioxidant

activity of açaí pulp is mainly related to its polyphenol content. High concentrations of phenolic compounds were found in immature fruits, especially flavones, such as orientin and homoorientin. Thus, extracts from immature fruits may also be attractive, due to their content of bioactive compounds (Gordon et al. 2012).

Jussara (*Euterpe edulis*), from Southern Brazil, showed high content of polyphenols (2,610 mg/100 g) and anthocyanins (1,080 mg/100 g) (Borges et al. 2011). Its pulp extracts showed strong antioxidant capacity, by the DPPH and FRAP methods, and significant protective effects on stress tests in vitro, compared to gallic acid controls (Borges et al. 2012).

Jabuticaba (*Myrciaria cauliflora*), which may be considered a Brazilian cherry, is known to be a rich source of anthocyanins, similar to other cherries, such as blackberry. Recently, it was demonstrated that frozen and dried jabuticaba peel can be a good source of phenolic compounds with 556.3 mg/kg fresh matter, possessing a very high antioxidant capacity (Leite-Legatti et al. 2012). Other fruits, which are good sources of flavonoids, are pitanga (*Eugenia uniflora*), acerola, and cashew, containing several flavonoids, such as myricetin, quercetin, and kaempferol (Hoffmann-Ribani et al. 2009). Recently, a study on red and white jabuticaba wines showed a higher antioxidant activity than grape wine, and close to the values of BHT (Barros et al. 2010).

In another study on Amazonian fruits, a high content of bioactive compounds in fruits of cutite (*Pouteria macrophylla*), followed by jambolão (*Syzygium cumini*), araçá (*Psidium guineense*), and murici (*Byrsonima crassifolia*), was found, and a great number of phenolic compounds, assigned as hydrolyzable tannins, proto-anthocyanins, flavonols, and flavonolols, were identified (Gordon et al. 2011).

The fruits of cutite (*Pouteria macrophylla*) were recently recognized as equivalents to other Amazonian fruits characterized by high nutritional value and can be considered as rich sources of polyphenols for human diet. Fresh fruits demonstrated a high antioxidant activity, when compared to other fruits widely consumed and marketed in Brazil (da Silva et al. 2012b).

A study on jenipapo (*Genipa americana*), umbu (*Spondia purpurea*), and siriguela (*Spondia purpurea*) revealed the presence of phenols, tannins, anthocyanins, proto-anthocyanins, flavonoids, leucoantocianins, catechins, flavonones, anthraquinones, anthrones, coumarins, triterpenoids, sterols, and saponins in samples of peels and seeds. The seeds and peels of seriguela and umbu showed the highest antioxidant activity and the lipid peroxidation assay indicated that jenipapo pulp is a promising source of antioxidants (Omena et al. 2012).

Other promising Brazilian palm fruits, regarding their antioxidant content, are the fruits of guariroba (*Syagrus oleracea*), jerivá (*Syagrus romanzoffiana*), and macaúba (*Acrocomia aculeata*). Among these fruits, jerivá can be considered a good source of carotenoids (1.2 mg/g), and all these fruits possess significant amounts of tocopherols (mainly α-tocopherol). Additional studies on antioxidant activity and toxicity of compounds present in these palm fruits were reported (Coimbra and Jorge 2011).

Bioactive compounds and antioxidant capacity of licuri (*Syagrus coronate*) were evaluated (Belviso et al. 2013). It was found that licuri fruits contain 1.21 and 2.78 mg/g of total polyphenols, in roasted and raw fruit samples, respectively. There was an increase in the antioxidant capacity when the fruit was roasted, due to the increased amount of phenolic compounds, particularly those belonging to the class of flavan-3-ols.

The mucuja chestnut (*Couma rigida*), inajá (*Maximiliana maripa*) jenipapo (*Genipa americana*), buriti (*Mauritia flexuosa*), and uxi (*Endopleura uchi*) showed high concentrations of phytosterols. The pulps of buriti and uxi contain large amounts of α-tocopherol and vitamin E, suggesting that these fruits could be interesting sources of bioactive compounds (da Costa et al. 2010).

In fruits of camarinha (*Gaylussacia brasiliensis*), a high amount of phenolic compounds (492.8 mg/100 g) and anthocyanins (240.4 mg/100 g fresh fruit) was observed. A correspondingly high antioxidant capacity was determined: 1.96, 1.66, and 0.67 mmol trolox equivalents/100 g fresh fruit, by ABTS, DPPH, and FRAP assays, respectively (Bramorski et al. 2011). A good correlation between the content of polyphenols and antioxidant capacity of the extracts was observed. The research highlighted the potential of this fruit as an important source of bioactive and nutritional compounds, available in this typical Brazilian plant.

The araçá-boi fruit (*Eugenia stipitata*), from Brazilian Amazon, is rich in terpenoids, volatile compounds, fiber, and vitamin C. The fruits, known for their antioxidant activity, were investigated and showed 184.5 mg/100 g phenolic compounds. The ethanol extracts of araçá-boi fruits showed high antimutagenic activity in vivo, suggesting their use as preventive agents against cancer (Neri-Numa et al. 2012).

In bacupari fruits (*Rheedia brasiliensis*), 7-epiclusianone was discovered. This substance, despite having a low antioxidant activity, was able to protect cells against mutagenic effect at doses of 5–15 mg/kg, and it can be used in the future as a potential agent in the prevention of cancer (de Carvalho-Silva et al. 2012).

Among 11 fruits produced in the Northeastern Brazilian region, it was verified that murici (*Byrsonima crassifolia*) and mangaba (*Hancornia speciosa*) possess a high antioxidant activity, determined by DPPH and ABTS assays, correlated with their polyphenol content, and were proposed as good sources of antioxidants (Almeida et al. 2011).

In one of the first studies with native plants of the Brazilian Cerrado, the aqueous and ethanolic extracts of pequi (*Caryocar brasiliense*) peel and ethanolic extracts of seeds of cagaita (*Eugenia dysenterica*) and ariticum (*Annona crassiflora*) pell showed significant levels of phenolics compounds (209.3, 208.4, 136.99, and 136.96 mg/100 g, respectively) and high antioxidant potential, requiring further studies (Roesler et al. 2007).

Pequi pulp (*Caryocar brasiliense*) is rich in lipids and dietary fibers and presents a high content of phenolic compounds (209 mg/100 g), indicating that it could be considered a food with high antioxidant capacity. A correlation between its high levels of unsaturated fatty acids with phenolic compounds and carotenoids was found (De Lima et al. 2007).

The pulp of cagaita (*Eugenia dysenterica*) shows 34.1 mg/100 g of vitamin C, contributing significantly to the daily needs of this vitamin, especially for families and vulnerable groups in Cerrado areas, characterized by high levels of food insecurity (Cardoso et al. 2011).

Moreover, among native fruits of Brazilian Cerrado, it was observed that the pulp of marolo (*Annona crassifolia*) presents a good antioxidant activity (13.16 mmol Trolox equivalents/100 g), a large amount of phenolic compounds (739.37 mg/100 g), and 59.05 mg/100 g ascorbic acid. These results were similar to jenipapo (*Genipa americana*), murici (*Byrsonima crassifolia*), graviola (*Annona muricata*), and sweet passion fruit (*Passiflora alata*) (de Souza et al. 2012). The lipid fraction of the seeds of *Annona crassifolia* presents significant amount of bioactive substances, especially phytosterols, tocopherols, and unsaturated fatty acids, presenting significant antioxidant capacity and oxidative stability (Luzia and Jorge 2013).

The pulp of several tropical fruits marketed in frozen form in southern Brazil contains high levels of polyphenols and good total antioxidant activity, especially acerola (*Malpighia glabra* L.) and mango (*Mangifera indica*). Among the fresh products, baguaçu (*Eugenia umbelliflora*) stands out as a powerful antioxidant, due to its content in anthocyanins (Kuskoski et al. 2006).

Three species of fruits native of southern Brazil, the ariticu-do-mato (*Rollinia sylvatica*), coquinho-azedo (*Butia capitata*), and mandacaru-de-três-quinas (*Cereus hildmannianus*), showed considerable amount of vitamin C and phenolic compounds, but *B. capitata* showed the highest antioxidant capacity, similar to some varieties of plum (Pereira et al. 2013).

In blackberry (*Rubus* sp.), another common plant in southern Brazil, it was found that some cultivars present high levels of phenolic compounds (600–1,000 mg/100 g) and considerable antioxidant activity. It was found that the content of phenolic compounds is not correlated with antioxidant activity of the fruit, which is probably related to vitamins and anthocyanins (Vizzotto et al. 2012).

The antioxidant capacity of four species of *Citrus* produced in Brazil was assessed. The peel of "Ponkan" tangerine showed the highest total antioxidant capacity, correlated with vitamin C and phenolic compound content. In addition to pulp, citrus peels are a good source of bioactive compounds and minerals, and their composition and properties should be explored (Barros et al. 2012).

The phenolic compound content and antioxidant activity of pomace from the winemaking of grape varieties, widely produced in Brazil, were investigated. Cabernet Sauvignon pomace was found to have the highest content of total phenolic compounds (74.75 mg/g), the highest antioxidant activity, and reducing power, while Bordeaux varieties showed the highest lipid peroxidation inhibition power. Thus, these varieties showed to be a good source of antioxidant compounds (Rockenbach et al. 2011).

1.3 Medicinal Plants

In a study on leaves, barks, and fruits of 15 Brazilian plants, murici (*Byrsonima crassifolia*), pata-de-vaca (*Bauhinia macrostachya*), embaúba (*Cecropia palmata*), cedro-cheiroso (*Cedrela odorata*), chapeu-de-sol (*Cordia exaltata*), cipó-de-carijó (*Davilla kunthii*), cipó-caboclo (*Davilla rugosa*), verônica (*Dalbergia subcymosa*), ingá (*Inga edulis*), and barbatimão (*Stryphnodendron barbatiman*) were considered good sources of antioxidants. A high correlation between flavonoid, phenolic compound content, and antioxidant activity was found (Silva et al., 2007). Subsequently, it was confirmed that extracts of murici (*Byrsonima crassifolia*), cipó-de-carijó (*Davilla rugosa*), and ingá (*Inga edulis*) can be considered good sources of polyphenols and that leaves of *I. edulis* are the best source of polyphenols with antioxidant properties (Souza et al. 2008).

Saratudo (*Byrsonima japurensis*), an Amazonian plant, popularly considered as a potent anti-inflammatory drug, and traditionally used for gastrointestinal and genitourinary diseases, was also recently studied. The aqueous extract of saratudo bark, obtained by infusion at 5 %, showed significant antioxidant activity ($IC_{50} = 42.5$ μg/mL), as determined from the inhibition of lipid peroxidation. This activity was higher than that showed by BHT, tested under the same conditions (Guilhon-Simplicio et al. 2012).

In another screening of medicinal species, it was found that angico (*Anadenanthera macrocarpa*), aroeira (*Astronium urundeuva*), jurema-branca (*Mimosa verrucosa*), and quixabeira (*Sideroxylon obtusifolium*) were effective in reducing the oxidation of DNA. The extracts of *A. macrocarpa* showed a high antioxidant activity ($IC_{50} = 54$ μg/mL) (Desmarchelier et al. 1999).

Moreover, in another screening on 71 extracts of 16 different species, the ethanolic extracts of leaves of angico (*Anadenanthera peregrina*) and monjolo-sabão (*Pseudopiptadenia contorta*) showed higher antioxidant activity than rutin ($IC_{50} = 14.16$), measured by the DPPH method. This study also highlighted the good antioxidant properties of ethanolic extracts of leaves of *Vitex polygama* and *Vitex litoralis*, leaves and flowers of *Lantana trifolia*, and aerial parts of *Hyptis tetracephala*, in comparison with the standardized extract of *Ginkgo biloba* leaves ($IC_{50} = 40.72$). The partition of the extracts in hexane, dichloromethane, ethyl-acetate, and n-butanol was also studied, and the leaves and flowers of *Lantana trifolia*, the leaves and bark of *Vitex poligama*, and the aerial parts of *Hyptis elegans* and *Raphiodon echinus* showed high antioxidant activity, when compared to rutin. Plants belonging to the Verbanaceae family, e.g., maria-preta (*Vitex polygama*) and gervãozinho-do campo (*Verbena litoralis*), showed lower IC_{50} values than other plant extracts (Mensor et al. 2001).

Further screening about the properties of extract from medicinal species, in particular from carnaúba (*Copernicia cerifera*), guariroba (*Syagrus oleraceae*), pata-de-vaca (*Bauhinia variegata*), and hortelã-bravo (*Hyptis fasciculata*), showed interesting antioxidant activity, e.g., $IC_{50} < 60$ μg/mL, suggesting the presence of good sources of DPPH free radical scavengers. Tests carried out on yeasts,

however, demonstrated that only the extracts from *C. cerifera*, *S. oleraceae*, and *Mauritia vinifera* presented protective effects against BHT incubation (Silva et al. 2005).

Carqueja (*Baccharis trimera*), widely used as anti-inflammatory, hypoglycemic, and remedy for digestive problems, was evaluated, as regards its antioxidant activity, by the DPPH method. The dried powder showed an $IC_{50} = 22.74$ μg/mL, comparable to vitamin E ($IC_{50} = 16.71$ μg/mL), suggesting good antioxidant properties (Dias et al. 2009).

In order to obtain purified fractions of polyphenols and antioxidant compounds, extraction processes from plant materials were considered. It was demonstrated that the most effective extraction process for obtaining antioxidants is represented by supercritical fluid extraction for cipó-de-são-joão (*Pyrostegia venusta*), common bean (*Phaseolus vulgaris*), nó-de-cachorro (*Heteropterys aphrodisiaca*), and ingá-cipo (*Inga edulis*), using ethanol as co-solvent, evidencing high recovery of antioxidant compounds and low manufacturing costs (Veggi et al. 2011).

The leaves of cashew (*Anacardium occidentale*) were studied about their phenolic compound content and antioxidant activity. Methanol extracts showed the highest amount of total phenolic content (307.3 mg/g dried mass), proving to be a powerful DPPH and ABTS scavenger, comparable to rutin and quercetin standards (Razali et al. 2008).

The leaves of ingá (*Inga edulis*) were also considered a promising source of antioxidants. In a recent study, a methanol–water extract of leaves was fractionated and phenolic compounds were identified as gallic acid, catechin, epicatechin, myrcetin-3-rhamnopyranoside, and quercetin-3-rhamnopyranoside. The crude dry extract showed a polyphenol content of 496.5 mg/g dry mass and an antioxidant capacity of 11.16 mmol trolox equivalents/g dry crude extract, measured by ORAC assay (Souza et al. 2007).

Silva et al. (2007) also found high antioxidant capacities in leaves and bark of *Byrsonima crassifolia*, *Inga edulis*, *Davilla kunthii*, and *Cecropia palmata*. According to authors, their great biomasses in the forest should stimulate further studies regarding their characterization and isolation of phenolic compounds.

Furthermore, some Brazilian spirits, popularly known as "cachaças," flavored by aging with woody plants, were studied. The spirits flavored with jatoba (*Hymenaea courbaril*) and chestnut (*Castanea* sp.) woods showed the highest levels of total polyphenols and tannins. The spirits flavored with louro-canela (*Aniba parviflora*), canela-sassafras (*Ocotea pretiosa*), and amendoim bravo (*Pterogyne* sp.) presented significant amounts of flavonoids, while they were more efficient in inhibiting lipid peroxidation than oak-flavored spirits used for comparison. However, oak spirits exhibited higher free radical scavenging capacity, against DPPH. The amendoim bravo (*Pterogyne* sp.) spirits proved to be the most potent antioxidant extract (Cardoso et al. 2008).

Finally, the antioxidant action of teas and most consumed Brazilian seasonings was evaluated by the DPPH method. Results showed that unfermented green tea (*Camellia sinensis*) was the most active ($IC_{50} = 0.14$ mg/mL), in which the main

antioxidant compounds are represented by epigallocatechins. The most active seasoning was cinnamon (*Cinnamomum*) ($IC_{50} = 0.76$ mg/mL), in which eugenol was the main antioxidant reported (de Morais et al. 2009).

1.4 Conclusions

Results and findings reviewed in this chapter show the importance of Brazilian plant product as potential sources of extremely useful antioxidants, both for industrial purposes and human health. The knowledge about the content and the quality of polyphenols present in fruits and vegetables in poorly studied plant products is rapidly growing and the aim of the present review was the collection and the report of results from the world scientific community about the polyphenol content and antioxidant activity in these plant materials. This knowledge can drive the population to properly choose products with higher medicinal and functional power. The results described in this work hopefully will stimulate the continuity of research to evaluate the antioxidant power of isolated substances of studied species. Of course, other detailed agronomical, biochemical, and chemical research must be performed in order to elucidate the role of these substances, from the original plant to human beings.

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Chapter 2
Quality and Potential Healthy Traits in Vegetables and Berries

Paolo Sambo and Carlo Nicoletto

Abstract In recent years, the sensitivity of consumers and producers towards the environment and health topics has increased significantly, and these issues are involving more and more the agricultural world. Much has been done in terms of cropping systems and technology, but the issues relating to the quality and nutritional value of products turn out to be more complex and sensitive. In this regard, the consumer is more aware of these issues thanks to the many suggestions offered daily both in the health and in a healthy diet.

In this sense, this chapter aims to provide a current status of the concept of quality in the context of vegetable products and highlight its importance especially in order to promote vegetables by improving the final consumer diet. In this regard, the indicative pattern of the chapter could include three main sections. The first concerns about the identification and exploration of the quality concept and its evolution over time with respect to all aspects that contribute to its perception by the consumer. Moreover the technical-agronomic factors and environmental factors that determine the product quality associated with pre-harvest until the ripening stage will be considered. Finally, in the last section of this chapter, we will refer to the quality maintenance in post-harvest considering the evolution of multiple physiological aspects (antioxidants, phenols, vitamins, macro- and micronutrients, etc.) to the hypothetical purchase of the product by the consumer. During all the steps described so far, in which quality is involved, we will consider the potential health traits and benefits relevant to the health of the consumer trying to provide a clear and complete view in this research field.

Keywords Food quality • Nutrition • Antioxidant • Phenols

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2.1 Introduction

The term "quality" and the different meanings that this word may assume have for some years occupied a key role in any discussion on the production and marketing of goods and services. As regards vegetables for fresh consumption, the concept "quality" has changed profoundly, passing from just commercial and organoleptic parameters to cover a much wider range from the sanitary to intrinsic health and nutritional characteristics, and also the "ethical" aspects linked to the production process. At international level, the accepted definition is "*the set of priorities and characteristics of a product or service that confer on it the capacity to satisfy the expressed or implicit demands of the consumer*" (Peri 2004). This is a wider concept than the traditional definition which referred principally to the aesthetic characteristics of the goods, rendering it necessary to adapt production to a system of quality that can meet all the needs of the market.

The fruit and vegetable production sector is no exception to this trend. In times of rapid social and economic changes and market globalization, success in international competition depends mainly on the quality of the produce.

It is therefore important to investigate the significance of quality and understand how this changes in the different circumstances and in the ambits of the actors in a supply chain that begins with the producer and ends on the consumer's table. Quality only exists in the mind of the observer, i.e. the consumer, and will consequently change over time with a frequency and intensity that depend on the consumer's developing tastes. In this context it would seem appropriate to discuss how the aspects of quality, applied to the fruit and vegetable market, have evolved over time, and which prevalent directions will be taken in the near future to comply with a consumer demand that is often steered by the advertising and commercial policy of the large-scale retail trade.

The quality of a product is the result of a series of factors, some of which are perceptible but cannot be measured and are therefore subjective (e.g. taste, aroma, etc.) and others that are measurable and consequently objective (e.g. sugar level, acidity, concentration of polyphenols, antioxidants, vitamins, nitrates and others).

Within this general context a precise definition of quality is not easy in the horticultural sector, as the food products (raw, cooked or in some way prepared and conserved) are obtained from annual or perennial herbaceous angiosperm plants that belong to more than a hundred species, as well as fungi. It should also be kept in mind that within the same family the parts of plants consumed are at times drastically different (e.g. flowers, leaves, shoots, stalks and roots in the *Brassicaceae*). In addition, within the same species, there may be a large number of cultivars that have the same edible organ but with different morphological characteristics. For example in the *Solanaceae*, the tomato may have berries coloured yellow, red or purple, of a globular shape (round or flattened, smooth or ridged), elongated (e.g. San Marzano), cherry or grape. In the *Cucurbitaceae*, melon fruits can be from more or less spherical to oval, a skin colour from green to red, with or without netting and more or less evident clove signs and a flesh

colour from white to yellow to different shades of orange. In the same family, the courgette has fruits that are more or less long or spherical, with skin colours from different shades of green to pale yellow, uniform or striped in various ways. Similar considerations can be made for the watermelon which has fruits that, in addition to differing colour and shape, can reach unitary weights from around 1 to 20 kg and more. From this summary it can be deduced that it is extremely difficult to identify generalizable qualitative requisites in botanical terms. Thus other parameters have to be identified so that homogeneous groupings can be made on which it might be easier to generalize a definition of quality.

The parameters that could allow sufficiently homogeneous classifications, used to guide the interventions destined to enhance the quality characteristics of the product, might, for example, be the type of marketable edible parts and the phenological stage at harvest.

In addition to this, there is an increasing need to adapt the productions to a quality system which can guarantee that all the requirements necessary to satisfy the demands of the consumer are present in the purchased good. In this case attention is paid to the genuineness, absence of treatment residues (pesticides), sanitary characteristics, healthiness, naturalness, seasonality and with a growing interest in produce from organic cultivations. Concerning the sanitary aspect, there are precise and accurate interventions which, when not statutory, are defined by the different businesses that commit themselves to undertaking appropriate methods of self-regulation of their activity following the HACCP methodology (Hazard Analysis Critical Control Point). This is done to provide guarantees for their clients, but also allows access to otherwise inaccessible markets. These certifications guarantee that at every step of the supply chain, which begins with the sowing of the crops and ends with the distribution and sale of the edible produce, all the technological and organizational measures have been implemented that are necessary to prevent possible health risks for the consumers. This new concept of quality involves all the protagonists who form the supply chain and, consequently, the interventions can no longer have the primary aim of resolving just the specific needs of the individual actor, but must fit into a much wider context in which the suggestions and requirements of the other sectors that take over in the various stages are also considered.

In order to facilitate an understanding of the entire supply chain quality, it is worth summarizing, in sequence, the succession of interventions during the salient stages of the itinerary that, starting with the choice of species to cultivate and the cultivar, ends on the table of the consumer. It is therefore obvious that there is an interaction of highly diversified and complex aspects. To simplify the various effects, it may be worth considering two stages, in the first of which the principal actor is the producer with his farm, while the second comprises the actors who will manage the produce in all the stages that must be passed through before reaching the end user, as shown in Fig. 2.1.

In the first stage, which is the time when the quality of the produce can be most directly determined, particular attention must be paid to the choice of cultivar, evaluation of the seed, preparation of the soil, planting methods and patterns,

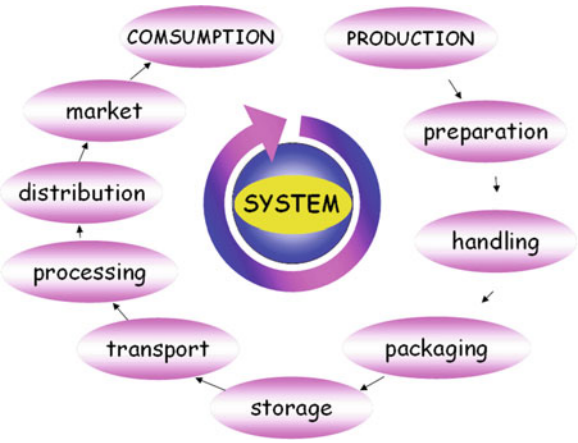

Fig. 2.1 Flow chart of the stages involved in the supply chain

fertilization, irrigation, pest and weed control or the guidelines for organic crops, until the most important goal is reached, the harvest. This is the moment when the production will be evaluated not only in terms of quantity, but more especially its quality, which will represent the maximum value reached with the technical–cultural methods applied. The second stage covers all the interventions necessary for product management; these include handling, transport, preparation, packaging, storage, transformation, distribution, the market and consumption. Extreme care must be taken in these steps because they can each have effects on the quality.

2.2 Some Chemical Components Determining the Quality of Vegetables

Currently, the high productions and vast areas growing horticultural crops cannot always guarantee a product with the characteristics that the market requires, especially in these last years. Consumers now demand a "high-quality" product from sundry points of view and, even better, one of "guaranteed quality". The concept of quality is very wide, difficult to define, firmly anchored to subjective evaluations and in continual evolution depending on the progressive shifts in the tastes, typical lifestyles and requirements of Western societies. According to an international definition, as previously mentioned, the quality represents "*the set of aspects and characteristics of a product or service that can satisfy the declared or implicit requirements*" (standard UNI EN ISO).

Quality can be divided into "structural quality" and "functional quality" (Mezzetti and Leonardi 2009): the former refers to the intrinsic characteristics of a product (e.g. sugar content in a fruit); the latter to the manifestation of these characteristics for the consumer (e.g. sweet taste of the fruit). In addition, reference has also been made more recently to the concept of "global quality", an expression

that covers multiple meanings in which aspects regarding both the product and the process coexist. Indeed, quality changes according to the point of view: for the farmer quality means high yield, disease resistance, simultaneity of maturation, ease of harvest and good appearance; for the trader it means resistance to handling and transport; for the wholesaler the long storability is important and lastly the consumer is interested in the flavour, the right price, the absence of residues and high nutritional content, rather than the exterior aspect that attracted him up until a few years ago. There has also recently been a tendency to widen the concept of quality and food safety beyond the intrinsic characteristics of the product, by taking the quality of the production process into consideration (Abbott 1999; Rico et al. 2007). In the choices of the consumer, his taste perceptions and nutritional needs are now combined and matched with his expectations regarding respect for the environment, the biosphere and the guarantees offered by the producers (Peri 2004). Therefore, in addition to the expectation to eat a product with optimal organoleptic and chemical–nutritional characteristics, new elements have appeared on the horizon, which do not refer to the product itself, but relate to the environment and production methods.

These elements may regard tradition and culture (the importance of geographical origin and strong ties between local foods and customs), the environmental impact of the production process and elements linked to the honesty, transparency and ethics of the producer (Menesatti 2000).

The quality of fresh vegetables has been discussed in many studies in recent years. This is due to the fact that they are foods that regulate metabolic activity through their supply of water, minerals, vitamins, fibres and other nutrients. Vegetables are increasingly appreciated for their high content of substances like vitamin C and polyphenols, compounds that protect against the onset of various types of tumour, cardiovascular disease, premature ageing of the cells, etc. (Vinson et al. 2001).

Unfortunately, at times the horticultural sector does not fully satisfy the consumer demands because knowledge of this aspect is scarce or completely lacking for some vegetables, especially for those which are not universally known and appreciated. This limits the expansion of many horticultural products that, if suitably characterized from a quality point of view, could offer a higher profitability. Among the many parameters that characterize this aspect of vegetables, the most interesting health-wise are the total antioxidase capacity, total phenols, ascorbic acid, pigments, sugars and protein nitrogen, but the potentially harmful components like nitrates and nitrites must also be taken into consideration.

2.2.1 Antioxidants

Many of the phytochemical compounds in fruit and vegetables have an antioxidant function that can offer a basic protection against some of the most widespread illnesses, such as cardiovascular diseases, cancer and many other degenerative

pathologies linked to ageing (World Health Organization 1990; Ames et al. 1993; Willet 1999; Chu et al. 2002). The term antioxidant generally means the property of a substance to prevent or inhibit oxidation, which is a chemical reaction that transfers electrons from a substance to an oxidant. These metabolites react with the free radicals produced by this reaction and thus interrupt the chain reactions that are initiated by intervening on the intermediate radicals, inhibiting other oxidation reactions and oxidizing in place of the oxidizable substrate. A wider definition of antioxidant is a substance that, added in low concentrations compared to that of the oxidizable substrate, can significantly delay or prevent the oxidation of that substrate (Cabras 2004).

The presence of antioxidants in food plays a significant role in the reduction of oxidative phenomena and in the relationship between this activity and the onset of pathologies such as arteriosclerosis (oxidation of the low density lipoprotein—LDL), tumours (oxidative damage to the DNA) and other pathologies, as has been demonstrated in epidemiological studies (Dalla Rosa 1996; Proteggente et al. 2002; Serafini et al. 2002; Kris-Etherton et al. 2002; Liu 2004). They can be divided into essential antioxidants, like some vitamins (A, C, E, folic acid), and non-essential antioxidants, including some secondary compounds of the plant metabolism (polyphenols, tannins, glucosinolates, metilxantine, ubiquinone, phytic acid, lipoic acid). Another classification takes into consideration the mechanisms of action of the antioxidants and, based on these, they are divided into primary and secondary antioxidants. The primary antioxidants are reducing substances, which oxidize in place of the food, thus protecting it from alteration; they are acceptors of free radicals and thus delay or inhibit the initiation or interrupt the propagation of the autoxidation reaction. The antioxidants of this type react with the lipid and peroxide radicals and convert them into more stable and non-radical compounds, adding a hydrogen atom. The secondary antioxidants are instead able to reduce the primary antioxidants, when these have reacted with the food, rendering them once again suitable to continue their activity. They slow down the speed of oxidation in different ways but do not convert the radicals into more stable compounds. The secondary antioxidants can chelate pro-oxidant metals and deactivate them (chelating antioxidants), restore hydrogen to the primary antioxidants (reducing agents), break the hydroperoxides down into non-radical species, deactivate the singlet oxygen quenchers, absorb ultraviolet radiations or behave as oxygen scavengers. These antioxidants are often defined synergically because they promote the activity of the primary antioxidants. It is plausible that the beneficial effects due to the consumption of plant products are determined by the presence of a mixture of antioxidant compounds that act in synergy, conferring a much higher antioxidant activity on the fruits and vegetables compared to the simple sum of the anti-radical action of the individual compounds (Tomás-Barberán and Espin 2001; Cabras 2004; Ismail et al. 2004).

The potential beneficial role of the antioxidant molecules is immediately understandable if we consider that our bodies are continuously exposed to the aggression of highly reactive chemical species that can damage cells and tissues, which are produced by the intermediate metabolism of oxygen and known as free

radicals. Some of these substances are produced during the normal metabolic cycles, while others are related to lifestyle or the result of different pathologies (Fogliano 2009). The free radicals become harmful when their production is higher than the capacity of elimination by the natural defence systems. To counteract these negative actions, the human body has developed a complicated defence system that uses endogenous enzymes and numerous substances with antioxidase activity that are directly or indirectly supplied by the diet. In addition to the fundamental action performed by the enzyme inhibitors of oxidation, such as superoxide dismutase, catalysis and glutathione peroxidase, various compounds can interact with the reactive species of oxygen and have a regulatory effect. These include vitamins C and E, carotenoids and all the phenolic compounds.

As regards vitamins, fruit and vegetables are the primary source of vitamin C or ascorbic acid, a water-soluble molecule that performs multiple functions in the body. Being a powerful reducing agent, vitamin C exerts a strong antioxidant action, reacting rapidly with the free radicals in diverse reactions and oxidizing to dehydroascorbic acid. Together with glutathione, ascorbic acid is an important reserve of reducing capacity and is accumulated to a certain extent in the body. However, excessive amounts are immediately eliminated so it is important to have a continuous intake of vitamin C with the diet (King et al. 1994; Padayatty et al. 2003). Vitamin E is instead the principal vitamin with a lipophilic structure and for this reason it is indispensable for protection of the cellular membranes and other subcellular lipid structures. It has demonstrated a reasonable antioxidant activity thanks to its capacity to block lipid peroxidation. This property is due to its transformation into a stable radical compound, successively regenerated by the intervention of vitamin C and glutathione (Rimm et al. 1993; Balz 1999).

To prevent oxidation reactions it is important to have various molecules available with different reducing potential or anyway able to prevent oxidation with multiple mechanisms. For this reason the presence of the greatest variety possible of antioxidant molecules ensures the best protection in the various tissues (Fogliano 2009).

Agronomic practices, seasonality and genetic improvement can significantly influence the presence of elements with antioxidant activity in the plant products, as can the post-harvest treatments (Dalla Rosa 1996; Chassy et al. 2006; Shao et al. 2008; Björkman et al. 2011).

Among chemical compounds with antioxidant activity the phenolic compounds, or polyphenols, represent one of the most numerous and widely distributed groups of substances in the vegetable kingdom, with more than 8,000 known phenolic structures. They derive from the secondary metabolism of plants and are all characterized by the presence of an aromatic ring endowed with one or more hydroxylic group (Urquiaga and Leighton 2000). The structure of polyphenols varies from simple molecules, like the phenolic acids, to highly polymerized compounds, like the tannins (Vinson et al. 1998; Harborne and Dey 1989). For simplification, polyphenols can be split into two large families:

– *Flavonoids* that in their turn include the anthocyanins (red or blue pigments), flavanols (yellow pigments), flavones and flavonols (white or ivory pigments). The tannins (brown or blackish in colour) derive from the flavanols.
– *Non-flavonoids* or *phenolic acids* that can be found in the form of benzoic acids (e.g. gallic acid, catechic acid and cinnamic acids (e.g. caffeic acid and coumaric acid). These latter can combine with the anthocyanins and with tartaric acid, forming condensed polyphenols (Taiz and Zeiger 2002).

The flavonoids represent a vast family of polyphenolic compounds of low molecular weight, the majority of which are found in the outer layer of plant tissues (Clifford 1999; Chu et al. 2000). From the quantitative point of view the polymers of the flavonoids are of great importance, especially catechin. Vegetables, together with some fruits, are the principal food sources of flavonoids. There are various theories relating to their role in plants; the most credible are protection from UV-B rays and defence against pathogen attacks (Takeda et al. 1994). Because flavonoids also play an active role in the photosynthetic processes (Middleton and Teramura 1993), their quantity is influenced by exposure to light, rising with an increase in light intensity and especially UV-B radiation (Takeda et al. 1994).

2.2.2 Pigments

In addition to the chlorophylls, the main pigments present in plants, carotenoids and anthocyanins, are extremely important for plants and also for our bodies (Bartley and Scolnik 1995; Lila 2004, 2009; Pangestuti and Kim 2011). The carotenoids, orange and red pigments, are compounds belonging to the family of the tetraterpenes. The presence of conjugate double bonds allows them to easily accept electrons and therefore function as oxidation inhibitors. The carotenoids take part in the energy transport chain during photosynthesis, while in non-photosynthetic organisms they have an important role as antioxidants. The carotenes, formed just of carbon and hydrogen, and xanthophylls, also containing oxygen, both belong to this family. From the nutritional point of view it is important to distinguish between the carotenoids that are precursors of vitamin A (mainly beta-carotene) and the non-vitamins. The principal carotenes are lycopene and beta-carotene, while the xanthophylls include lutein and zeaxanthin.

2.2.3 Nitrates

Among the many bioactive compounds useful for the human body, some are a potential hazard. Nitrate (NO_3) is widespread in nature, in the soil, plants and water (Trinchera 2001; Addiscott and Benjamin 2005; Lundberg et al. 2004) and is the most important source of nitrogen for plants, which allocate a significant part of

their carbon and energy reserves to its absorption and assimilation (Buchanan 2003). Nitrate is not introduced as such into the organic compounds, but must first be reduced to ammonium through a process in two stages. First of all nitrate is reduced to nitrite (NO_2) by nitrate reductase, then nitrite is reduced to ammonium by nitrite reductase (Buchanan et al. 2003) and lastly the ammonium is assimilated through various metabolic pathways in the organic compounds, first and foremost the amino acids (Gonnella et al. 2002).

For humans, the three main sources of nitrate are in the order: vegetables, water and salami/sausages (Santamaria 1997; Hord et al. 2009). In fact nitrate and more especially nitrite are used as food additives in prepared and preserved meats because of their antimicrobial action (Santamaria 2006). The presence of nitrate, especially in vegetables, is considered a serious threat to human health (Santamaria 2006).

From the toxicological point of view, nitrate in itself has an extremely low acute toxicity (Speijers 1996). The main problem is linked to the fact that in humans 5–10 % of the nitrate ingested is reduced to the more toxic nitrite in the saliva and gastrointestinal tract (Walters and Smith 1981) through the reduction from nitrate to nitrite by bacterial enzymes (Santamaria 2006). Even more worrying is the fact that nitrite can react with amines and amides to form N-nitroso compounds, which are toxic and can lead to serious pathologies in humans (Santamaria 2006). The principal effect produced by nitrite is oxidation of the haemoglobin in the blood, which is transformed into methaemoglobin, a compound no longer able to transport oxygen to the tissues. Lower oxygen transport has consequences, especially in babies up to 6 months old as it causes methaemoglobinemia, also known as "blue baby syndrome", which results in the bluish coloration of the extremities (fingers, nose) due to the poor oxygenation of the blood (Santamaria 2006). To evaluate the carcinogenicity in laboratory animals, around 300 N-nitroso compounds have been studied: 85 % of the 209 nitrosamines and 92 % of the 86 nitrosamides have resulted as being carcinogenic in more than 40 animal species (Gangolli et al. 1994). These include mammals, birds, reptiles and fish, so there is no reason to suppose that humans would be the only ones resistant (Hill 1999). Numerous genetic, environmental and cropping factors influence the absorption and accumulation of nitrate in plant tissues. Among the studied factors, both the intensity and duration of light have been identified as the main influence on the nitrate content in vegetables (Santamaria et al. 2002; Pimpini et al. 2005; Nicoletto and Pimpini 2010). In fact, both affect the activity of nitrate reductase that can regulate the accumulation of nitrates as it stimulates the triggering of the nitrogen assimilation process. Essentially, the greater the light intensity and length of the photoperiod, the lower the content of nitrates in the plant tissues (Pimpini et al. 2005). A variation of the nitrate content in leaves during the day derives from this, with the minimum values found around sunset and maximum at dawn (Minotti and Stanley 1973). This is of practical interest as it suggests the best times for harvesting, which should not be underrated given that the nitrate content is now one of the main characteristics evaluated within a context of high-quality production.

Temperature can also affect the nitrate concentration in the tissues, as it influences the processes of absorption, translocation and assimilation, often in a combined way (Gonnella et al. 2002). Behr and Wiebe (1992) found that photosynthetic activity is inversely correlated to the temperature of the environment, with an increase in nitrate accumulation as the temperature rises. In rocket, for example, the results from trials conducted by Ventrella et al. (1993) showed that the leaves had higher nitrate contents at a temperature of 15 °C than 10 °C. Another factor to be taken into consideration is the water content in the soil. A good availability of water favours the absorption of nitric ion and its accumulation (Paradiso et al. 2001), but it can also increase the loss of nitric nitrogen by percolation towards the water soil layer (Patruno 1987). Furthermore, according to Maynard et al. (1976), water stress should be avoided because, in these conditions, the plant continues to absorb nitrate even when the nitrate reductase activity has already been interrupted and this causes an obvious increase in the nitrate concentration in the tissues. Lastly, post-harvest storage also has a strong effect on the nitrate content in the edible parts (Pimpini et al. 2005). In general, high temperatures, scarce oxygenation (atmosphere rich in CO_2) and high relative humidity increase the formation of nitrites (Santamaria et al. 2002).

Nevertheless, the most important cropping factor that can determine the amount of nitrates in plant tissues is nitrogen fertilization. Different aspects linked to fertilization can assume a determining role in the accumulation of nitrates in the edible parts of vegetables. First of all, it has been found that the nitrate concentration generally increases with the increased availability of nitrogen in the fertilizer (Bonasia et al. 2002). However, a high availability of N does not always correspond to an increase in production (McCall and Willumsen 1998). On the contrary, the plants continue to absorb nutritional elements in excess, storing them in the vacuoles, in order to still guarantee growth when the amounts of fertilizer diminish (Koch et al. 1988). This leads to an excessive accumulation of nitrates, a condition that is difficult to verify if it is not a soilless crop. In addition to nitrogen dose, the NH_4/NO_3 ratio has particular influence. The higher the ammoniacal rate, the lower the content of nitrates (Pimpini et al. 2005). The problem is that NH_4 is not the form usually preferred by plants (Salsac et al. 1987); moreover, if absorbed in excess, it can cause toxicity.

Regarding fertilization type, the slow-release fertilizers, given the risks of the release of nitrogen, should only be used after a careful evaluation of the application time and length of the cropping cycle to avoid high nitrate concentrations in the plants at harvest (Pimpini et al. 2005). With reference to planting density, an excessive number of plants per unit surface area must be avoided because the competition leads to a reduction of the light intensity at crop level. High densities also result in phenomena of high growing, with an abnormal lengthening of the leaf and an increased proportion of petiole, where the greatest nitrate concentration is found, in the edible product. Indeed, it has repeatedly been proved that the nitrate content varies in the different parts of the plant in this decreasing order: petiole, leaf, stem, root, inflorescence, tuber, bulb, fruit and seed. The different capacities of nitrate accumulation may be correlated to the localization of the nitrate reductase

enzyme as well as to the different level of nitrate absorption and transfer in the plant (Santamaria 2006).

At least another two factors influence the nitrate content in vegetables: the botanical family and type of leaf. Regarding the former, the vegetables that accumulate more nitrate belong to the families of the *Chenopodiaceae* (e.g. spinach), *Brassicaceae* (e.g. white cabbage), *Apiaceae* (e.g. carrot) and *Asteraceae* (e.g. radicchio and lettuce) (Santamaria et al. 1999). As regards the latter, the inner leaves of lettuce accumulate less nitrate than the outer leaves. This may be due to the fact that the outer leaves have lower photosynthetic efficiency than the inner ones and contain larger vacuoles (accumulation sites of the nitrates) (Santamaria et al. 1999).

At regulatory level, given the fact that vegetables provide the most significant contribution to the intake of nitrates, the European Commission's Scientific Committee for Food (SCF) proposed the introduction of maximum limits for nitrate content and the adoption of cropping techniques aimed at reducing its concentration in vegetables (Santamaria and Gonnella 2001). Starting from the SFC proposals, and in order to protect public health, the European Commission Regulation no. 194/97 came into force on 15 February 1997, giving the maximum acceptable nitrate contents in lettuce and spinach in all states of the European Union (Santamaria et al. 2002). The regulation set higher nitrate levels for crops grown in winter than those in summer. For lettuce, higher limits were given for crops grown in the greenhouse with respect to outdoor crops (there is higher light intensity and lower temperature in the field; consequently the nitrate content is lower). On 2 December 2011 the European Commission substituted Regulation no. 1881/2006 with Regulation no. 1158/2011. This introduced some changes and set new maximum acceptable limits for the nitrates contained in the large leaf vegetables, such as spinach and lettuce. The limits are expressed in milligrams per kilogram of fresh produce and vary from 2,000 mg for lettuces grown outdoors to 4,500 mg for lettuces cultivated in a protected environment. This new regulation lays down that the member states of the Union must make regular checks on the nitrate content in vegetables, in particular green leaf vegetables, communicating the results to the Commission by 30 June of each year. In addition, the Department of Community Policies and the SCF have established an Acceptable Daily Intake (ADI) for NO_3 that should not be above 3.7 mg kg^{-1} of body weight.

2.3 The Quality as a Function of the Process Variants

As many factors affect the quality of products as those that control the yield levels. Their effects on the quality parameters can appear in different and sometimes contrasting ways. Within the same crop, in fact, the product quality depends on genetic factors, environmental factors, agronomic practices and aspects connected to the harvest and post-harvest stages. In general these factors may have direct or indirect effects in relation to their influence on the processes of assimilation, water

absorption, nutritional status and the repartitioning of the photosynthates in the edible parts of the plant (Mezzetti and Leonardi 2009).

2.3.1 Genetic Aspects

For fruit and vegetable crops, the choice of genotype has always been of primary importance in relation to the effects on the quality characteristics of the produce that derive from the continual diffusion of new cultivars characterized by a high productive potential or suitable to cope with specific stress conditions. The variability of the quality due to genotype can be one of the most direct strategies for producing quality characteristics that meet specific consumer demands.

In the case of programmes aimed at enhancing the nutritional quality, for example, it would first be necessary to understand the processes that determine the efficacy of the bioactive compounds; therefore, it is indispensable to identify them chemically and verify their stability in the various conservation stages (Finley 2005).

The nutritional quality of different fruit and vegetable species can be enhanced using a traditional approach of genetic improvement only if genetic resources are available that can provide effective progresses in the different generations of crosses. The transgenic system has been successfully applied for some vegetables such as tomatoes (Davoluri et al. 2005) and this demonstrates the possibility of using these technologies to increase the content of specific bioactive components in the plants even if the interventions are rather complex.

2.3.2 Climate

Apart from what has already been reported for nitrates and nitrites, the variability of the climatic conditions is undoubtedly an important parameter for product quality. Climatic factors can have effects on the assimilation processes and nutritional status of the plant (Anttonen et al. 2006). Furthermore, the creation of "modified" climatic conditions through the use of more or less advanced protected environments may also have effects on the quality of the produce.

Among the many aspects that define climate, light and temperature play an important role in influencing quality because they interact strongly with the biochemical processes of the plant (Wang et al. 2003). They have different effects on the assimilation processes, because high light levels favour the accumulation of photosynthates, whereas high temperatures accelerate their demolition.

The influence of these two parameters on the quality must be considered in terms of the many definitions and attributes of quality. Extreme temperatures, for example, can have effects on the processes of macro- and micro-sporogenesis (Subodh

and Munshi 2001), assimilation (Zhang et al. 2007) and the synthesis and demolition of pigments (Hamauzu et al. 1994).

Instead, low light intensity can have positive effects on some quality parameters in leaf vegetables (e.g. high wateriness of the tissues, attenuation of the green colour, etc.), while it can worsen the health characteristics of the produce because of the high nitrate accumulation (Santamaria et al. 2002; Santamaria 2006). The photoperiod is also of interest for some horticultural products (e.g. radicchio) as it may be the determining factor for phase transition, with a consequent decline in the product quality due to the plant going to seed (Pimpini et al. 2007; Pimpini and Nicoletto 2008). Relative humidity can also be a critical factor for the quality because it can determine a slowing down or activation of water exchange and its different allocation in the various parts of the plant (Leonardi et al. 2000).

2.3.3 Fertilization and Irrigation

Relevant effects on the quality of horticultural produce are mainly exercised by irrigation and fertilization (Ferrante et al. 2008). A regular water supply generally improves their quality through an increased water content in the edible parts. Yet a high water content may, in some cases, compromise the storability of the products or their tastiness. In general lines it can be stated that the quality is impaired by a lack of water for the species with vegetative organs and by excesses for those with reproductive organs (La Malfa 1988).

The effects of mineral nutrition depend on the role which the different elements have on the plant metabolic processes that synthesize and translocate the many biochemical compounds. If on the one hand, high fertilizer doses may be the prerequisite for an improvement in the positive factors of quality, on the other they may also involve the accumulation of nitrates in the edible parts (Malaguti et al. 2001).

2.3.4 Harvest and Post-harvest

The aspects that affect quality undoubtedly include those relating to the harvest and post-harvest. In the former case these refer to the maturation stage of the product, environmental conditions and harvesting methods. For the majority of fruit and vegetable products, the best quality characteristics are reached at the time of harvest; in the successive stages there is a progressive decline of the quality that can only be slowed down with opportune conservation strategies. Therefore the storage conditions and processing techniques can be of great importance during post-harvest as the products may suffer mechanical damage or pathogen attacks that alter the metabolic situation of the product (Almirante and Colelli 1994).

From what has been described so far, the need emerges for the horticultural sector to adapt to the new scenarios that have been appearing in the last years, through technical-agronomic and organizational innovation, as well as paying increasing attention to consumer expectations. Indeed, consumers are showing a growing mistrust of products that come from a production process characterized by a high level of intensification. In addition, they are increasingly aware of healthy products, obtained with eco-compatible techniques. These interests are due to the above-cited progressive change that is taking place in the concept of quality for horticultural produce. Therefore, one of the main aims to pursue in order to maintain the competitiveness of the sector is that of the qualification and characterization of the productions.

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Chapter 3
Unit Processing Operations in the Fresh-Cut Horticultural Products Industry: Quality and Safety Preservation

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Abstract The current high demand of minimally processed or fresh-cut fruit and vegetables results from the consumer's desire for healthy, convenient, fresh, and ready-to-eat plant food derived commodities. Human nutritional research has increasingly shown that a well-balanced diet, rich in fruits and vegetables, promotes good health and may reduce the risk of certain diseases. These new elaborates show similar characteristics to the whole original product, contain exclusively natural ingredients, are of good quality with relatively low price, and do not need time for preparation. Indeed, they are elaborated by using mild unit processing operations, to decrease the product deterioration ratio, and packaged with suitable polymeric films, usually under active or passive modified atmosphere packaging while its shelf life is under refrigerated conditions. The most important goal to preserve quality and safety is releasing the microbial spoilage flora since every unit operation involved will influence the final microbial load. For that reason, the implementation of a proper disinfection program, together with the development of new molecular tools for microbial diagnosis, should be the main concern. Sanitation in the washing step is the only operation able to reduce microbial load throughout the production chain. Chlorine is widely used as an efficient sanitation agent, but some disadvantages force to find emerging alternatives. It is necessary to deal with aspects related to sustainability because, apart from reducing its use, it could positively contribute to the net carbon balance. Several eco-friendly innovative techniques seem to reach that target. However, industrial changes for replacing

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conventional with innovative technologies request a fine knowledge of the benefits and restrictions as well as a practical outlook. This chapter describes the general steps used in the fresh-cut industry and reports emergent techniques to preserve quality and safety of minimally processed horticultural products.

Keywords Minimally processed • Ready-to-eat-Quality • Safety • Sanitizers • Innovative technologies

3.1 Introduction

Since consumers increasingly perceive fresh food as healthier than heat-treated food, it motivates a general search of food production methods with reduced technological input, including agrochemicals. In that way, the current high demand for minimally processed or fresh-cut fruit and vegetables is a result of the consumer desire for fresh, healthy, convenient, and ready-to-eat horticultural products. In fact, the worldwide fresh-cut produce industry has grown rapidly in recent years to a multibillion dollar sector. After USA, and within Europe, UK and France are the main producers and consumption countries. Fresh-cut products are fruits and vegetables prepared with slight processing operations (peeling, cutting, slicing, shredding, trimming, sanitizing, etc.) and packed in suitable polymeric semipermeable films under active or passive modified atmosphere packaging (MAP) and kept under a refrigerated shelf life. They show similar characteristics to the whole original product, contain exclusively natural ingredients with a good quality and freshness at a relatively low price, and do not need time for preparation (Artés et al. 2009). It has been suggested that minimal processing techniques have emerged to replace traditional harsher methods of food preservation as they retain better the nutritional and sensory quality (Ohlsson 2002). While conventional food-processing methods extend the shelf life of fruit and vegetables, the minimal processing to which fresh-cut fruit and vegetables are subjected renders products highly perishable, requiring a proper temperature management to ensure a minimum shelf life (Rico et al. 2007). Human nutritional research has increasingly shown that a well-balanced diet, rich in fruits and vegetables, promotes good health and may reduce the risk of certain diseases (Meng and Doyle 2002). In that way, fresh-cut products are an important source of antioxidants and other phytochemicals, which play important roles in human nutrition due to free radical scavenging activities and induction of genes encoding anticarcinogenic enzymes. Therefore recommendations of an equilibrated diet must include the consumption of fresh fruits and vegetables, which in fact are a very important part of the diet around the world (Robles-Sánchez et al. 2009).

After wounding living tissues begin a flow of metabolic reactions that starts with increased respiration rate and can result in texture changes, accelerated ripening and/or senescence, off-flavors, discoloration, and other undesirable events (Toivonen and Brummell 2008). Handling and processing also result in an

increased ethylene production which promotes ripening and senescence. Microbiologically, removing the protective peel of fresh produce leaves a cut surface that is covered with water from the cell contents, which makes it convenient for microbial development. Since the minimal processing damages plant tissues, leading to additional quality losses, the derived fresh-cut commodities are in fact more sensitive to disorders than the original ones. Therefore, the deterioration of fresh-cut fruits and vegetables is mainly due to further physiological ageing, biochemical changes, and microbial spoilage which turn the product unmarketable. The adverse changes affecting minimally fresh processed fruits and vegetables are off-flavors, discoloration, browning, softening, water loss, and contamination (Artés et al. 2007).

During minimal processing, products are subjected to mechanical damages that stimulate fast physiological and biochemical answers, which are recognized by an increase in their metabolism. Wounding is produced when processing increases the respiration rate of the plant tissue, probably as a consequence of induced ethylene (C_2H_4) biosynthesis, which stimulates respiration (Tsouvaltzis et al. 2006). In fact, the introduction of new cultural practices, cultivars, harvesting and handling methods, postharvest treatments, and packaging determines the effect of C_2H_4 on minimally processed fruits and vegetables.

Browning is one of the major causes of quality loss and spoilage of fresh fruits and vegetables because it is a frequent problem during postharvest handling, processing, and storage. It reduces produce quality and very often is the factor limiting shelf life and marketability of minimally fresh-cut produce. This phenomenon can be due to enzymatic and nonenzymatic reactions. Enzymatic browning or oxidative browning requires different components: enzymes (such as polyphenol oxidase—PPO and peroxidase—POD), a substrate, and co-substrates such as O_2 and H_2O_2 (Vamós-Vigyázó 1981; López-Gálvez et al. 1996; Hodges and Toivonen 2008). Browning takes place at the cut surface of fruits and vegetables because of decompartmentation that occurs when cells are broken, allowing substrates and oxidizers to come in contact. The brown color development is related primarily to oxidation of phenolic compounds including monophenols, triphenols, and *o*- and *p*-diphenols to *o*-quinones, a reaction catalyzed by PPO and POD (Artés et al. 1998). The oxidation products of these reactions, *o*-quinones, polymerize with each other and react with NH_2 or SH groups from amino acids and proteins, and with reducing sugars, giving complexes of high molecular weight polymers of unknown structure which leads to the formation of dark brown or black pigments (Vamós-Vigyázó 1981). It was reported that wounding also induces synthesis of some enzymes involved in browning reactions or substrate biosynthesis (Brecht 1995). In some fruits like apple, lipoxygenase (LOX) may be also responsible for the browning. LOX activity during storage has been investigated in the core, flesh, and peel. Activity was always highest in the core and peel. On storage, activity was increased in each part of the fruit but especially in the core and peel. Increase in LOX preceded the browning of the core (Baysal and Demirdöven 2007). LOX activity may also promote synthesis of desirable or undesirable aroma volatiles. In cut, bruised, or senescent plant products this oxidative reaction occurs readily,

minimally processed commodities being highly susceptible to oxidative browning reactions. Considerable research has been devoted to inhibit this disorder (Cliffe-Byrnes and O'Beirne 2008).

3.2 Factors Affecting Quality and Safety

Several factors affect the shelf life and microbial quality of fresh-cut vegetables like agricultural practices at the farm, hygienic practices during harvesting and handling, quality of washing water, processing technologies, packaging methods and materials, and transportation, processing, and storage temperatures (Ahvenainen 2000; Nicola et al. 2009; Francis et al. 2012). The distribution chain is generally composed of many different steps in storage and transportation until final consumption, and traceability is still today a key concept (Allende et al. 2004).

3.2.1 Preharvest Factors

Attaining the optimum postharvest quality of fruits and vegetables actually begins very early in the farm planning process. The effects of preharvest factors on postharvest quality are often overlooked and underestimated. However, many of the decisions made during the crop production can greatly influence the postharvest quality of crops. The first factor is the plant itself: large genotypic variation given by many different botanical species and genetic type or variety has been described. Secondly, there are external factors. Although the factors that can define the external background are very different, those who have been most studied are the environmental (climatic conditions), the agronomic (cultural practices), and the physiological conditions. The first one includes temperature, relative humidity, rainfall, wind, soil, etc. In the second one it should be mentioned fertilization, watering, pruning, etc. and in the third the maturity stage at harvest could be considered as the most relevant. This set of factors determines not only the quality of the fruit, but also affects postharvest behavior, especially when the produce will be stored for a long term or when they are the raw material for minimal processing. There are few postharvest disorders that are completely independent of preharvest factors. Even incidence of disorders induced specifically by storage conditions will be modified by preharvest environment and cultural practices (Ferguson et al. 1999).

Each product has a certain combination of compositional and physical characteristics and will have specific growing, harvesting, and processing practices and storage conditions. Different cultivars vary in several attributes including size, color, flavor, texture, nutritive value, pest resistance, processing suitability, eating quality, and yield. Since it all starts with the seed, the cultivar selected for fresh-cut processing has a very critical effect on product shelf life and overall quality. Variety

selection may also affect the nutritional value. Susceptibility to browning may also widely differ from one cultivar to another (Francis et al. 2012).

The possible use of genetic engineering to develop higher production and more resistant plant foods is relatively well known. Currently this technology is being used to introduce desirable attributes such as improved color, aroma, flavor, and taste of different fruits and vegetable products. Although the huge advance of these techniques was in the last decade, there is still a lack of published information about the development of genetically modified fruits and vegetables which overcome some relevant problems of the postharvest science such as chilling injury resistance, longer storage duration, and pathogen resistance. Therefore much more effort should be done in this area, and recent advances in functional genomics should bring candidate genes to manipulate (Rodov 2007).

The protected culture system of raw material for the minimal processing industry, when can be used, shows several advantages compared to the open field system, among others, a protection from adverse weather conditions, a reduction in evapotranspiration rate, an increase in photosynthesis rate, an advance in the harvest date, and higher internal air temperature. All these factors commonly improve plant health and raw material quality, yield and safety (Nicola et al. 2009). Moreover, adequate soil nitrogen supplies allow for the optimal development of vegetable color, flavor, texture, and nutritional quality. Excess soil nitrogen can be problematic as well. Research has shown that too much soil nitrogen can reduce the vitamin C content of leafy vegetables while excessive nitrogen may lower fruit sugar content and acidity (Ferguson et al. 1999).

3.2.2 Processing Operations

The unit operations employed during processing of minimally processed produce (i.e., peeling, slicing, shredding) cause the destruction of surface cells, stress tissues, and in the case of fruits, remove natural barriers such as cuticles and skins, which make tissues more susceptible to water loss and decay (Brecht 1995). In that way, processing promotes a faster physiological deterioration, biochemical changes, and microbial degradation of the product even when only slight processing operations can be used (O'Beirne and Francis 2003), which may result in degradation of the color, texture, and flavor.

3.2.3 Packaging

Modified atmosphere packaging (MAP) uses low O_2 and enriched CO_2 concentrations within packages to preserve quality of fresh-cut produce. The beneficial effects of MAP include a reduction in respiration rate, ethylene production, enzymatic reactions, and of some physiological disorders, thereby enhancing

product quality and shelf life (Ahvenainen 1996). The use of a low O_2 concentration (5 kPa) and a high CO_2 concentration (10 kPa) under refrigerated storage is proposed by many researchers as optimal conditions for fresh-cut fruits and vegetables to maintain their sensory as well as microbial quality during shelf life. By matching permeation rates for O_2 and CO_2 with the respiration rate of the packaged fresh-cut produce an equilibrium modified atmosphere can be established inside the package (Artés et al. 2012).

3.2.4 Temperature Management

A quick precooling just after harvest and maintaining strict low temperatures (<5 °C) during processing, especially after peeling or cutting, transportation, and retail sale of fresh-cut product is a critical parameter for quality preservation. Temperature strongly affects respiration rate, dehydration, enzymatic browning, and permeability of gases through packaging films, which implies changes in atmospheres within MAP packages. Moreover, temperature is one of the most important of factors affecting survival and growth of pathogens on fresh-cut produce (Artés et al. 2009).

3.2.5 Produce Contamination and Diagnosis

The pathogens of major concern in fresh-cut produce are *Listeria monocytogenes*, pathogenic *Escherichia coli* mainly O157:H7, and *Salmonella* spp. However, a number of important human pathogens can contaminate fresh-cut produce and there has been an increase in the number of produce-linked food-borne outbreaks in the recent years. Produce contamination can occur during agricultural production (via animals or insects, soil, water, dirty equipment, and human handling), harvesting, processing (cutting, shredding, washing, contaminated work surfaces/equipment, hygiene practices of workers), packaging (contaminated packaging materials/equipment), and transportation and distribution (Tomás-Callejas et al. 2011). The development of new molecular tools for microbial diagnosis of pathogenic bacteria has improved both diagnostic efficiency and knowledge about spread of such microorganisms. The main advantage of microbial identification by genetic markers is the relative stability of the genotype rather than the phenotype. Nucleic acids and proteins can act as a "fingerprint" of a microbial species, allowing more precise identification as well as traceability (Francis et al. 2012). DNA-based monitoring procedures promise a fast quantification and identification of microorganisms by applying real-time polymerase chain reaction (PCR) and melting point analysis. The 23S ribosomal DNA (rDNA) is one of the genome target regions valuable for genotyping a wide range of food-borne bacteria (Manchado-Rojo et al. 2008). However, the cost of molecular diagnosis is still too high to be

supported by small and medium companies so more research should be conducted in that sense.

In addition, to ensure the quality and safety of all incoming raw materials, implementation of a quality management standard such as ISO9000:2000 has been recommended as a basis for an agreement between the supplier and the fresh prepared produce manufacturer which should include a hazard analysis of critical control points (HACCP) to identify what could go wrong with incoming produce (Artés et al. 2009). Finally, it is necessary to evaluate rapidly and nondestructively the quality of plant raw materials at receiving in the factory for safety aspects like pesticide residues, microbial load, toxic metals, naturally present undesirable compounds, and plant growth regulators (Yildiz 1994).

3.3 Unit Processing Operations

The traditional processing procedure for obtaining fresh-cut products usually consists of a sequence of unit operations like peeling, trimming, shredding, dicing, cutting, washing/disinfection, drying, and packaging. In general, the extension of the shelf life depends on a combination of proper temperature management throughout the entire cold chain, dips in anti-browning solutions, optimal packaging conditions (usually MAP), and good manufacturing and handling practices in well-designed factories (Artés et al. 2009).

The main objective of the fresh fruit and vegetable processors throughout all processing operations involved in the production of fresh-cut produce is food safety, quality optimization, and loss reduction. For that reason a relationship between Industry–Academia–Government with common research concerning would be ideal to enhance food safety (Osterholm et al. 2009). Common practices consist of the protection of the produce from damage caused by poor handling or machinery functioning, foreign body contamination, and/or pest infestation (Day 2000). In addition, contamination by human handling during handling, washing, drying, and packaging processing may occur from unhygienic personnel (Hurst 1995). Therefore, although worker sanitation is an aspect that is too often neglected in the minimal processing of fresh plant products, good manufacturing practices must be practiced and all food handlers must be supervised and trained in food hygiene matters related to their work activities (Brackett 1992).

The sequence of steps needed in a typical industrial factory of fresh-cut fruits and vegetables has similarities, although both of them require specific and differentiated steps (Artés-Hernández et al. 2010). Figure 3.1 illustrates the general unit operations and the maximum recommended temperatures to each processing step in the production line of leafy vegetables.

The first step in the fresh-cut factories is generally sanitation of whole products to eliminate unwanted dirt, pesticide residues, plant debris, soil, insects, and foreign matter and retardation of the enzymatic discoloration reactions. Sodium or calcium hypochlorite and other salts are widely used for surface sanitation and sterilization

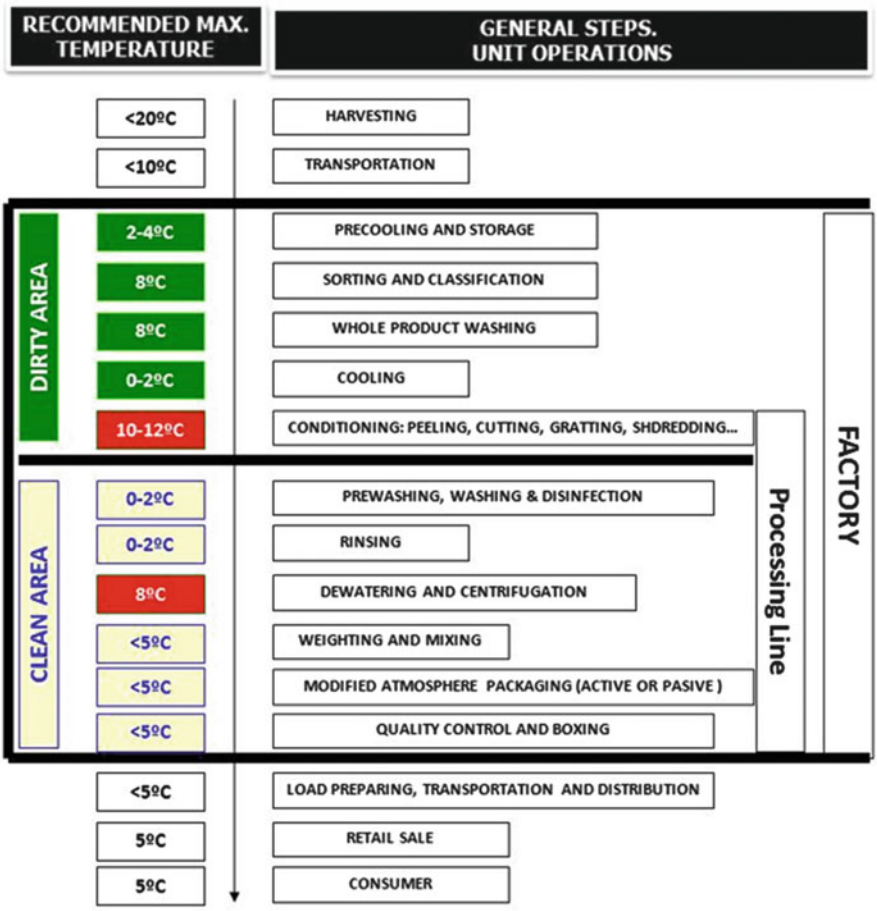

Fig. 3.1 General unit operations and maximum recommended temperatures for each step in the fresh-cut horticultural produce industry

of fruits to prevent microbial inoculation, although after pathogens have infected their host, chlorination is not very effective (Hong and Gross 1998).

Peeling and cutting steps constitute a critical hygienic point in the processing line, and the equipment used in this operation needs to be cleaned, disinfected, and sharpened at regular intervals every working day to avoid buildup of organic residues. The extent of wounding is affected by the number of cuts and the severity of the cutting treatments or the sharpness of cutting blades. Portela and Cantwell (2001) showed that melon pieces cut with a blunt blade exhibited increased ethanol concentrations, off-odors, and electrolyte leakage compared to pieces processed with a sharp blade. Similarly, the use of sharp cutting blades reduced the wound response, lignin accumulation, white blush, softening, and microbial growth in fresh-cut carrots (Barry-Ryan and O'Beirne 1998). The physical damage,

physiological stress, and increase of microbial growth caused in this step are well documented. These changes are mainly due to the increase of wound respiration and C_2H_4 production due to mechanical injuries which results in the release of intracellular oxidizing enzymes and substrates and leads to various biochemical deteriorations, such as browning, and increased availability of cell juice and nutrients. The fluid exudation of bruised and cut surface tissues can lead to a high difficulty for keeping quality and shelf life of some fresh-cut produce (Artés et al. 2007). Meanwhile, cutting appears to have a dramatic effect on nutritional value, overall quality, and shelf life of minimally fresh processed fruits and vegetables (Barry-Ryan and O'Beirne 1999; Artés et al. 2009). Many different peeling machines are commercially available, like mechanical, chemical, or high-pressure steam peelers, but peeling is normally manually accomplished. A great number of machines are commercially available for cutting, grating, chopping, shredding, or slicing fresh produce into pieces of several shapes and sizes.

Just after peeling and/or cutting, washing is a critical point since disinfection takes place, being the only step throughout the production chain where a reduction in the microbial load can be obtained, thus minimizing populations of potential pathogens (Beuchat 1998; Nguyen-The and Carlin 1994; Gómez-López et al. 2008; Artés et al. 2009). However, published efficacy data indicate that these conventional, time-consuming methods are not capable of reducing microbial population on produce by more than 90–99 %, which is insufficient to ensure microbiological safety (Sapers 2001). Sanitizers are primarily used to maintain bacteriological quality of the water rather than the produce (Brackett 1999). Washing can be achieved very simply by spraying with potable water, although it generally involves the immersion of the product in cold (1–5 °C) sanitized water in a bath or wash-tanks usually containing between 50 and 150 ppm of sodium hypochlorite (NaClO) solution and acidified with about 150–200 ppm of citric acid to manage pH values between 6.5 and 7.5 for optimizing the chlorine efficacy. Systems that ensure mixing of the wash water and product will improve washing performance, because the turbulence generated by the aeration allows eliminating almost all traces of soil and foreign matter without damaging the product (Artés et al. 2009). Among the different industries, the food industry ranks third in water consumption and wastewater discharge rates coming after the chemical and refinery industries. The adoption of less water consuming systems is required for improved water management (Ölmez and Kretzschmar 2009). It is necessary to deal with aspects related to sustainability because, apart from reducing the use of a limited resource, it could positively contribute to the net carbon balance.

A great number of antimicrobial washing solutions have been reported, but probably the most widely used is a NaClO solution containing 40–150 ppm of available chlorine and quaternary ammonium compounds. When chlorine gas (Cl_2) or hypochlorite salt [e.g., NaOCl or $Ca(OCl)_2$] is added to water, they will generate Cl_2, hypochlorous acid (HOCl), which is the active form, or hypochlorite ions (OCl^-) in various proportions, depending on the pH of the solution. However, the antimicrobial activity of NaClO solutions is related to the concentrations of undissociated OCl^- and HOCl (Adams et al. 1989). There is no unified criterion about

the recommended free chlorine concentration and contact time for the disinfection water. Therefore, the USFDA recommends 50–200 ppm total chlorine and contact times of 1–2 min for this purpose (USFDA 1998), and the International Fresh-Cut Produce Association (IFPA 2001) Model HACCP Plan for shredded lettuce suggests a maximum chlorination of 100–150 ppm total chlorine at pH 6.0–7.0 and the maintenance of 2–7 ppm free residual chlorine after contact (Soriano et al. 2005). It is remarkable that chlorinated washing produce can effectively remove sand, soil, and other debris from fresh fruits and vegetables but should not be relied upon to completely remove organisms (Brackett 1992). Since the disinfection efficacy is greatly affected by the quality parameters of water itself, it is also important to understand the relation between the pH, temperature, turbidity, and organic matter contents of water and the efficacy of disinfection (López-Velasco et al. 2012).

Additionally, some authors have proposed the use of edible coatings in combination with anti-browning compounds to improve the color preservation of fresh-cut fruits (Olivas and Barbosa-Cánovas 2009). Edible coatings provide a partial barrier to gas exchange (including water vapor) and help to maintain or improve color, texture, mechanical integrity, and volatile flavors and to reduce microbial growth (Han et al. 2004). The choice of coating used on fresh-cut products is very critical due to the hydrophilic nature of cut surfaces where some coatings may not adhere. In addition, some coatings may not resist water vapor pressure. Lipids confer important water barrier characteristics but may give a gummy mouthfeel to the product, while hydrophilic polymers have less barrier properties, especially with high RH values. In addition, antioxidants, fungicides, and preservatives may be added to the emulsion mix to increase the coating performance, while adding minerals or vitamins may enhance the nutritional value of fresh-cut products (Baldwin et al. 1996; Rojas-Argudo et al. 2009).

Dewatering is the next critical processing operation. Drying or dewatering wet surfaces must be carried out carefully to avoid unnecessary damage to the plant tissues, reducing the product moisture content and removing the cell leakage that can support microbial growth and enzymatic activity. Dewatering systems include draining systems, gentle removal with cheesecloth, centrifugal spin dryers, vibrating racks, rotating conveyors, hydro-sieves, forced air, and spinless drying tunnels (Gorny et al. 2002).

The high centrifugal force not only removes water, but also cracks and crushes the tissues (Ahvenainen 2000). Forced cold air, or heat just during the first drying phase and then cold air, has been recently applied as an alternative to the conventional dewatering systems. However, their main inconvenience is the low efficiency to dry high volumes of product. Another new technique that has recently been developed is the use of infrared light to dry the fresh processed commodities. However, this technology still has two main problems for its application at industrial scale, such as the high initial financial investment and the large area needed in the processing plant to install the device. Additionally between the drying and packaging steps it could be interesting to introduce techniques already applied in the clean room technology by installing a filtered air system that is able to ensure

the presence of less than 70 particles with a diameter higher than 5 μm and less than 10,000 with a diameter higher than 0.5 μm (Artés-Hernández et al. 2010).

Fresh-cut products should be kept below 5 °C under modified atmosphere packaging (MAP) to achieve the needed commercial shelf life. The aim of MAP is to create an atmosphere around the packaged produce, which retards their respiration and deterioration rates in such a way that the tolerated minimal O_2 or maximum CO_2 concentrations are not exceeded, in order to avoid a shift towards fermentation or other metabolic or biochemical disorders (Toivonen and Brummell 2008). The design and selection of the appropriate polymeric film for trays or bowls as well as for sealing are crucial. In this way, when temperature abuse (over 5 °C) during transport, distribution, and, particularly, retail sale could commonly occur, the use of little perforations or microperforations in MAP polymeric films should be suggested. It is also worth to mention that even when from one side, atmospheres of high CO_2 and low O_2 levels could control microbial growth, on the other side the risk of recontamination of commodities within packages increases (Artés et al. 2006, 2012).

It is well known that temperature is the most important environmental factor that influences the deterioration rate of harvested commodities (Artés et al. 2007). Knowledge about the time–temperature conditions in the cold chain of fresh processed fruit and vegetables is necessary to determine the influence of the actual cold chain on the quality loss and the shelf life of these products. Although throughout the distribution chain commodities must be kept at 1–5 °C to ensure quality and shelf life, it is almost impossible to guarantee that this temperature will be maintained during transit, distribution, and retail display (Orsat et al. 2001). In fact, it is frequently seen that these products are often subjected to temperature abuse (>10 °C) in the display cabinets for a long time during retail sale.

3.4 Alternative Technologies for Keeping Quality and Safety

Although chlorine is the most common chemical used for disinfection, it has been shown that many microorganisms exhibit resistance to chlorine treatments (Nguyen-The and Carlin 1994), leaving the food industry to seek alternative agents (Gómez-López et al. 2008; Artés et al. 2009). The use of chlorinated water has also raised questions due to some facts, such as even when used at low concentration, it may cause taste and odor defect in treated product, the possibility of health hazards due to the potential toxicity, carcinogenicity, and mutagenicity of chlorinated water and chloro-organic compounds formed by reaction with food components, and the processing cost and problems associated with disposal of waste chlorinated water (Dychdala 1991; Singh et al. 2002; Delaquis et al. 2004).

Understanding the ecology of pathogens on fresh produce is essential for development of methods to eliminate them from these products. Therefore, future research should focus on factors affecting survival, attachment, and internalization of human pathogens in fresh produce. Indeed, conventional sanitizing methods are

ineffective for removing internalized bacteria and improved sanitizing may develop from studies examining synergistic interactions between sanitizers. Pathogen contamination of fresh produce may originate before or after harvest, but once contaminated produce is difficult to sanitize. The prospect that some pathogens invade the vascular system of plants and establish "subclinical" infection needs to be better understood to enable estimation of its influence upon risk of human illness (Olaimat and Holley 2012). Many studies of biofilms formation on produce have revealed that they are resistant to commonly used sanitizers and disinfectants. In that way knowledge about the efficacy of current disinfectants used to decontaminate microorganisms present in biofilms of produce biofilms and control the produce-related outbreaks by alternative approaches is quite interesting (Kabir and Ha 2012). A recent frontier in pathogen detection is represented by biosensors. Nucleic acid based sensors play an increasingly important role in the detection of pathogenic organisms in health care, environment monitoring, and food safety (Moldenhauer 2008). Diagnostic biosensors are a group of devices and technologies that use a biologically derived material immobilized on a detection platform to measure the presence of one or more analyte. For applications in food microbiological analysis, an ideal biosensor would be a self-contained, automated system capable of pathogen detection directly from a food matrix without pre-enrichment and also capable of differentiating live from dead cells (Ivnitski et al. 2000).

There is a real need to find alternatives for preservation of fresh-cut fruit and vegetables in order to improve the efficacy of washing treatments. In order to achieve fresh-cut plant produce with fresh-like quality, safety, and high nutritional and sensory quality, the industry needs to implement improved ecoinnovative techniques which include chemical coadjutants like antimicrobial solutions, acidulants, antioxidants, etc. In that way, it has subsequently reviewed the main emergent techniques which can be used at industrial level for keeping quality and safety of fresh-cut plant commodities which have been recently reviewed (Artés-Hernández et al. 2010; Oms-Oliu et al. 2010; Artés et al. 2009, 2011).

3.4.1 Antimicrobial Solutions

The superficial microbial load of the plant material will be effectively reduced by the washing and disinfection steps. Some alternatives to chlorine are described as follows:

Peroxyacetic acid: Peroxyacetic acid (CH_3COOOH) is a promising sanitizer due to its dissociation in water in acetic acid and H_2O_2. Its breakdown products, water, O_2, and acetic acid are biodegradable. It is applied for surface cleaning in concentrations ranging from 85 to 300 ppm, and the U.S. Food and Drug Administration has set a minimum of 85 ppm. Because of peroxyacetic acid tolerance to several factors like temperature, pH (from 1 to 8), and hardness and soil contamination, its current main area of application is in fruit and vegetables processing.

Ozone (O_3): Ozone is a highly unstable tri-atomic oxygen molecule (O_3) formed by the addition of an oxygen atom ($O^{\bullet}$) to a molecular diatomic oxygen (O_2) and acts as a strong oxidizing agent effective in destroying microorganisms. O_3 destroys microorganisms by the progressive oxidation of vital cell components, preventing the microbial growth and extending the shelf life of many fruit and vegetables. Washing with ozonated water has been suggested as an interesting alternative to traditional sanitizers due to its efficacy at low concentrations and short contact times as well as the breakdown to nontoxic products.

Chlorine dioxide (ClO_2): ClO_2 is a stable eco-friendly dissolved gas, with a higher oxidation and penetration power than NaClO, being more effective against spores, bacteria, and viruses. Moreover, it is less corrosive than NaClO. With minimal contact time, it is highly effective against pathogenic organisms such as *Legionella*, *amoebal cysts*, *Giardia cysts*, *E. coli*, and *Cryptosporidium*.

Acidified sodium chlorite—ASC ($NaClO_2$): ASC chemistry is principally that of chlorous acid ($HClO_2$), which forms on acidification of chlorite. Once formed, $HClO_2$ gradually decomposes to form chlorate ion, chlorine dioxide, and chloride ion. It is hypothesized that the mode of action of ASC derives from the uncharged $HClO_2$, which is able to penetrate bacterial cell walls and disrupt protein synthesis by virtue of its reaction with sulfhydryl, sulfide, and disulfide containing amino acids and nucleotides.

Organic acids and calcium salts: Organic acids have been largely applied for the prevention of enzymatic and nonenzymatic browning and microbial growth at levels that did not adversely affect taste and flavor of plant commodities. They are more effective for bacteria than for molds and yeast due to the low pH (between 2.1 and 2.7) at which they are applied. Calcium is related to maintain cell wall structure and firmness of plant commodities by combining with pectin to form calcium pectate.

Electrolyzed water (EW): EW has a strong bactericidal effect against pathogens and spoilage microorganisms, more effective than NaClO due to its high redox potential. Hypochlorous acid is present in EW at 6.8 pH and it is generated by electrolysis of a dilute salt solution, usually NaCl at about 0.1 % in an electrolysis chamber where anode and cathode are separated by a membrane. HCl is formed at the anode site which neutralizes the NaOH at the cathode site. Safety is the main advantage of EW. In contrast with the NaClO problems EW is not corrosive to skin, mucous membranes, or organic material. In addition, when EW comes in contact with organic matter, or is diluted by tap water or reverse osmosis water, it becomes ordinary water again, being more eco-friendly than NaClO.

3.4.2 Prepackaging: UV-C Radiation and Intense Light Pulses

The use of nonionizing and germicidal ultraviolet light at 190–280 nm (UV-C) could be effective for surface decontamination of fresh-cut products. It has been

reported that UV-C affects several physiological processes in plant tissues and damages microbial DNA reported that 0.5–20 kJ UV-C m^{-2} inhibited microbial growth by inducing the formation of pyrimidine dimers which alter the DNA helix and block microbial cell replication. The effectiveness of UV-C seems to be independent of the temperature (5–37 °C) but depends on the incident irradiation determined by the structure and surface of the product. However, UV-C can change the cell permeability increasing electrolytes, amino acids, and carbohydrates leakage, which can stimulate bacterial growth. The crucial point is whether a safe dose could be found which would greatly impair pathogen growth without damaging the product. Intense light pulses (ILP) are an innovative decontamination method for food surfaces approved by the US-FDA that could be suitable for sanitizing fresh-cut plant commodities. ILP kills microorganisms using short-time (85 ns to 0.3 ms) high-frequency pulses (0.45–15 Hz) and energy per pulse (3–551 J) of an intense broad spectrum, rich in UV-C light.

3.4.3 Nonconventional Packaging

An alternative active MAP by the use of superatmospheric O_2 (>75 kPa O_2) has been described as effective to inhibit enzymatic browning, prevent anaerobic fermentation, moisture, and odor losses and reduce aerobic and anaerobic microbial growth. The combined high O_2 level and 10–20 kPa CO_2 may provide adequate suppression of microbial growth and prolong shelf life of several fresh-cut plant commodities. The use of nonconventional gases like Ar, He, Xe, or N_2O has also been proposed for improving quality of selected fresh-cut plant commodities.

3.4.3.1 Hurdle Technology

The hurdle technology concept results from the combination of different preservation techniques as a quality and safety preservation strategy. The most important hurdles are based on controlling temperature, dehydration, water activity, acidity, redox potential and the use of natural preservatives, modified atmosphere, and competitive microorganisms. By combining hurdles, the intensity of the individual preservation techniques can be kept comparatively low, minimizing the loss of quality, while the overall impact on microbial growth may remain high. A great attention should be paid when selecting hurdles. There are more than 60 potential hurdles for foods that improve the stability and/or quality of minimally processed products. Combined stresses and enhanced exposure of bacterial cells to chemicals would result in higher lethality. However, more systematic studies on multi-synergistic effects should be conducted in real food systems (Leistner and Gould 2002).

It should be remarked that when comparing the alternative disinfection methods above reviewed should not be actually a straightforward procedure. A successful

comparison should be made on the basis of the life cycle assessment (LCA) for each of the recommended alternatives. This is necessary for being able to make a comparison in terms of not only the antimicrobial efficacy or shelf-life prolonging effects but also on the basis of their environmental impacts in the long-term (Ölmez and Kretzschmar 2009).

3.5 Conclusions

Ready-to-eat fruit and vegetable market has grown rapidly in recent years due to the health benefits associated with these foods. However, these products are very perishable and highly susceptible to deterioration. Many factors affect their shelf life and microbial quality, including good agricultural practices, good hygienic practices during harvesting and handling, quality of washing water, processing technologies, packaging methods and materials, and processing, storage, transportation, distribution, and retail sale temperatures. Common practices consist of avoiding the commodities from damage caused by poor handling or machinery and contamination.

The traditional product processing usually consists of a sequence of unit operations (trimming, peeling, cutting, washing/disinfection, drying, and packaging) and, generally, the extension of the shelf life depends on a combination of keeping a low temperature throughout the whole production chain, dips in antimicrobial and antioxidant solutions, optimal MAP conditions, and good manufacturing and handling practices. However, the fresh-cut processing industry is currently seeking ecoinnovative emerging alternatives to prolong the shelf life preserving fresh-like quality attributes. Further studies should be conducted in this field since industrial changes for replacing conventional with pioneering techniques request a fine knowledge of the benefits and restrictions as well as practical outlook and, of course, they have to be within the frame of the regulations.

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Chapter 4
Analytical Aspects for Tropical Meat Quality Assessment

Luis Artur Loyola Chardulo, Antônio Carlos Silveira, and Fabio Vianello

Abstract Meat tenderness is the most important quality attribute influencing consumer satisfaction and, therefore, meat consumption. The beef meat market in Brazil is going through transformation, and some quality traits, such as meat tenderness, begin to increase importance in the consumer choice at the time of purchase. However, even if the slaughterhouse industry applies criteria for carcass selection, in only 80 % of cases it correctly selects meat for tenderness. Consumer choice is guided by the observation of phenotypic characteristics, such as the amount of intramuscular, marbling score, and subcutaneous fats. Currently, Brazilian herds are basically composed by the Nellore breed (*Bos indicus*) and their crossbreeds, and it is very important to understand the influence of meat quality criteria on meat tenderness in Nellore cattle. Nellore breed animals possess a great capacity to accumulate subcutaneous fat. However, they do not have the ability to deposit marbling fat. Studies showed that the most important problem of Zebu breeds (*Bos indicus*) is represented by meat toughness, caused by a high activity of the calpastatin enzyme, an inhibitor of the proteolitic calpain system. The meat of zebu derived animals possess a high concentration and activity of

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calpastatin, which could explain the large variation of the meat tenderness of these animals. Animals subjected to intensive production systems in self-accelerated phases of the growth tend to present a higher meat tenderness. In this growth phase, muscle protein degradation is reduced, occurring with low protein turnover, and the activity of calpastatin is increased. It is observed that in all bovine breeds, some animals do not produce tender meat, even under favorable environmental conditions. In zebu breeds (*Bos indicus*), specifically in Nellore, the number of these animals tends to increase as they advance in age, and are slaughtered in the self-decelerating phases of growth.

Keywords Beef cattle • Carcass characteristics • Meat tenderness • Proteolytic enzymes

4.1 Introduction: The Brazilian Meat Market

Meat tenderness is a major quality attribute, which can influence consumer satisfaction and, therefore meat consumption. The demand for quality products is remarkable, since insufficient tenderness creates marketing problems for the meat industry. The beef market in Brazil, although accustomed to low trading prices, undergoes transformations, where the difficulty in the recognition of high quality products leads consumers to try new experiences, even if with a significant increase in selling prices.

The slaughterhouse industry, in turn, observes wide variations of animal standards and of fatness of carcasses at slaughter (backfat end point). Furthermore, the predictable seasonality of the quality of meat products leads to mechanisms for carcass classification, which are not always suitable as tools for predicting the final meat quality. When meat industry classifies animals, and therefore their carcasses, as of superior quality, in about 80 % of the cases, it successfully ranks meat cuts as corresponding to the quality desired by the consumer at the time of purchase.

In a recent work on zebu animals (*Bos indicus*) in several slaughterhouses in the State of São Paulo—Brazil, we observed the effects of carcass classification at the slaughterhouse as an element of prediction of meat quality. Three classes of carcasses were established before slaughter: (A) animals characterized by appropriate weight, age, and backfat end point, (B) animals characterized by suitable weight and backfat, but advanced age, and (C) animals characterized by inadequate weight and backfat and advanced age (Fig. 4.1).

The Myofibrillar Index Fragmentation, MFI, is generally used for the classification of beef tenderness from these animals (Culler et al. 1978). MFI values account for more than 50 % of the variation in meat tenderness, with high positive correlation ($rg = 0.75$) with the tenderness and negatively ($rg = -0.72$) with shear force values (Culler et al. 1978).

As it can be observed in Fig. 4.1, regardless of the selected carcass category, variations of meat tenderness may reach very high levels, even with approximately

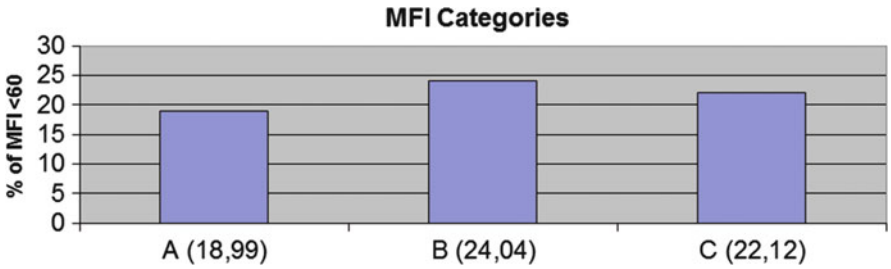

Fig. 4.1 Relationship between meat tenderness measured by the myofibrillar fragmentation index (MFI) and different carcass categories of bulls, *Bos indicus*. *Source*: Silveira Consultancy/ Nutrideal

20 % error, in the determination of this important sensory characteristics, evidencing the inability of this rating system in predicting the quality of the final product. The presence of a wide variety of genetic groups (and their crossbreeds) and production systems in Brazil are certainly among the main factors responsible for this fact.

4.2 Quality Characteristics of Tropical Meats

The main aspects to be considered at meat purchase are, in order of importance, food safety, where the origin and product branding are fundamental, type and size of cutting, coloring, fat meat content, and meat tenderness. The tenderness cannot be considered by the consumer as a limiting factor for the decision of buying the product, because there are no practical mechanisms that can ensure reliable phenotype of this feature at the time of purchase.

For the classification of meat with the higher possibility of being tender, the consumer uses to observe the most important phenotypic traits, such as meat color and cut size, and, particularly, the amount of intramuscular (marbling scores) and subcutaneous fat. These ratings are quite relevant in the choice, since the deposition of subcutaneous fat, also known as backfat end point, is of extreme importance to ensure the meat quality after cooling (Tait et al. 2005). The backfat end point, as quality parameter, reduces the impact caused by a rapid cooling of the carcass, preventing the shortening of muscle fibers (cold shortening), with the consequent lowering of meat tenderness in postmortem.

Meat marbling represents a process resulting from the deposition of intramuscular fat, and it is considered as one of the main factors influencing the consumption habits. This process gives the meat flavors and juiciness, and it actually changes the value of the final product (Killinger et al. 2004). It is also possible a positive relationship between price cuts and tenderness, which confirms the fact that this feature is a major component of consumer satisfaction (Savell and Shackelford

1992), even though the market demand for meat with less subcutaneous and marbling fats is increasing, for health reasons. Anyway, this parameter is essential to classify important meat characteristics, such as tenderness, juiciness, and flavor (Silveira et al. 2010).

Besides backfat end point of the carcass and marbling, numerous other factors such as animal age, sex, breed, pre- and post-slaughter management, enzymatic activity in muscle fibers during postmortem storage, composition, and preparation of the product by the consumer can influence meat tenderness.

4.3 The Meat Quality of Nellore Cattle

Among all zebu breeds present in Brazil, Nellore genotype outstands for its strong presence. It was first imported from zoos in Europe in 1874, as Ongole breed. These animals began their massive occupation of Brazil between 1900 and 1920, when significant breed imports were carried out directly from their country of origin, India, more specifically the Nellore region, hence its current name. Interestingly, all Ongole animals, which were exported to Brazil, came from the Nellore region. Thus, Nellore cattle found its ideal habitat in Brazil, considering the presence of vast areas of grassland, tropical weather, and short growing needs. However, all these factors did not impose this breed as one of the main protagonists in the global meat production scenario for many years. Things changed starting from the 1990s. For these reasons currently, about 80 % of animals slaughtered annually coming from Brazilian herds are basically composed of Nellore or Nellore-Zebu crossbreeds (Fig. 4.2).

Currently, problems with meat quality are evident in Brazilian production, when tenderness is considered as one of the most important meat quality characteristics for the classification of first class products (Chardulo 2000).

Some researchers reported on the existence of a negative relationship between the percentage of zebu (*Bos indicus*) and animal meat tenderness (Wheeler et al. 1994). Thus, animals with less than 25 % of zebu genotype are similar to *Bos taurus*, regarding meat tenderness. One explanation can be due to the higher levels of calpastatin showed by Nellore cattle, with respect to *Bos taurus*, which possibly results in the decrease of the flesh softness (Wheeler et al. 1990; O'Connor et al. 1997).

Thus, it is mandatory to identify the mechanisms that underlie the growth and meat quality of Nellore cattle, with the aim to identify intervention processes to improve the production of meat tenderness and high quality products. This is particularly important since these animals form the basis of Brazilian cattle, and due to their adaptability to the specific environmental conditions.

Fig. 4.2 Herd of Nellore females in a farm in the Mato Grosso (MT) state. *Source*: Silveira Consultancy/Nutrideal

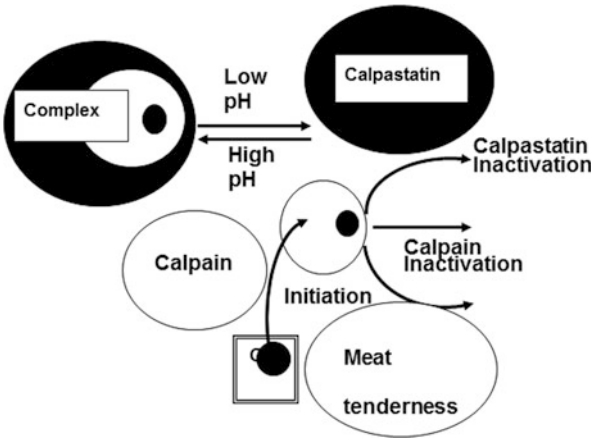

Fig. 4.3 Model for calcium activation and inactivation of calpains by calpastatin. Adapted from Dransfield (1994)

4.4 Meat Tenderness in Nellore Cattle

The calcium-dependent proteolytic system, called calpain–calpastatin, is considered as a limiting factor in the synthesis and degradation of myofibrillar proteins (protein turnover). Thus, the knowledge of the characteristics and properties of this enzymatic system should allow its modulation, which can result in changes in growth rate and degradation of myofibrillar proteins (Koohmaraie et al. 2002), fundamental factor for obtaining proper meat tenderness.

This calcium-dependent system (Fig. 4.3) has been regarded as the most important proteolytic system in cell cytosol (superior to lysosomal system) and can be characterized by the presence of three components: a proteolytic enzyme requiring low calcium concentration (*μ-calpain*), another requiring high calcium concentrations (*m-calpain*), and a specific calpain inhibitor, calpastatin (Dransfield 1994). It is noteworthy that meat tenderness is a result of the contribution of two

Fig. 4.4 Nellore cattle in feedlot in the state of Mato Grosso (MT) and carcasses with suitable fat thickness, standardized by intensive production system. *Source*: Silveira Consultancy/ Nutrideal

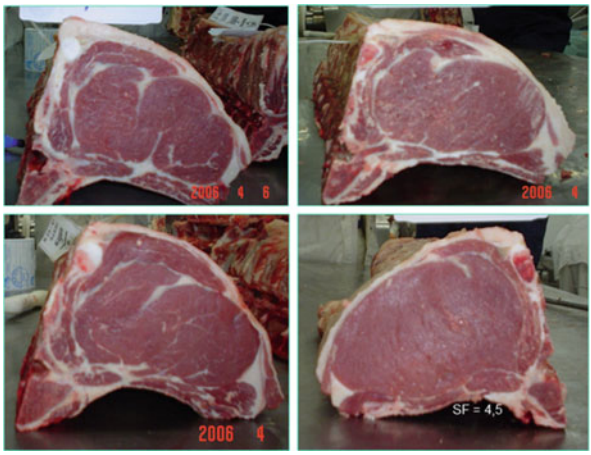

Fig. 4.5 Samples of *Longissimus dorsi* muscle in the 12th and 13th ribs from Nellore cattle (*Bos indicus*) males, showing variations in the values of shear strength, by Warner Bratzler Shear Force (SF), in animals subjected to the same production system. *Source*: Silveira Consultancy/Nutrideal

main muscle components: connective tissue and the loss of structural integrity of sarcomere, the main apparatus of skeletal muscle contraction, during postmortem period (Sawdy et al. 2004).

Zebu cattle presents high variations of calpastatin concentrations and consequently of calpain proteolytic activity (Wheeler et al. 1990; O'Connor et al. 1997; Rubensam et al. 1998). This explains the wide variations of meat tenderness characteristic observed within Nellore populations in the world. According to Morales et al. (2006), despite Nellore shows specific growth patterns when subjected to intensive production systems, thus providing quite homogeneous carcasses characteristics, large variation of meat tenderness, as measured by shear force test, was observed in these animals (Figs. 4.4 and 4.5).

In a study on Nellore cattle (*Bos indicus*) and its crossbreds with Aberdeen Angus (*Bos taurus*) animals in feedlot system for at least 120 days, Hadlich (2007) observed, despite few variations of morphological characteristics observed between the different genetic groups at slaughter age (Table 4.1), differences in meat tenderness that were evidenced when animals reached advanced ages (21 months for Nellore and 24 months for crossbred animals—50 % Nellore and 50 % Aberdeen Angus) (Table 4.2).

Table 4.1 Quality characteristics of the Nellore bulls meat (*Longissimus dorsi* between 12th and 13th ribs) and crossbreed with Aberdeen Angus (50 % Nellore and 50 % Aberdeen Angus) slaughtered at different physiologic ages

	Genotypes and slaughter ages				
	50 % Nellore and 50 % Aberdeen Angus		Nellore		
Charactersitics	13 months	21 months	15 months	24 months	Significativity
Backfat (mm)	3.30	5.80	4.17	4.20	**
Marbling[a]	2.0	2.9	1.5	1.2	*
Shear force (kg)	3.58	2.99	3.30	4.08	*

*$p < 0.05$; **$p < 0.01$

[a]Marbling: practically devoid = 2.0–2.9; traces = 3.0–3.9; slight = 4.0–4.9. Adapted from *USDA Quality Grade* 2000 (Hadlich 2007)

Table 4.2 Shear force of meat (*Longissimus dorsi* between 12th and 13th ribs) at different post-slaughter ages of Nellore bulls with 15 (NEL15) and 24 (NEL24) months of age at slaughter and Aberdeen Angus crossbreed steers with 13 (AA13) and 21 (AA21) months of age at slaughter

	Shear force (kg)			
	Time after slaughter			
Experimental treatments	24 h	7 days	14 days	Average
AA13	4.56^{aA}	3.19^{bA}	3.00^{bB}	3.58^{A}
AA21	3.26^{aB}	2.89^{aA}	2.82^{aAB}	2.99^{A}
NEL15	4.45^{aA}	2.97^{bA}	2.49^{bA}	3.30^{A}
NEL24	4.35^{aA}	4.00^{aB}	3.89^{aC}	4.08^{B}
Average	4.15^{a}	3.26^{b}	3.05^{b}	

Means followed by the same lowercase letters, in the same row do not differ ($p > 0.01$) by the Student Newman Keuls test. Means followed by the same uppercase letters, in the same column do not differ ($p > 0.01$) by the Student Newman Keuls test (Hadlich 2007)

Differences between genotypes about the morphological meat characteristics reflect variations of the genetic group, when compared to the same physiological age at slaughter, demonstrating that, even with higher fat thickness (backfat). Nellore animals showed a lower amount of intramuscular marbling fat. A typical Nellore characteristic, that is low ability to deposit fats within the muscles, can be observed.

However, considering the values of shear force (Table 4.2), small variations between Nellore and Aberdeen Angus crossbreed can be observed, especially at younger ages. Considering 7 and 14 days meat aging at 1 °C, only Nellore animals with an average age of 24 months presented shear force values higher than the crossbreeds.

These data (Hadlich 2007) evidence a typical characteristic of meat production from zebu cattle in feedlot, specifically of Nellore breed, where individuals present a higher ability to deposit subcutaneous fat at the expense of lower muscle growth, when fed with diets with high protein density and high energy.

Fig. 4.6 Nellore breed (*Bos indicus*) animals under semi-intensive systems (*left*) and feedlot production (*right*). *Source*: Silveira Consultancy/Nutrideal

Fig. 4.7 Nellore animals (*Bos indicus*) in feedlot, at 12–13 months of age, treated with diets containing 80 % of concentrated food. *Source*: Silveira Consultancy/Nutrideal

Another important point to be emphasized refers to the fact that Nellore animals do not have high capacity to accumulate marbling fat in muscles. This does not reflect on the most important sensory characteristic for the consumer, that is tenderness. However, *Bos indicus* animals, slaughtered starting from 24 months of age, begin to show higher variations of this feature, which undoubtedly increases the average meat toughness (Hadlich et al. 2006).

According to Silveira et al. (2010), the production of young animals, characterized by carcasses with good subcutaneous fat thickness, regardless if *Bos indicus* or *Bos taurus* sub-species, represents one of the key factors in determining the final product quality, especially tenderness (Fig. 4.6).

According to the same authors (Silveira et al. 2010), rapid animal growth and the consequent slaughter of young animals, with good amount of subcutaneous fat (in the 4.0–5.0 mm thickness range, measured in the region of *Longissimus dorsi* muscle between the 12th and 13th ribs), provide significant improvements in the Nellore meat quality, mainly of meat tenderness feature (Fig. 4.7).

Animals, grown in intensive production systems, when slaughtered in self-accelerated growth phase, tend to show a higher meat tenderness, confirming studies which evidenced that, when muscle protein degradation is reduced, e.g. under low protein turnover, the activity of the calpastatin is increased (Morgan et al. 1993).

It was reported that, approximately 46 % of meat tenderness variations, observed in beef cattle from several breeds, are due to genetic factors, while the remaining 54 % variations can be explained by the effect of the environment. Moreover, when

the same analysis was performed on a single breed, genetic factors explain only 30 % of variations of meat tenderness, while 70 % depended on the environment (Koohmaraie 2003).

Thus, it can be summarized that all breeds present animals which do not produce tender meat, even under favorable environmental conditions, and that in zebu breeds (*Bos indicus*), specifically in Nellore, the number of these animals increases as they advance in age and are slaughtered in the self-decelerating growth phase.

4.5 Conclusions

The wide variety of breed production systems may be an obstacle for the slaughterhouse industry in the selection of meat products characterized by high quality.

The use of animals with superior genetics, selected for muscle growth, the applications of intensive production systems, such as feedlots, and the slaughter of young animals, equipped with proper backfat end point, represent, without any doubt, some of the most accurate tools in the production of high quality carcasses of Nellore animals, leading to an increased potential for the production of tender meat and good acceptance by domestic and export markets.

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Chapter 5
Lycopene Bioavailability and Its Effects on Health

Ana Lucia A. Ferreira and Camila Renata Corrêa

Abstract Lycopene is a lipophilic carotenoid which is responsible for the red color in various fruits and vegetables and is commonly found in tomatoes. Lycopene is one of the most potent antioxidants among the dietary carotenoids mainly due to its many conjugated double bounds, and it also has the strongest singlet oxygen-quenching ability compared to other carotenoids. Besides acting as antioxidant, other mechanisms such as immune system stimulation, cell cycle regulations, gap junction communication enhancement, mutagenesis reduction, tumor cell proliferation inhibition, antitumor immune response improvement, and anti-inflammatory action have also been identified with this carotenoid. Lycopene, as a dietary source of a carotenoid, has received considerable scientific interest in several chronic diseases including cancer, cardiovascular diseases, osteoporosis, and diabetes. It is one of the major carotenoid in the diet of North Americans and Europeans. Besides tomato, lycopene is found in watermelon, guava, papaya, and apricot. The amount of lycopene in fruits and vegetables varies according to the season, stage of ripeness, variety, geographical and climatic effect, planting location, and postharvest handling and storage; in general, the more reddish the food, the greater the concentration of lycopene. The highest concentrations of lycopene are generally in the bark of food sources as compared to the pulp. Its largest concentration is found in food produced in regions with warm climates. Several factors affect the bioavailability of lycopene, such as the food processing. Ingestion of cooked tomato in oil medium increased human serum lycopene levels than consumption of unprocessed tomato juice.

Keywords Lycopene • Bioavailability • Tomato • Oxidative stress

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Table 5.1 Lycopene content of fruits and vegetables

Food	State	Concentration (μg/100 g wet weight)	References
Tomatoes	Fresh raw	2,937	Scott and Hart (1995)
Tomatoes	Fresh, cooked	3,703	Scott and Hart (1995)
Tomatoes	Sauce canned	6,205	Scott and Hart (1995)
Tomato	Concentrated canned sauce	6,500	Mangels et al. (1993)
Tomato	Fresh raw	3,100	Mangels et al. (1993)
Tomato	Canned juice	8,580	Mangels et al. (1993)
Tomato	Ketchup	9,900	Mangels et al. (1993)
Apricot	Dehydrated	864	Mangels et al. (1993)
Apricot	Canned	65	Mangels et al. (1993)
Apricot	Raw	5	Mangels et al. (1993)
Guava	Juice	3,340	Mangels et al. (1993)
Guava	Raw	5,400	Mangels et al. (1993)
Watermelon	Fresh raw	4,100	Mangels et al. (1993)
Papaya	Fresh	2,000–5,300	Ong and Tee (1992)

5.1 Introduction

Lycopene is a carotenoid that gives red color to many fruits such as tomatoes, guava, and watermelon among other foods. The lycopene amount in fruits and vegetables varies according to the season, stage of ripeness, variety, geographical and climatic effect, planting location, and postharvest handling and storage. In general, the more reddish the food is, the greater the concentration of lycopene (Table 5.1). The highest concentrations of lycopene are generally in the bark of food as compared to the pulp, and its largest concentration is found in foods produced in hot climate regions (Moritz and Tramonte 2006).

Tomato is the most abundant source of lycopene. The consumption of tomatoes has not been reported in Europe and USA until the sixteenth and eighteenth centuries, respectively. However, before the sixteenth century, the tomato was originated in Peru and Ecuador, where it was introduced and cultivated as food by the Incas. Around 1529, the Aztec emperor Montezuma offered tomatoes as a gift to a Spanish conquistador, Hernando Cortés. This act was probably responsible for the introduction of lycopene in the USA (Texas, Arizona and California), Mexico, and Europe (Gerster 1997).

Lycopene is a natural pigment synthesized by plants and microorganisms to absorb light during photosynthesis (Moritz and Tramonte 2006). It is an unsaturated symmetrical and acyclic hydrocarbon with a molecular formula of $C_{40}H_{56}$ and a molecular weight is 536.85 daltons. It occurs naturally as all *trans* form and its chain containing 13 double bonds of which 11 are conjugated (Fig. 5.1). It may undergo isomerization (Fig. 5.2) from *trans*- to mono-*cis* or poly-*cis* when exposed to high temperatures, light, oxygen, acids, and metal ions. The *trans* form

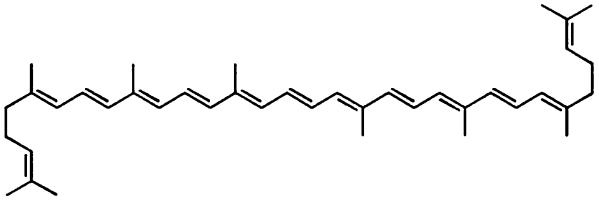

Fig. 5.1 Chemical structure of lycopene

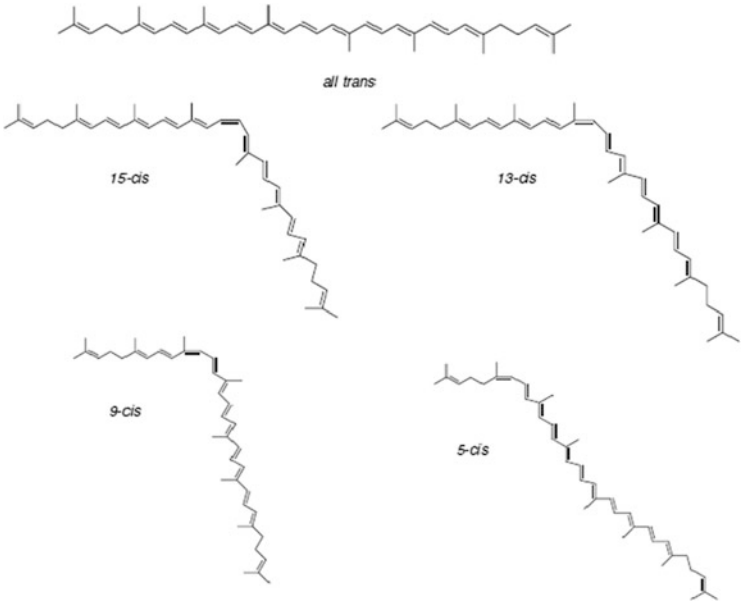

Fig. 5.2 Structures of *cis* and *trans* isomers of lycopene

is considered to be more stable and is the most common form present in foods (Rao et al. 2006).

Lycopene is a lipophilic compound with hydrophobic characteristics due to its acyclic structure and 11 linear conjugated double bonds that make it more soluble in organic solvents such as acetone, chloroform, hexane, benzene, methylene chloride, and petroleum ether (Agarwal and Rao 2000). This system of conjugated double bonds is a chromophore responsible for its ability to absorb light in the visible range, consequently by its coloring power, being responsible for the orange-red coloration of plants. At least seven conjugated double bonds are necessary for a carotenoid to be colored. When the conjugated system is extended, the color is also intensified (Rodriguez-Amaya 2002; Niizu 2003).

To be absorbed by the body, lycopene needs to be released from food, solubilized in the intestine in the presence of fat and bile acids, and incorporated into

dietary lipid micelles. It is absorbed by passive diffusion through the intestinal mucosa cell. After its uptake by cells of the small intestine, lycopene is rather carried in the plasma by low density lipoprotein (LDL) (60 %), HDL (25 %), and VLDL (15 %) (Parker 1989). It is stored in sites rich in LDL receptors (liver, testis, prostate, ovary, adrenal, and adipose tissue). The excretion of this carotenoid is performed mainly via fecal. Twelve to 33 days is the estimate period to eliminate the lycopene ingested (Rock et al. 1992).

Lycopene is present in plasma (~0.5 μmol/L) and varying amounts in human tissues. Adrenals and testes have higher concentrations of lycopene (~20 nmol/g wet tissue) (Stahl and Sies 1996). In fact, study of plasma samples and tissue obtained at autopsy showed that the highest levels of lycopene were found in the testes, adrenal glands, and liver. Brain showed carotenoid concentration below the levels of detection by HPLC (Stahl et al. 1992; Kaplan et al. 1990). Samples of human prostates obtained by prostatectomy showed 0.6 and 0.9 nmol of lycopene per g wet tissue regions in malignant and benign prostate, respectively (Kaplan et al. 1990). The authors speculated that the reason for this difference is due to the fact that tissue derived from normal prostate is metabolically less active than in cancerous tissue in uptake carotenoids from plasma (Clinton et al. 1996). A cross-sectional study examined healthy adult (12 women and 13 men) by dietary carotenoid intake, serum, and adipose tissue biopsies (abdomen, buttock, inner thigh). The carotenoid with the highest median concentration in adipose tissue was *cis*-lycopene, regardless of whether the adipose tissue was taken from the thigh, buttocks, or abdomen. Lycopene median serum concentration was 405.8 nmol/L (*trans*- + *cis*-). Moreover, serum concentrations of *trans*-lycopene were significantly correlated with their levels in buttock adipose tissue ($p < 0.02$) (Epstein et al. 2009).

Although lycopene lacks provitamin A activity, this carotenoid has attracted attention in part due its antioxidant properties (Clinton 1998; Stahl and Sies 1996; Gerster 1997; Giovannucci 1999; Rao and Agarwal 2000) by protecting against oxidative damage implicated in the pathogenesis of several human chronic diseases. However, other mechanisms such as immune system stimulation, cell cycle regulations (Rao et al. 2006), gap junction communication enhancement (i.e., increasing cell–cell communication) (Clinton 1998; Zhang et al. 1991), mutagenesis reduction, tumor cell proliferation inhibition, antitumor immune response improvement (Zhang et al. 1991), and anti-inflammatory action (Luvizotto et al. 2013; Marcotorchino et al. 2012; Bignotto et al. 2009) have also been identified.

Lycopene is one of the most potent antioxidants among the dietary carotenoids mainly due to its many conjugated double bounds (Stahl and Sies 1993), and it also has the strongest singlet oxygen-quenching ability compared to other carotenoids (Di Mascio et al. 1989). This singlet oxygen-quenching ability of lycopene is twice as high as that of β-carotene and ten times higher than of α-tocopherol (Agarwal and Rao 2000). Besides quenching singlet molecular oxygen and peroxyl radicals (Stahl and Sies 2003), strong interaction of lycopene has been shown to occur with other ROS such as H_2O_2 (Wang et al. 2004), which can generate the hydroxyl radical, known to induce membrane lipid peroxidation and DNA strand scission (Lu et al. 1995).

The toxicity of lycopene is minimal. The toxicity (as irritation reactions only) in skin and eyes was identified in Sprague-Dawley when lycopene dose was higher

than 5,000 mg/kg body wt/day. The tolerable intake (45 mg/kg body wt/day) for humans was calculated using the value of 10 as a safety factor for intraspecies differences and factor 10 for interspecies difference. Therefore, the lethal dose (LD_{50}) for humans is 45 mg/kg/day of lycopene (Matulka et al. 2004).

5.2 Bioavailability

Lycopene is not synthesized by body and therefore its levels in plasma and human tissues reflect the dietary intake. Factors that influence the bioavailability of lycopene are its release from the food matrix due to processing, presence of dietary lipids, and heat-induced isomerization from the all-*trans* to *cis* form. They all enhance lycopene absorption into the body. Other events affect the absorption of dietary lycopene including age, gender, hormonal status, body mass and composition, blood lipid levels, smoking, alcohol, and the presence of other carotenoids in the food (Rao et al. 2006; Khachik et al. 2002; McClain and Bausch 2003; Bramley 2000).

The products derived from tomatoes are the richest source of lycopene. The amount of lycopene is directly related to the ripening of tomato (Pangaribuan and Irving 2006). The absorption of lycopene appears to be higher in baked products using tomatoes and influenced by the amount of dietary fat. Furthermore, some fibers, such as pectin, can reduce the absorption of lycopene due to increased viscosity (Lugasi et al. 2003). During cooking tomato, lycopene losses are minimal. Actually, ingestion of cooked tomato juice in corn oil (1 %) for 1 h enhances significantly (2–3 times) serum lycopene as compared to unprocessed tomato juice (Stahl and Sies 1992).

Current knowledge of the bioavailability of lycopene in humans is limited due to the inability to distinguish newly administered lycopene from the body reserves of lycopene. A quantitative method to assess the absorption and relative bioavailability of newly absorbed synthetic or natural lycopene was developed using two deuterated lycopene sources, in conjunction with an advanced liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry (LC/APCI-MS) to analyze newly absorbed lycopene in blood samples of study subjects. We previously evaluated the bioavailability of lycopene (deuterium-labeled lycopene, $^{2}H_{10}$ lycopene) obtained either from intrinsically labeled tomatoes (steamed and pureed) grown hydroponically (16.3 and 17.4 μmol lycopene) or chemical synthesis (11 μmol lycopene) in humans. Our results showed that the relative bioavailability of synthetic lycopene in oil was three times more bioavailable than that from tomatoes, up to 34 days after taking $^{2}H_{10}$ lycopene dose. It is well known that tomato processing, such as cooking in oil, can increase the absorption and bioavailability of lycopene from tomato. Thus, although our preparation involved steaming the tomatoes for 10 min and giving the dose with a liquid diet containing fat, the tomatoes were not heated with oil, and this might not have been optimal for absorption (Tang et al. 2005).

The structure and physical and chemical properties of lycopene in foods determine their use by organism (Gartner et al. 1997). The bioavailability of lycopene is also related to its isomeric forms. Although lycopene is present in foods, mostly in the form of *trans*- (80–97 %), the *cis*- seems to be better absorbed and found in the human body due to its short length and its better solubility in micelles (Boileau 1999). The acidic pH of the stomach seems to contribute with small part in the conversion from all-*trans* to *cis*-isomers of lycopene. The improved bioavailability of *cis*-isomers was demonstrated in a study by Boileau (1999) who compared the bioavailability of lycopene in different isomeric forms in vivo. It was reported the presence of 52 % *cis* isomers in ferret serum 2 h after the intestine was perfused with lycopene [LycoRed in soybean oil (40 mg/kg body wt)] that contained 91 % all-*trans*-lycopene (Boileau 1999).

We have previously demonstrated (Ferreira et al. 2000) the sample preparation and animal species also can interfere on extraction efficiency of lycopene isomers. Oral treatment with 4.6 mg lycopene/(kg body wt/day) for 9 weeks resulted in the appearance of lycopene in plasma and all tissues studied in both rats and ferrets. Ferret plasma contained 11.2 nmol/L total lycopene, 33 % of which was present as *cis* isomers. With the exception of the rat testes, sample saponification resulted in a higher extraction efficiency of lycopene isomers from the tissues of both animals. All-*trans*-lycopene was the major isomer detected in the rat tissues, except for the prostate, either by saponification or by direct extraction. In marked contrast to rats, ferret tissues had predominantly *cis*-lycopene in most tissues, whereas all-*trans*-lycopene was the major isomer in the prostate and plasma. The study also showed rats absorbed lycopene more effectively than ferrets. Thus, there are species differences in the ability to absorb and store lycopene in vivo and in the ability to absorb and concentrate the various lycopene isomers in specific tissues.

There are also some indications of in vivo *trans* to *cis* isomerization reactions. Very little is known about the in vivo metabolism of lycopene. In 1996, Clinton et al. suggested the occurrence of in vivo isomerization of lycopene, since they detected higher amounts of *cis*-lycopene than all-*trans*-lycopene in human serum and in both benign and malignant prostate tissue (Clinton et al. 1996). Using the post-mitochondrial fraction of rat intestinal mucosa, we have investigated lycopene metabolism (Ferreira et al. 2003). The incubation media was composed of cofactors and lipoxygenase (soybean). The addition of lipoxygenase (LOX) into the incubation significantly increased the production of lycopene metabolites. The enzymatic incubation products of deutered lycopene ($^{2}H_{10}$ lycopene) were separated using high performance liquid chromatography (HPLC) and analyzed by UV/Visible spectrophotometer and LC/APCI-MS spectroscopy. We have identified two types of products: cleavage products and oxidation products. The cleavage products were likely: 3-keto-apo-13-lycopenone (or 6,10,14-trimethyl-12-one-3,5,7,9,13-pentadecapentaen-2-one) and 3,4-dehydro-5,6-dihydro-15,15′-apo-lycopenal (or 3,7,11,15-tetramethyl-2,4,6,8,12,14-hexadecahexaen-1-al). The oxidative metabolites are likely: 2-apo-5,8-lycopenal-furanoxide; lycopene-5, 6, 5′,6′-diepoxide; lycopene-5,8-furanoxide isomer (I); lycopene-5,8-epoxide isomer (II); and 3-keto-lycopene-5′,8′-furanoxide. Our incubation procedure produced significant amount of

cis isomers (peaks lycopene-5,8-furanoxide isomer (I) and lycopene-5,8-epoxide isomer (II)) from the original *all-trans* lycopene. Although the biological importance of these lycopene metabolites, including their *cis–trans* isomers, is still unknown, the health effect attributed to lycopene in humans may be due to the activity of some of these oxidation products (Ferreira et al. 2003). Metabolism of lycopene has also been studied in pathological conditions. We have investigated lycopene metabolism in post-mitochondrial fraction of intestinal mucosa from rats treated with doxorubicin (a chemotherapic agent; multiple doxorubicin doses; cumulative dose, 16 mg/kg body wt). As previously demonstrated (Ferreira et al. 2003), we added LOX to obtain maximum production of the metabolic products. Deuterated lycopene ($^2H_{10}$) was used with a characteristic enrichment profile that helped us to identify the lycopene cleavage products. Lycopene metabolites consisted of both enzyme-catalyzed cleavage products (3-keto-apo-13-lycopene) as well as oxidative products (2-ene-5,8-lycopene-furanoxide, *cis*-2-ene-5,8-lycopene-furanoxide, *cis*-lycopene 1,2,5′,6′-diepoxide, lycopene-5,6,5′,6′-diepoxide, *cis*-lycopene-1,6-epoxide, and lycopene-1,6-epoxide). When compared with our previous study, new oxidative products were found, such as *cis*-lycopene 1,2,5′,6′-diepoxide, *cis*-lycopene-1,6-epoxide, and lycopene-1,6-epoxide. This difference may be due to the different rat strain used. The quantification of these products revealed that, when compared to control group (rats treated with saline), intestinal mucosa from doxorubicin group had significantly higher amounts of intact lycopene and lower oxidative cleavage products, suggesting that doxorubicin may have contributed to preventing the lycopene breakdown process and therefore preserving lycopene in its intact form (Ferreira et al. 2007a). From these data, we cannot suggest that cancer patients under doxorubicin therapy should or not eat tomato products. Our results indicated that doxorubicin seems to retard lycopene metabolism (i.e., preserves $^2H_{10}$ lycopene in its intact form (all-*trans*-) and lowers oxidative cleavage products of $^2H_{10}$ lycopene). Considering that the intact lycopene has higher antioxidant activity as compared to those of its metabolites, and that there is no known toxicity of lycopene, it may be beneficial for cancer patients with doxorubicin therapy (especially in the acute treatment) to consume lycopene-rich foods such as tomatoes or tomatoes products (Ferreira et al. 2007a).

5.3 Amount of Lycopene in Food Sources

The amount of lycopene in fruits and vegetables varies according to the season, stage of ripeness, variety, geographical and climatic effect, planting location, and postharvest handling and storage. In general, the more reddish the food, the greater the concentration of lycopene. The highest concentrations of lycopene are generally in the bark of food sources, when compared to the pulp, and its largest concentration is found in food produced in regions with hot climates (Moritz and Tramonte 2006). Tomato and its derivate products, guava, watermelon, and papaya are the main sources of lycopene (Table 5.1).

Latin America has a wide variety of foods with high concentrations of different carotenoids. Besides tomato, lycopene is the predominant carotenoid in papaya, guava and red cherry. Climatic and geographical differences can interfere in lycopene amount (Rodriguez-Amaya 1999).

The concentration of lycopene of tomato also displays great variation, particularly with regard to color, ripeness, and the planting site climate. It is considered that summer generates fruit with more lycopene content than winter or spring (Stahl and Sies 1993). Studies have shown different results for the same analysis of a variety of tomatoes (*Lycopersicon esculentum*). The colors of tomato species range from yellow to orange-red due to the reason lycopene/fruit carotene. Ripe tomato contains higher amounts of lycopene *trans* beta-carotene (Giovannucci 1999). Several Brazilian vegetables show expressive content of lycopene (μg/g of food), such as canned concentrate tomato (23,500), ripe pitanga (7,600), pink guava (6,900), mamao formosa (2,600), caja pulp (560), bocaiuva (170), and acerola (70–160) (Rodriguez-Amaya 2002).

Thus, we can notice that there are a wide range in concentration, amount, and bioavailability of lycopene in foods. This carotenoid is the one which takes action by itself, not being a precursor of vitamin A. Tomato, tomato-based sauces, and its juices are the most abundant sources of this compound for human (Giovannucci 1999).

High performance liquid chromatography (HPLC) system is considered gold standard for measurement of lycopene concentration in foods, blood, and tissue. One of the most used methods of extraction was described by Riso and Porrini (1997) and chromatographic conditions were established by Yeum et al. (1996). Lycopene is a nonpolar soluble substance with a retention time 33 min in HPLC (Fig. 5.3).

5.4 Lycopene and Diseases

Given the importance of oxidative stress in the pathogenesis of chronic diseases, several therapeutic strategies using antioxidants have been tested with the aim of reducing reactive oxygen species (ROS) and nitrogen (RNS) overproduction (Ford et al. 2005). Various observational studies have shown that diets rich in fruits and vegetables are correlated with reduced risk of chronic diseases onset (Hung et al. 2008; Neuhouser et al. 2002). Thus, it is likely that antioxidant nutrients present in these foods can prevent damage caused by ROS and RNS.

There is a positive correlation between lycopene intake and health. It plays an important role in preventing several diseases, including cancers. Lycopene is the most efficient oxygen and free radicals scavenger among carotenoids. Moreover, it controls cell cycle and activates phase II detoxification enzymes. Epidemiological studies confirm its significant role in the diseases preventing (Bramley 2000). An important and prospective cohort study of 47,367 U.S. male health professionals (dentists, optometrists, osteopaths, podiatrists, pharmacists, and veterinarians), aged 40–75, were followed for 12 years. During this period, 2,481 men developed prostate cancer. Results showed that frequent tomato sauce intake was associated

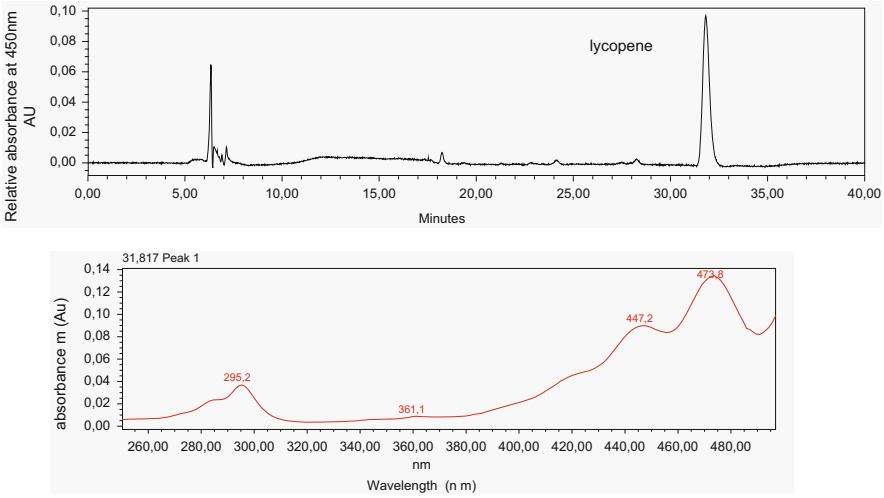

Fig. 5.3 Lycopene extracted from tomatoes by HPLC at 450 nm with its respective spectrum

with a great reduced risk of prostate cancer (organ-confined, advanced, or metastatic). Interestingly, the authors did not observe a substantial association between tomato sauce intake and risk of prostate cancer in men diagnosed when younger than 65 years. Possibly, prostate cancers presenting at an early age may represent an accelerated process of carcinogenesis influenced more by genetic or endogenous factors (Giovannucci 1999).

The relation between lycopene and cardiovascular disease has been examined in several epidemiological studies (Wu et al. 2003; Kardinaal et al. 1993; Kohlmeier et al. 1997; Kristenson et al. 1997). Lycopene protection against the oxidation of DNA bases has recently been demonstrated in cardiomyocytes from rats subjected to cardiotoxicity and supplemented with lycopene in oleoresin (Ferreira et al. 2007b). Examining men ($\leq$70 years) from ten European countries, an important study showed an inverse association between risk of acute myocardial infarction (AMI) and adipose tissue level of lycopene. The results also showed that lycopene was the only carotenoid with independent association for low risk of AMI (Kohlmeier et al. 1997). Other authors reported decreased lycopene levels in plasma from patients (men and women, average age 55 years) with dyslipidemia (total cholesterol $\geq$ 240 mg/dL; triglycerides $\geq$ 250 mg/dL) (Araujo et al. 1995). The effect of supplementation with lycopene in attenuating disease has also been examined. In men (30–35) supplemented with lycopene (60 mg/day/3 months), a study showed a significant reduction in plasma LDL. The authors also showed that the addition of lycopene to a macrophage culture resulted in decreased synthesis of important coenzyme in cholesterol [3 macrophage-hydroxy-3-methyl glutaryl coenzyme A (HMGCoA) reductase] (Fuhrman et al. 1997). Supplementation with lycopene as tomato extract (15 mg licopeno/day/8 weeks) resulted in improvement of systolic and diastolic pressure and LDL oxidation (induced by $CuSO_4^{-}$) of

patients (30–70 years) with hypertension (Engelhard et al. 2006). Another study has showed that there was no effect on DNA damage after supplementation of individual carotenoids (15 mg/12 weeks of α/β carotene, lutein, or lycopene) in men and women (25–45 years) as compared with placebo group. However, there was interesting inverse correlation between serum carotenoids and oxidized pyrimidines (Collins et al. 1998). Another study using the same amount of carotenoids adopted by Collins and collaborators (Collins et al. 1998) during shorter period (1 week) showed a significant increase in DNA repair in young women (24–34 years) after individualized supplementation with lutein, carotene, or lycopene (Zhao et al. 2003). Men and women with normal BMI who made use of Lyco-O-Mato 6 % oleoresin (5–7 mg of lycopene) for 26 days showed significant reduction in DNA damage in lymphocytes subjected to oxidative stress (Porrini et al. 2005).

An experimental study found that lycopene (at doses of 10, 30, 60, and 90 mg/kg) administered to adult hyperglycemic Sprague Dawley rats resulted in several improvements (decreased glucose and lipid peroxidation; increased insulin and antioxidant enzymes) in a dose-dependent manner (Ali and Agha 2009). The results suggest that supplementation with lycopene can contribute to attenuation of oxidative stress in this model. Adult hyperlipidemic Sprague Dawley rats supplemented with tomato powder, paste, and ketchup (10 or 20 mg lycopene/kg diet) showed improvement in all lipid parameters. In addition, this study demonstrated that the lowest dose of lycopene (10 mg/kg diet) tomato paste achieved a better atherogenic index and a significant increase in high density lipoprotein cholesterol (HDL) in these animals (Ibrahim et al. 2008). In a model of ischemia and reperfusion in the heart from adult Wistar rats was observed that lycopene decreased the damage caused by lipid peroxidation, increased the concentration of antioxidant enzymes, and improved hemodynamic parameters by suppressing oxidative stress and reducing myocardial injury (Bansal et al. 2006). In hypercholesterolemic mice was observed that the concentrate tomato juice added to the diet (20 g of lycopene/100 g diet) prevented atherosclerosis by protecting the plasma lipid oxidation (Suganuma and Inakuma 1999).

Besides acting as an antioxidant, lycopene has also been reported to display anti-inflammatory effects in adipocytes (Marcotorchino et al. 2012) and liver (Bignotto et al. 2009). Evidence is increasing that lycopene or tomato preparations can lower inflammatory markers (Hung et al. 2008; Gouranton et al. 2011; Ghavipour et al. 2012) and may improve diseases with chronic inflammatory backgrounds such as obesity (Ghavipour et al. 2012). Such anti-inflammatory role of lycopene in adipocytes was demonstrated by its inhibitory action on the transcription factor kappa B in producing pro-inflammatory cytokines (Bramley 2000).Our recent study have also showed lycopene supplementation (10 mg lycopene/kg body wt/day/6 weeks) significantly decreased leptin, resistin, and IL-6 gene expression in adipose tissue and in plasma concentrations from obese animals (Luvizotto et al. 2013), suggesting that dietary lycopene may be proposed as an effective strategy to reduce the inflammation in obesity.

Lycopene is a carotenoid that has recently received considerable attention, and it is hypothesized to play a preventative role in a variety of diseases. Although the

chemistry and in vitro properties of lycopene have been known for several years, little is known about its biodistribution, metabolism, and bioavailability in humans and its bioactivity. The products derived from tomatoes are the richest source of lycopene and ripening and cooking in oil medium are factors that enhance its bioavailability. However, current knowledge of the bioavailability of lycopene in humans is limited due to the inability to distinguish newly administered lycopene from the body reserves of lycopene. Thus, research utilizing labeled tomato will contribute to clarify the lycopene effect on nutritional modification and disease prevention. Although several questions still remain to be answered, it would be prudent to consider including dietary lycopene as part of a healthy diet.

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Chapter 6
The Postharvest of Tropical Fruits in Brazil

Patrícia Maria Pinto and Angelo Pedro Jacomino

Abstract Brazil is the largest producer of tropical fruits in the world, showing expanding production, and aiming to improve internal and external trade due to numerous growth opportunities. Besides bananas, pineapples, papayas, melons and mangoes, Brazil has a huge variety of native fruits from the Amazon and the Cerrado regions such as açaí, abiu, cupuaçu, camu-camu, and buriti. These fruits have a great marketing potential and may meet consumer interests in unique products and in foods characterized by high amount of bioactive compounds. Thus, recently, the research on fruit postharvest increased in order to improve our knowledge of the physiological and biochemical aspects of these fruits to develop appropriate techniques for fruit handling and storage.

Keywords Brazilian fruits • Production • Quality • Technology • Physiology • Preservation

6.1 Introduction

6.1.1 An Overview of Brazilian Fruit Production

With a land area of approximately 8.5 million km^2, Brazil is the third largest fruit producer in the world, following China and India, with a production of approximately 45 million tons (ABF 2012). The main fruits produced in Brazil are considered tropical (Table 6.1). Brazil produces the most tropical fruits in the world and is the largest producer of red guava and passion fruit, the second largest

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Table 6.1 Estimates of Brazilian fruit production for 2012

Fruits	Volume (tons)	Area (ha)
Oranges	19,059,890	818,685
Bananas	6,861,719	505,665
Pineapples	3,187,463	62,868
Watermelons	2,198,624	98,501
Coconuts	1,912,319	271,633
Papayas	1,854,343	35,881
Grapes	1,455,056	84,339
Apples	1,338,270	38,077
Mangoes	1,249,521	76,391
Lemons "Tahiti"	1,126,736	47,528
Tangerines	1,004,727	53,303

Brazilian Annual Report on Fruit Production (Anuário Brasileiro da Fruticultura - ABF 2012)

producer of papaya, the fourth largest producer of bananas, and the fifth largest producer of pineapple (FAO 2012).

In Brazil, the state of São Paulo is the largest fruit producer with a production volume of almost 20 million tons of fruit per year. Together with other states in Southeast Brazil, fruit production in this region accounts for 52 % of the country's production. The Northeast region is prominent for tropical fruit production, mainly because of its good weather. This region is the main production area of pineapples, bananas, cashews, guavas, mangoes, and melons, and accounts for approximately 25 % of all fruit production in Brazil. The remaining regions, the South, North, and Midwest, correspond to 13, 7, and 3 % of the national fruit production, respectively (IBRAF 2012).

Brazilian soil and climatic conditions throughout the entire territory are favorable for the commercial production of various native and exotic tropical fruits. Because fruit production requires large quantities of skilled laborers, it promotes job creation in regions where fruit production is established (Santos-Serejo et al. 2009). National fruit production plays an important role in the distribution of national income and generates approximately 4 million jobs (Sacramento and Barreto 2012). The greatest employability is in the agricultural sector, which acts to improve the quality of life in communities and creates production centers in interior regions, thus advancing urban and regional economies.

Moreover, there has been a recent increase in the economic exploration of products and by-products of various fruits because of the growing consumer concern with healthy eating (Yahia 2010). The world population is becoming aware that food is not only for nourishment but is also a source of biologically active compounds or elements that provide additional health benefits. It is understood that there is a relationship between the intake of fruits and decreased risk of developing various chronic diseases mediated by the action of free radicals (Avello and Suwalsky 2006).

Thus, the projected fruit demand indicates growth of the domestic and foreign markets. The international tropical fruit market presents numerous opportunities for growth. In 2012, fruit export amounts increased by 1.73 % compared to the

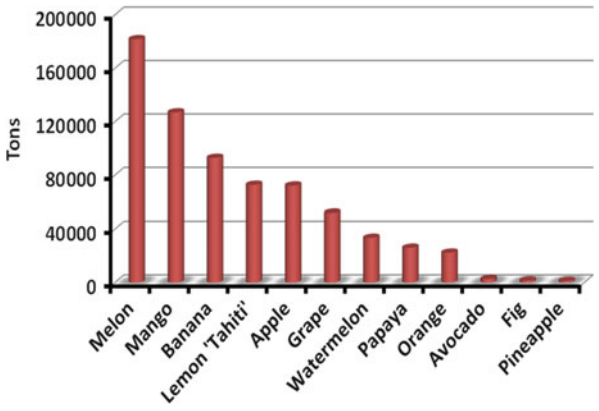

Fig. 6.1 The exportation of fruit produced in Brazil in 2012 [Brazilian Fruit Institute (Instituto Brasileiro de Frutas - IBRAF 2012)]

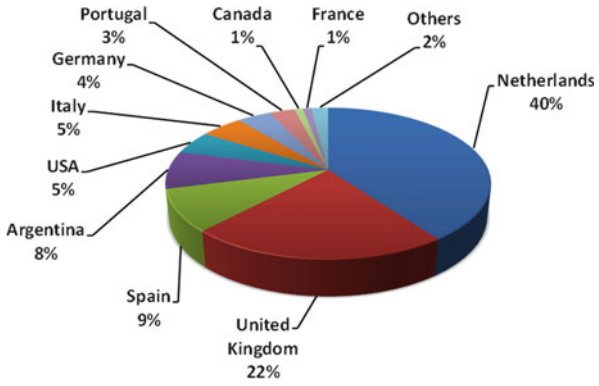

Fig. 6.2 The main destinations for Brazilian fruit exports in 2012 [Brazilian Fruit Institute (Instituto Brasileiro de Frutas - IBRAF 2012)]

previous year with an export of approximately 693,000 tons of fruit. The most exported fruit was melon, which reached approximately 182,000 tons and represented 26.22 % of the total exports, followed by mangoes, bananas, and lemons (Fig. 6.1) (ABF 2012; IBRAF 2012).

Brazil significantly exports approximately 25 different fruits to 56 nations, and the Netherlands remains a major gateway for Brazilian products in Europe. Approximately, 40 % of the total shipments in 2012 were destined for the Netherlands and then spread across the continent. The European market absorbs approximately 85 % of Brazilian exports (Fig. 6.2) (ABF 2012; IBRAF 2012).

It should be noted that the incorporation of a wide variety of unexplored tropical fruits, including caja, umbu, mangaba, camu-camu, *Annona squamosa*, soursop, and pitanga, into large-scale agriculture should constitute an excellent long-term strategy for increasing the market share occupied by Brazilian fruit production on the international market (Cardoso and Souza 2000).

However, the exportation of fruits produced in Brazil has fallen short of expectations, partially reflecting the absence of technological mastery over fruit production, particularly in relation to physiology and postharvest technology. The European Union is the world's largest importer of fruits. In 2011, 7.7 billion euros

were spent to purchase 19 of the main fruits obtained from outside the EU, according to data from the Statistical Office of the European Communities (Eurostat). According to analysts at the Center for Advanced Studies on Applied Economics (Centro de Estudos Avançados em Economia Aplicada—Cepea) of the "Luiz de Queiroz" School of Higher Education in Agriculture/University of São Paulo (Escola Superior de Agricultura "Luiz de Queiroz"/Universidade de São Paulo—ESALQ/USP), Brazil received only 6.07 % of this amount, which is equivalent to 468.9 million euros. Thus, we must identify the barriers that slow the growth of Brazilian fruit exports, mainly tropical fruits, and provide technologies that enable their cultivation, including breeding, propagation techniques, cultural practices, and phytosanitary and postharvest aspects (CEPEA 2012).

6.2 The Postharvest of Tropical Fruits

Studies on the postharvest of tropical fruit remain underdeveloped compared to those of traditionally temperate fruits; however, this scenario has been recently changing. Many studies seek to develop appropriate techniques for pre- and postharvest, such as determining the physiology of tropical fruits and ideal harvest times for each species. Furthermore, handling techniques and postharvest storage using technologies involving refrigeration, modified atmosphere, and blocking the action of ethylene, which is the hormone responsible for ripening fruits, are also being studied to reduce postharvest losses, increase commercialization, and advance tropical fruit production.

6.2.1 Physiological Aspects

The postharvest physiology of plant products has a large influence on the maturation process and maintenance of these products relative to their quality for fresh or processed consumption. Knowledge of this developmental phase of tropical fruits is significant for the provision of technical inputs that seek to increase storage time without altering physical, sensory, or nutritional characteristics.

Respiratory activity is essential in the fruit ripening process because several reactions coupled to respiration are responsible for the synthesis of compounds such as pigments and phytohormones (Purvis 1997).

The respiratory activity pattern of fruits can be divided into climacteric and non-climacteric. Climacteric fruits, such as bananas and mangoes, are characterized by an increase in respiratory activity, i.e., the production of CO_2 followed by ethylene production. In non-climacteric fruits, such as pineapples and cashews, this behavior is not observed. An increase in the ethylene concentration in climacteric fruit can occur before the increase of the internal CO_2 concentration, concomitant with the increase of CO_2 or following an increase in respiration in some fruits.

There is an acceleration of fruit ripening after the climacteric peak, which leads to senescence (Rhodes 1980, Lelièvre 1997, Chitarra and Chitarra 2005).

No stimulus is necessary to end flower bud dormancy in some tropical species, which occurs in temperate species that flower after a certain number of hours in the cold. In species such as papayas and passion fruit, flowering is subjected to the availability of climatic conditions that allow vegetative growth. Thus, these species continuously produce throughout the year. This condition of continuous harvesting leads to great variability in the quality and postharvest physiology of fruits and makes it difficult to standardize the quality throughout the year and for postharvest techniques to be adopted. Therefore, knowledge of the physiological aspects is fundamental for the preservation of tropical fruits.

6.2.2 Ideal Harvest Time

The harvest time of vegetables determines their potential for postharvest preservation and quality when offered to the consumer. This point is difficult to define in some tropical fruits, as is the case for mangoes and papayas.

The major obstacle in defining the ideal harvest time of these fruits is because of their multicolored peels. Deciding on the ideal harvest time of these fruits, which results in lower losses, must reconcile the desired postharvest shelf-life, normal ripening of the fruits, and maximum benefit of preservation techniques and processes during packaging. Some of these processes are even requirements of important markets, such as hydrothermal treatment to control fruit flies in mangoes, which is required by American and Japanese importers. Thus, the correct evaluation of fruit maturity is critical to ensure the quality and benefits of the techniques and processes used after harvest.

6.2.3 Handling and Postharvest Preservation

After harvest, quality losses increase with damage mainly caused by inadequate transportation and storage. The absence of knowledge of the physiological processes of fruits and inappropriate infrastructure and distribution logistics are the main factors responsible for the high level of postharvest losses observed in Brazil (Azzolini 2002). Postharvest losses of traditionally commercialized tropical fruits reach between 20 and 50 % of the entire amount produced. In many cases, the rate of quality deterioration is related to the modification of flavor with a loss in firmness, change in texture and appearance, and rot incidences (Kader 2002). Additionally, mild or severe losses may be because of mechanical injuries during an inadequately performed harvest, transport, or handling.

The postharvest handling practices are as important as cultural practices in the field. Many problems related to significant quality loss and food spoilage are the

result of successive and cumulative damage the fruits suffer across the supply chain. Thus, the preservation potential of a fruit is directly related to genetic factors (variety selection), preharvest environmental factors (climatic conditions and cultural practices), maturity state, harvest method, and in particular, proper handling during the postharvest, which involves preservation techniques (Chitarra and Chitarra 2005).

6.2.3.1 Preservation Techniques

Refrigeration. Refrigerated storage is the main method for preserving fruits because it reduces the intensity of vital processes using appropriate conditions, which reduces the normal metabolism and incidence of disease by inhibiting the growth of microorganisms, restricts enzyme and respiratory activity and inhibits water loss and freshness, without altering the physiology of the fruit, thus avoiding rapid deterioration (Damiani 2008).

When properly applied, refrigeration is one of the most effective methods of maintaining the quality of fruits and extending their marketing period; the function of refrigeration is to delay metabolic processes without causing physiological disorders (Awad 1993).

However, exposure to low temperatures for extended periods can lead to physiological injuries in some cases, which is common in tropical fruits stored in refrigeration (Kluge 1996). Tropical fruits are highly sensitive to the cold. Most tropical species and varieties suffer damage when stored at temperatures below 7–10 °C (Table 6.2). Thus, the benefits from cooling are limited, unlike temperate species that are stored and transported at temperatures near 0 °C.

Thus, low temperatures alone may be insufficient to delay changes in fruit quality in some cases, and it is necessary to determine other preservation methods.

Modified Atmosphere/Controlled Atmosphere. This method consists of extending the postharvest life of products by controlling or modifying the gas composition in the storage medium. The preservation of fruits in modified and controlled atmosphere conditions indicates storage in conditions in which the atmospheric composition is different from that of normal atmospheric air. Increased CO_2 and reduced O_2 levels can delay the ripening of fruits, reduce respiration activity and ethylene production, diminish water loss, and decelerate several metabolic reactions associated with senescence (Lana and Finger 2000).

In melons, in addition to refrigeration, atmosphere modification has been successfully used at exportation. Studies with bananas, lemons, and other tropical fruits have been performed to improve the marketing in domestic and, particularly, foreign markets (Table 6.3).

1-Methylcyclopropene (1-MCP). 1-MCP is a gaseous compound that blocks the action of ethylene through competition for binding sites with receptors on cell membranes and prevents their physiological stimuli (Blankenship and Dole 2003).

Table 6.2 The storage temperature conditions of various tropical fruits

Fruits	Temperature (°C)
Bananas	13
Lemons "Tahiti"	10
Mangoes	10–12
Melons	7–12
Papayas	7
Pineapples	6–10
Star fruits	9–10
Passion fruits	7–10

Adapted from Chitarra and Chitarra (2005)

Table 6.3 The recommended conditions for the storage of tropical fruits under a modified/controlled atmosphere

Fruits	Min. O_2 (%)	Max. CO_2 (%)
Bananas	5	2
Mangoes	10	3
Papayas	8	2
Lemons "Tahiti"	10	5
Pineapples	10	2
Melons	15	2

Adapted from Cantwell (2003)

1-MCP has different effects on the ripening and quality of climacteric or non-climacteric fruits.

This regulator has been demonstrated to extend the postharvest life of many fruits because of its ability to inhibit ethylene's action on various plant tissues. The beneficial effects of 1-MCP include the reduction of respiration activity and ethylene production, maintenance of the firmness and color of the peel, and extension of the postharvest life (Blankenship and Dole 2003).

1-MCP is infrequently used on tropical fruits at a large scale, although it is traditionally used on temperate fruits. However, there is potential for its use primarily because of the high perishability of tropical fruits, in which the utilization of postharvest techniques is essential (Selvarajah et al. 2001).

Some studies with guava showed the efficiency of 1-MCP as a tool for storage after harvest. In guava treated with 900 nL L^{-1} of 1-MCP for 12 h, the shelf life was greater than 10 days of storage without refrigeration (Fig. 6.3).

The application of 1-MCP on papayas has also been studied in Brazil. Research shows that 1-MCP increased the shelf life of papayas because it inhibited the action of ethylene in the fruits and delayed ripening (Jacomino et al. 2002).

The increased storage period under ambient conditions is important, considering the high perishability of papaya and other tropical fruits after harvest. A gain of 2–3 days of shelf life may permit the transportation of fruits to greater distances and expand their marketing period.

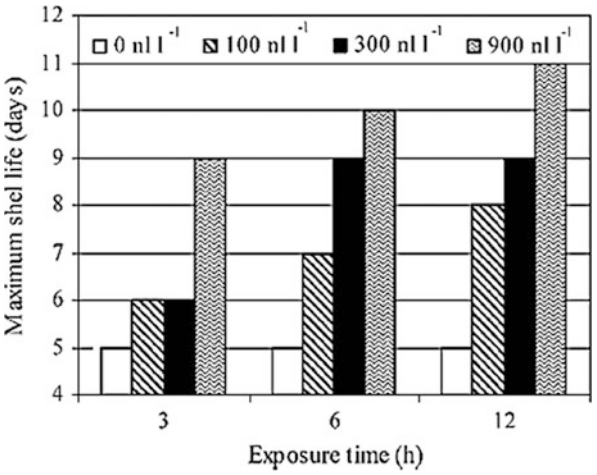

Fig. 6.3 The shelf life of guavas treated with 1-MCP and stored at 25 °C (Bassetto et al. 2005)

6.3 Export Potential

Despite the absence of knowledge on the postharvest of many tropical fruits, in which little technology has been researched, there are a large number of species with commercial potential, which is the case for fruits from the Amazon and Cerrado.

Fruit species from these typical Brazilian biomes contain high concentrations of bioactive compounds that function against free radicals (Avello and Suwalsky 2006). Nontraditional fruit species (acerola, cashews, açaí, camu-camu, among others) produced in Brazil present a significant opportunity for regional producers to conquer market niches and provide consumers with exotic products, rich in bioactive ingredients that may contribute to health.

One example is the açaí, which has boosted the economy of the Brazilian State of Pará and become the fourth most important economic activity in the state (ABF 2012). Most species of the Amazon and Cerrado are not marketed *in natura* but in the form of pulp, juices, liqueurs, and jams. Processing is necessary because of the great distances to consumer markets. However, with advancements in postharvest technology, it is possible to store these fruits *in natura* for the domestic market and particularly, the external market. For example, the camu-camu, which has the highest ascorbic acid content known, can be stored at 10 °C for 10 days without any quality loss, which enables marketing and the exportation of the fresh fruit in the future (Pinto 2012).

Another option for the exploration and marketing of tropical fruits is the minimal processing technique, which is a form of marketing that responds to changes in society's profile with the potential for exploration, particularly in European and Asian markets.

Minimally processed products are physically modified fruits or vegetables that retain their fresh state. Minimally processed fruits are products with higher value

when compared to fruit purchased *in natura*. These fruits may also present additional advantages to the consumer such as convenience and 100 % utilization of the purchased product. These fruits also have the status of fresh products and represent the response of the food industry to the modern socio-economic situation and an increase of female participation in the labor market and a reduction in meal preparation time, in which acquiring a fresh, safe, and nutritionally balanced product is emphasized. Additionally, there is an extreme practicality for fast food chains, restaurants, and various institutions to save physical space in kitchens and labor during preparation (EMBRAPA 2012).

Minimally processed fruits in Brazil remain recent but represent a growing market niche for a specific national consumer profile.

There are some foreign-funded enterprises located in Brazil that minimally process tropical fruits and export them to Europe by air using well-adjusted logistics, which allows the fruit to reach the consumer in less than 24 h after processing. This processing strategy in the country of origin provides numerous benefits compared to the exportation of the entire fruit for processing at the destination country because it allows better quality fruits to be processed, a lower processing cost, and fewer quarantine problems. However, the greatest difficulty lies in the logistics of air transport because of limited refrigerated transportation.

6.4 Final Considerations

Brazil is the world's largest producer of tropical fruit with a growing production and is seeking international commerce, which presents numerous growth opportunities. In addition to bananas, pineapples, papayas, melons and mangoes, Brazil has an immense variety of fruits native to the Amazon and Cerrado, such as açaí and camu-camu, which have great potential for commercialization and consumers interested in differentiated products rich in bioactive compounds. Thus, research on the postharvest of fruits is increasing, particularly relative to the knowledge of the physiological aspects of tropical fruits and development of appropriate techniques for handling, preserving, marketing and maintaining quality until reaching the final customer.

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Part II
Food Safety

Chapter 7
Impact of Animal Feeding on the Nutritional Value and Safety of Food of Animal Origin

Lucia Bailoni and Mirko Cattani

Abstract The quality, traceability and safety of food of animal origin are affected by several factors, but animal feeding plays one of the most important roles. The basis of the relationship between animal feeding and food quality is the carry-over of some nutrients, tracers and/or contaminants from feed to tissues and, consequently, to food (meat, milk and eggs). As regard the nutrients, an increasing number of papers report the possibility of improving the proportion of some beneficial components in products of animal origin through different dietary strategies. An enrichment of food with omega 3 fatty acids, conjugated linoleic acids, vitamin A and E and selenium could be obtained including feeds with a high concentration of these nutrients in the animal diet. In addition, some specific tracers (i.e. terpenes or volatile compounds) can be identified and quantified in products of animal origin to establish their geographic origin. Finally, as the demand for safer products is growing not only in EU, but worldwide, all actions to prevent and control the contaminants along the food chain must be implemented. The mycotoxin contamination of seeds and forages represents an emergent problem that can be worsened by the globalisation of trades and global warming. The carry-over of mycotoxins from feed to food of animal origin must be monitored to maintain the content of mycotoxins under the maximum levels established by regulation. In conclusion, animal feeding can exert a great impact on the quality, traceability and safety of food products, in order to satisfy the growing requirements of consumers.

Keywords Food safety • Food quality • Traceability • Animal feeding • Carry-over

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Abbreviations

AFB1	B1 aflatoxin
AFM1	M1 aflatoxin
CLA	Conjugated linoleic acid
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
LA	Linoleic acid
MUFA	Monounsaturated fatty acids
PUFA	Polyunsaturated fatty acids
Se	Selenium
SFA	Saturated fatty acids
SPME	Solid phase micro extraction

7.1 Animal Feeding and Quality of Food of Animal Origin

The "Quality of food of animal origin" is a highly complex concept because it involves the whole food chain, starting from the field (i.e. knowledge of pasture, forages, cereal production, etc.) to animal breeding (feeding, genetics and management), and to food processing (treatments, packaging, etc.). All production phases and operators are involved in the improvement of the food quality.

The concept of "quality" in food of animal origin can assume different meanings in relation to the stakeholders and countries, and it has been subjected to significant changes over time (Hocquette and Gigli 2005). Usually, all characteristics of food products evaluable by the consumers (i.e. nutritional traits, sensorial properties, social considerations) can be considered within the holistic and wide concept of "food quality". This chapter discusses the improvement of nutritional value of foods and, in particular, the increase in certain nutrients with beneficial effects on human health will be investigated.

7.1.1 Fatty Acids Profile in Food of Animal Origin

Animal fat contained in food is the chemical component that is more involved in human health due to its high content of saturated fatty acid (SFA). The World Health Organisation (WHO) and Food and Agriculture Organization (FAO) emphasise the need to decrease the intake of fat from food of animal origin, in order to reduce the incidence of more common pathologies in the developed countries such as obesity and cardiovascular diseases. The WHO and FAO (2003) provide data which suggest that by 2020 chronic diseases will account for almost 75 % of all

Table 7.1 Fatty acid profile (g/100 g of total fatty acids) of different food of animal origin

	Recomm.[a]	Milk	Trout	Chicken	Pork	Beef	Eggs
SFA[b]	25	63–71	28–29	33–36	36–40	41–48	45
MUFA[b]	15	23–33	41–43	32–47	43–47	43–45	37
PUFA[b]	60	4–6	29–30	20–32	13–21	7–16	18

[a]Recommended nutritional supply for human health
[b]The range of variation is due to different cuts

deaths worldwide with the vast majority being related to cardiovascular disease (CVD).

The recommended fatty acids ratio in human diets is 25:15:60 (SFA:MUFA:PUFA), but fat of milk is very high in SFA (>60 % of fatty acids).

Among meats of different species, the fatty acid profile of chicken is preferable to pork and beef. Fish products are the richest food in PUFA, and in particular in omega 3 fatty acids (about 24–25 % of fatty acids) (Table 7.1).

Some industrial processes can reduce the fat content of animal origin (i.e. milk skimming, ham trimming) but the partial or total removal of fat is not realisable for some products (e.g. cheese or egg). In addition, the lipid component of food of animal origin has a large number of substances with a bioactive role such as n-3 fatty acids (EPA and DHA acids), conjugated linoleic acid (CLA) and fat-soluble vitamins (A and E vitamins) (Givens 2010). In recent years, several attempts have been made to improve the fatty acid profile of animal origin foods by decreasing the proportion of harmful components (mostly SFA) in favour of PUFA such as omega 3 and various isomers of conjugated linoleic acids that have a beneficial effect on human health.

7.1.1.1 Omega 3 Fatty Acids

Several experiments have shown that supplementation of dairy cow diets with oilseeds rich in omega-3 fatty acids (i.e. flaxseed, rapeseed or soybean) is an effective strategy for improving the nutritional value of milk fat (reviewed by Glasser et al. 2008). However, literature reports that the effects of flaxseed on the milk fatty acid profile tend to be minimal (Kennelly 1996; Glasser et al. 2008). In this regard, Kennelly (1996) reported that the PUFA content of milk produced by cows fed flaxseed does not exceed 3–4 % of total fatty acids. Glasser et al. (2008) indicated that flaxseed promotes only slight increments of omega 3 in the content of milk (<1 % of total fatty acids). From a meta-analysis of published data, the same authors concluded that the beneficial effects exerted by flaxseed on the milk fatty acid profile are dose-dependent, as the magnitude of these effects was negligible at inclusion levels exceeding 600 g/head/day. Accordingly, a recent experiment (Cattani et al. 2013) observed that supplementation of 500 or 1,000 g extruded flaxseed/head/day led to comparable increments in the omega-3 content of milk and ripened cheese (Table 7.2). However, despite these shortcomings, flaxseed was found to be an effective source for improving the nutritional value of milk fat.

Table 7.2 Fatty acid profile (g/100 g of fatty acids) of milk and cheese obtained by cows fed different levels of extruded linseed (Cattani et al. 2013)

	Dietary treatment[a]			*P* values[b]	
	CTR	EF500	EF1000	CTR vs. EF	EF500 vs. EF1000
Milk					
SFA	72.5	72.9	71.7	0.83	0.34
MUFA	22.7	22.1	22.8	0.70	0.50
PUFA	3.59	3.93	4.29	0.09	0.20
n-6	2.74	2.80	2.98	0.31	0.31
n-3	0.30	0.52	0.61	0.03	<0.05
n-6:n-3	9.68	5.54	5.16	0.38	<0.05
Cheese					
SFA	71.4	70.4	69.6	0.32	0.53
MUFA	25.0	25.5	26.1	0.50	0.64
PUFA	3.65	4.07	4.35	0.12	0.38
n-6	2.79	2.88	2.98	0.26	0.47
n-3	0.31	0.53	0.63	0.06	0.30
n-6:n-3	9.38	5.53	4.94	<0.05	0.60

[a]CTR, control diet without extruded flaxseed; EF500, diet with 500 g/head/day of extruded flaxseed; EF1000, diet with 1,000 g/head/day of extruded flaxseed
[b]Orthogonal contrasts: CTR vs. EF500 + EF1000; EF500 vs. EF1000

Consequently, the milk of dairy cows fed with flaxseed displayed low atherogenic and thrombogenic indexes, thereby indicating low ratios between some SFA deleterious for human health (myristic, palmitic and stearic acids) and PUFA exerting health benefits as omega-3 and omega-6 fatty acids (Caroprese et al. 2010; Hurtaud et al. 2010).

Similar results were reported for sheep milk (Branciari et al. 2012). Even if flaxseed represents the most used source to improve the fatty acid profile of dairy products and meat, other oilseeds have been employed with the same scope. By supplementing cottonseed and soybean to dairy cows, some authors (Dhiman et al. 1999, 2000; Solomon et al. 2000) found positive responses on the CLA content of milk and cheese. Bailoni et al. (2004) observed that feeding dairy cows with extruded and toasted full-fat soybeans reduced the total proportion of SFA in milk and increased the total PUFA (in particular linoleic and α-linolenic acids) compared to soybean meal. More specifically, full fat soybeans reduced the proportion of palmitic acid (C16:0), which was found to be responsible for increasing cholesterol concentration in blood. Other studies reported that oilseed supplementation was also effective in improving the fatty acid profile of butter or cheese produced using milk of different species (Dhiman et al. 1999; Luna et al. 2005; Nudda et al. 2005; Gómez-Cortés et al. 2009; Hurtaud et al. 2010; Mele et al. 2011). Cattani et al. (2013) found that omega-3 and omega-6 fatty acids were efficiently recovered (>90 %) in curd during the cheese-making process, providing evidence that the supplementation of extruded flaxseed to dairy cows could represent a valid strategy for improving the nutritional quality of cheese fat.

In recent years, several studies have been conducted on beef cattle to manipulate the fatty acid composition of meat by the dietary inclusion of oilseeds. Recently, Mach et al. (2006) investigated the effects of three increasing levels (50, 80 and 110 g/kg of dry matter) of whole canola seeds and whole flaxseed on the fatty acid profile of 54 Holstein bulls and observed that the concentration of omega-3 fatty acids in the *Longissimus dorsi* muscle increased linearly with the supplementation level. Several studies investigated the effects of physical form of seeds on the fatty acid profile of milk and meat. Gonthier et al. (2005) did not observe differences when raw, micronised and extruded flaxseed were offered to dairy cows. Similarly, Raes et al. (2004), in a study conducted on Belgian Blue bulls, found that whole soybean and flaxseed (extruded or crushed) exerted comparable effects on the fatty acid profile of meat.

Regarding non-ruminant species, some recent studies showed that supplementation of extruded flaxseed could represent a promising strategy to enrich eggs (Shapira et al. 2008) and rabbit meat (Kouba et al. 2008) with omega-3 fatty acids.

7.1.1.2 Conjugated Linoleic Acids

The term "conjugated linoleic acid" (CLA) refers to a mixture of positional and geometric isomers of omega-6 essential fatty acids (cis-9, cis-12, C18:2, LA) (Kelly 2001). In ruminants, these isomers are mostly synthesised by some bacteria in the rumen as intermediate compounds of the bio-hydrogenation process, and, partly, in the mammary gland from the endogenous conversion of transvaccenic acid by Δ9-desaturase. In recent years, CLA aroused great interest in the scientific community because several in vivo and in vitro studies highlighted not only its anti-carcinogenic activity but also its anti-atherogenic, anti-obesity, anti-diabetic and immune-stimulating properties (McGuire and McGuire 1999). Cis 9, trans 11 CLA is the biologically more active isomer and accounts for 80–90 % of the total CLA present in milk or meat.

Even if food products derived from ruminant animals, milk in particular, are commonly rich in CLA (Bailoni et al. 2005), attempts are being made at further enriching their contents by means of nutritional strategies (Antongiovanni et al. 2003). The CLA content in milk or meat varies greatly from 0.1 to 2 % of fatty acids (Khanal and Olson 2004). The content of CLA in milk is mainly influenced by the amount and quality of forages. Cows fed with pasture, better if high hill pasture, produced milk with a higher content of CLA than those fed hay or silage (Bortolozzo et al. 2003) (Fig. 7.1). If pasture or fresh forages are not available, fats or fatty feeds can be added to the diet but, in order to avoid the process of bio-hydrogenation in the rumen, these supplements must be ruminally protected. The best protection is their saponification to calcium salts. As an alternative, full-fat oilseeds can be used, provided that they are adequately treated (i.e. extrusion) in order to protect lipids from rumen degradation (Bailoni et al. 2004) (Fig. 7.2).

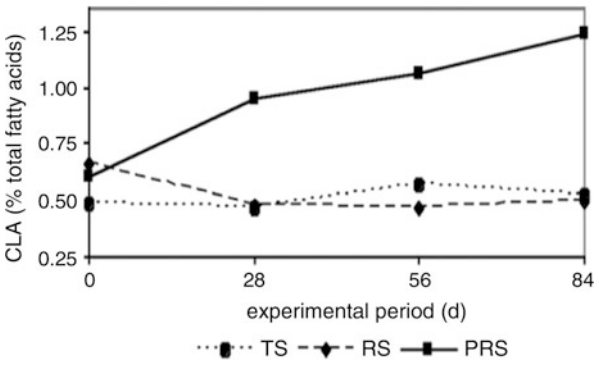

Fig. 7.1 CLA content of milk obtained by cows at pasture (PSR) and with total mixed ration with the addition of toasted (TS) or raw (RS) soybean (Bortolozzo et al. 2003)

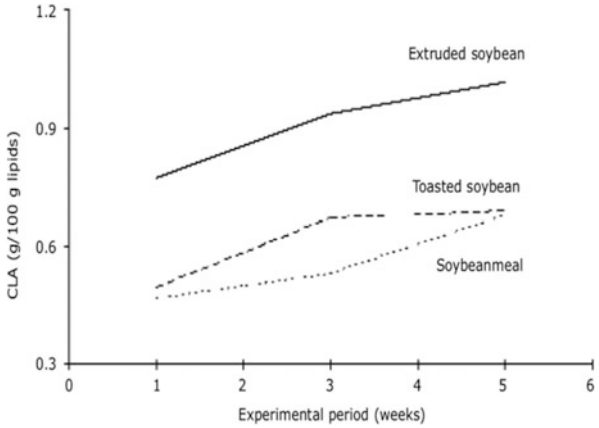

Fig. 7.2 CLA content of milk obtained by cows fed extruded or toasted soybean seeds and soybean meal (Bailoni et al. 2004)

In order to increase the CLA content of cattle meat, rumen-protected CLA can be administered directly to fattening animals. Schiavon et al. (2011) carried out an experiment on double-muscled Piemontese bulls to evaluate the effects of two rations differing in crude protein density (HP = 14.5 % DM and LP = 10.8 % DM) and top dressed or not with 80 g/day of rumen protected CLA for a long period (336 days). The authors observed that the concentrations of both cis9, trans11 CLA and trans10, cis12 CLA strongly increased in all tissues ($P < 0.01$) of bull-fed rumen-protected CLA (dosage of 80 g/day) compared to the control group (Fig. 7.3).

7.1.2 Selenium

Selenium (Se) is an essential trace element for both animals and humans. As a component of selenoamino acids (i.e. selenomethionine and selenocysteine), Se plays important roles in the maintenance of the thyroid function (WHO 2004)

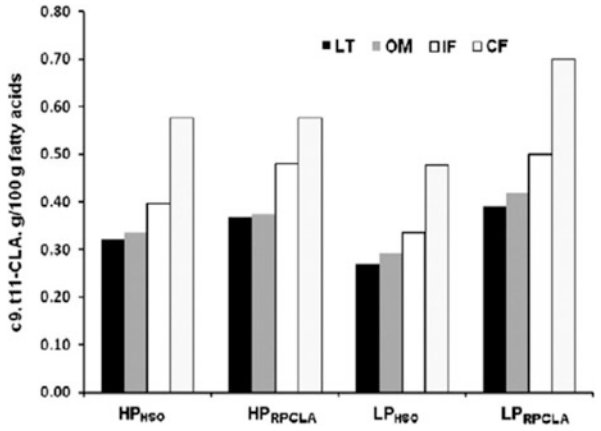

Fig. 7.3 Cis9, trans11 CLA concentration in the lipids of different tissues (LT, *Longissimus thoracis*; OM, other muscle; IF, intermuscular fat; CF, cover fat) in bulls fed two diets (HP = 14.5 % of dietary crude protein; LP = 10.8 % of dietary crude protein) and two top-dressings (HSO = 65 g/day of top dressed hydrogenated soybean oil, RPCLA = 80 g/day of top dressed rumen protected conjugated linoleic acid)

and the prevention of infertility (Ursini et al. 1999) and cancer (Corcoran et al. 2004; Whanger 2004).

Differently from fish and other seafood that are usually rich in Se, milk and dairy products are the poorest sources of Se (Matek et al. 2000). Usually, selenium concentration in milk ranges between 5 and 56 mg/l, depending on the selenium content of vegetable sources fed to animals and of soil where plants were cultivated (Underwood 1971). Recently, several studies have examined the validity of increasing the selenium content of milk by supplementing dairy cow diets with different levels and forms (inorganic or organic) of Se. Supplementation levels investigated by the literature ranged from a minimum of 0.10 mg Se/kg DM (Ortman and Pehrson 1999; Muñiz-Naveiro et al. 2006) to a maximum of 8 mg Se/kg DM (Moschini et al. 2010). Results showed that the transfer of Se from the ration to milk was nonlinear and decreased at increasing supplementation levels (Knowles et al. 1999; Moschini et al. 2010). In this regard, NRC (2001) indicates that selenium should be added to diets of lactating cows at a level of 0.30 mg/kg DM. Regarding the supplemented form, the use of organic Se (selenised yeast from *Saccharomyces cerevisiae*) for animal feeding has been recently introduced by the European Union (Commission Regulation: 2006/1750/EC). Selenised yeast was found to be rapidly effective, as a concentration of Se in milk reached the plateau only 1 week following the beginning of the supplementation period (Ortman and Pehrson 1997). Furthermore, several contributions (Conrad and Moxon 1979; Aspila 1991; Malbe et al. 1995; Ortman and Pehrson 1997; Givens et al. 2004) highlighted that organic Se is more effective than inorganic (sodium selenite or selenate) in increasing the selenium content of milk. A recent meta-analysis (Ceballos et al. 2009), considering the results of 42 different trials, reported

that, on average, supplementation with Se promoted an increment of Se concentration in milk of 13 µg/l; however, when organic selenium was used, the magnitude of this response increased up to 29 µg/l. Wu et al. (2011) outlined that the greater effectiveness of organic Se compared to the inorganic form is due to a higher availability of selenomethionine that is better absorbed by the tissues than inorganic forms. However, Weiss (2005) specified that inorganic forms should be preferred when the dietary content of sulphur, an antagonist of organic Se, exceeds 2 %.

Regarding dairy products, the literature showed the possibility of improving the selenium content of cheese, as there was a high recovery of Se in the curd during the cheese-making procedure (Knowles et al. 1999; Moschini et al. 2010).

Other studies conducted on poultry showed that egg and egg products can also be enriched with Se by adding this microelement to the diets of laying hens; as observed for milk, even in this case greater responses were achieved using organic Se (Payne et al. 2005; Skrivan et al. 2006; Invernizzi et al. 2013).

Fewer attempts have been directed at improving the selenium content of meat, especially as this food is, generally, a good source of selenium (Matek et al. 2000). Recently, Juniper et al. (2008), in a study conducted on beef cattle, found that the deposition of organic selenium was greater in the kidney (4.5–6.4 mg/kg DM) and lower in the liver, heart and skeletal muscle. Accordingly to what was described in the case of dairy and egg products, the effectiveness was higher for organic Se compared to inorganic.

7.2 Animal Feeding and Traceability of Food of Animal Origin

Traceability means the *ability to trace and follow a food, feed, food-producing animal or substance intended to be, or expected to be incorporated into a food or feed, through all stages of production, processing and distribution* (Reg. EC 178/2002). This concept of traceability is considered as a pre-condition for a successful food policy. For consumers traceability of food is a credence characteristic (Dolushitz and Engler 2005; Van Rijswijk and Frewer 2008) and is mainly associated with "food identity" in terms of its geographical origin and with animal rearing systems, with particular attention to animal welfare and the environmental impact (Kelly et al. 2005).

In this regard, several analytical tools have been recently developed to quantify specific compounds (tracers) in food and to evaluate the origin of the products or the feeding regimen of animals. These procedures are based on plant biomarkers (i.e. carotenoids, terpenes, flavonoids), metabolic markers (i.e. fatty acid profile, volatile compounds) or physical markers (i.e. isotopes of hydrogen or oxygen to assess the geographical origin and isotopes of carbon and nitrogen to evaluate the feeding regimen of animals). Multi-element and isotopic analyses have been applied to a range of foods to develop methods able to establish their geographical

origin, as summarised by Kelly et al. (2005). Other methods are being implemented to evaluate the traceability of food of animal origin using a genomic approach. In the following paragraph the use of terpenes and other volatile substances as tracers of milk and cheese origin will be discussed.

7.2.1 Terpenes

Several researches have been published on the possibility of identifying the provenance of animal origin foods, in particular cheese, through the analysis of specific chemical components. Some papers have turned their attention towards differentiating cheeses of mountain or lowland origin by examining a particular class of substances, namely the terpenes (Mariaca et al. 1997; Viallon et al. 1999). Terpenes are lipophilic aliphatic compounds present in particular herbaceous species and typical of highland pastures. Mariaca et al. (1997) identified terpenes as markers of cheese origin, in terms of altitude, by the sequence plant–animal–milk–cheese. Favaro et al. (2005) established the traceability of Asiago mountain cheese by analysing samples of herbaceous species, milk and cheese of mountain origin using the head-space solid-phase micro extraction (SPME) sampling procedure coupled with gas chromatography–mass spectrometry. Several sesquiterpenes, in particular beta-caryophyllene and beta-humulene, were found in mountain herbage, milk and cheese produced in the mountains, confirming the possibility of using these chemical compounds as suitable markers to discriminate cheese produced from animals grazing on mountain pastures. Figure 7.4 shows the presence of sesquiterpenes in milk samples obtained by grazing cows (from 49 to 54 min of retention time in the chromatograms called Laste Manazzo and Mandrielle); on the other hand, these compounds were absent in milk samples collected at plain in the same range of the retention time of the chromatogram designed at Agripolis.

7.2.2 Volatile Compounds of Milk and Cheese

In addition, Bugaud et al. (2001) found a relationship between the flavour and chemical composition of Abondance cheese and its production from animals grazing on mountain pastures. Therefore, also volatile compounds, which are responsible for the flavour of milk and cheese, can be used as markers to discriminate the origin of a food product. Bailoni et al. (2000) reported the effect of alpine pasture grazing on the flavour of milk produced by a local breed of cows (Rendena). The flavour components were determined by purge and trap techniques coupled with gas chromatography. Milk was collected in 15 farms before and after the grazing period (at plain) and during the alpine pasture (at mountain). The levels of some flavour components (exanal and dimethylsulfide) were over the perception threshold in samples collected at pasture and under this threshold in samples

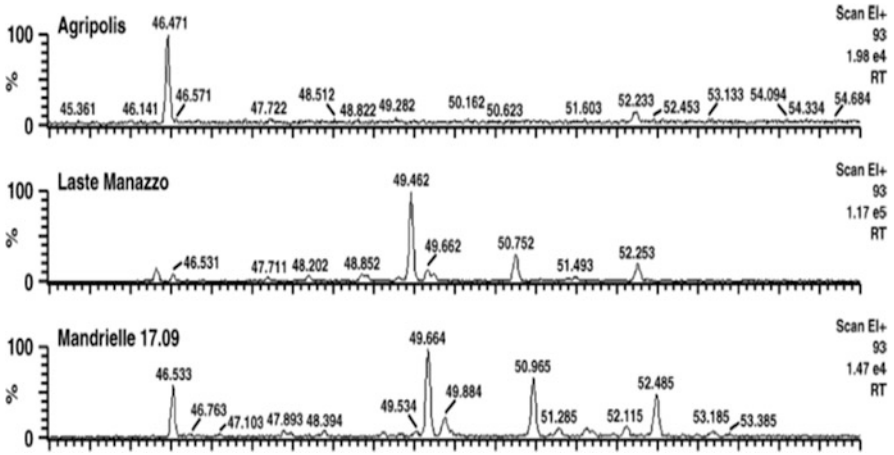

Fig. 7.4 Chromatograms of three milk samples obtained with solid-phase microextraction–gas chromatography–mass spectrometry [Agripolis = lowland sample; Laste Manazzo and Mandrielle = mountain samples (Favaro et al. 2005)]

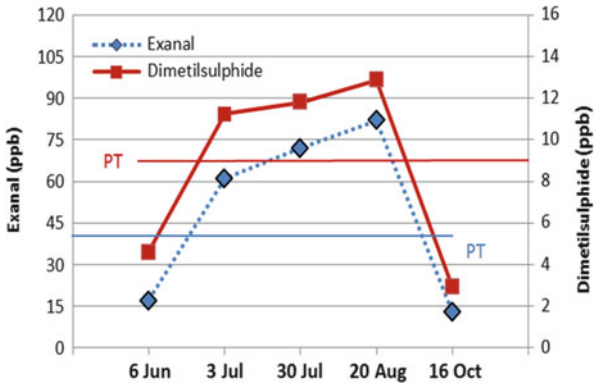

Fig. 7.5 Level of exanal and dimethyl sulphide in milk collected at mountain farms (intermediate samplings) and before and after grazing (6 June and 16 October). *PT* = perception thresholds

obtained by non-grazing cows (Fig. 7.5). These results suggest that a discrimination between milk produced on the alpine pasture and milk produced in the plain is possible using flavour components.

On the basis of previous works conducted on wine, Versini et al. (2000) proposed a new tool to characterise the volatile profile of typical alpine cheeses, using a headspace solid phase micro extraction (SPME) enrichment and gas chromatography coupled by a mass spectrometry (HRGC-MS) procedure. Figure 7.6 reports the chromatograms obtained for the "Puzzone di Moena" and "Nostrano" cheeses. Different amounts of ramified acids are present in two cheeses. This chemical profile can be used to characterise the fermentative pattern of each product and, consequently, their traceability.

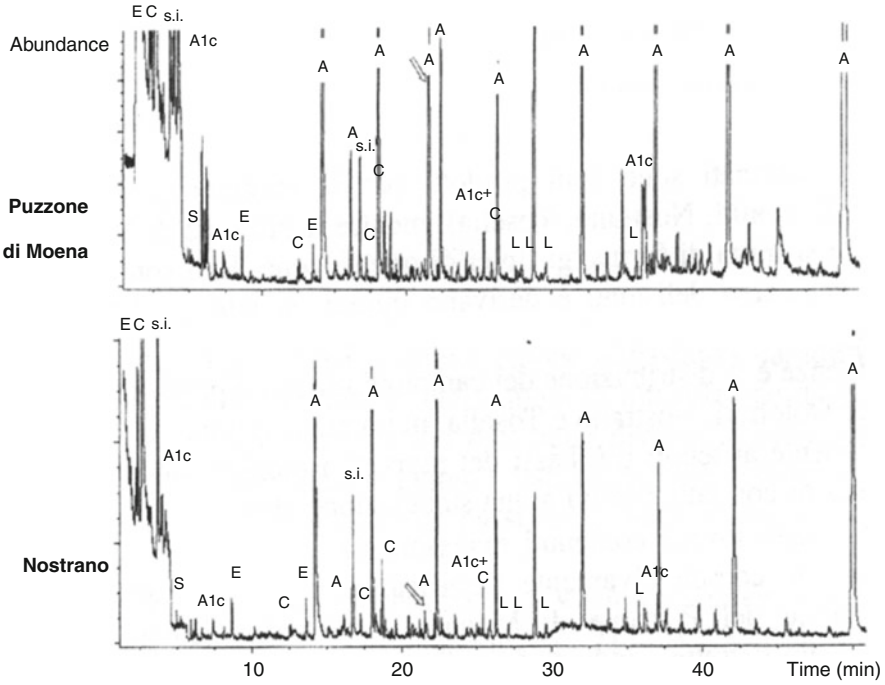

Fig. 7.6 Fatty acid profile of two cheeses (Puzzone di Moena and Nostrano)

7.3 Animal Feeding and Safety of Food of Animal Origin

Food safety refers to the absence of adverse health effects due to the presence of biological and chemical contaminants in food products. As reported in the White Paper on Food Safety (Commission of the European Community 1999), "*assuring that the EU has the highest standards of food safety is a key policy priority for the Commission*". The White Paper proposed a comprehensive and integrated approach to food safety, involving the whole food chain (from farm to fork), all food sectors (production, transport, processing, storage, etc.) and all Member States. Fourteen out of 84 specific actions involve animal feeding, indicating its relevance to guaranteeing safe products for consumers.

Contaminants can have a direct adverse effect on animal health and performance and, because of the carry-over from animal feeds to foods, they may represent a risk also for humans. In the following paragraphs the example of aflatoxin carry-over from feed to food is described.

7.3.1 *Aflatoxins in Milk*

Among all the food risks, the presence of natural toxic compounds (i.e. mycotoxins) in animal products represents an actual risk, even if their perception by the consumers is very low. The introduction of mycotoxins in the food chain is greatly determined by the ingestion of contaminated feeds by livestock and the subsequent carry-over into animal products for human consumption, particularly into milk and dairy products. At the present time, milk is the only product of animal origin subjected to a EU regulation in terms of mycotoxins and, in particular, aflatoxin M1 (AFM1).

Due to their genotoxic and carcinogenic effects, aflatoxins are considered to be the most dangerous mycotoxins for human health. Aflatoxins are produced principally by *Aspergillus flavus* and *A. parasiticus* mainly in tropical and subtropical regions where the temperature and humidity conditions are optimal for the growth of the moulds. These fungi can produce aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) on many feed products such as corn, cotton and peanuts. The AFB1 is considered to be one of most potently known natural hepatic-carcinogens for mammals. The exposure to AFB1 occurs mainly with the ingestion of contaminated feeds. Although ruminants are globally more resistant to mycotoxins than most of monogastric animals, as rumen microbes are capable of partially detoxifying mycotoxins, aflatoxin degradation in the rumen is less than 10 % for the contamination level which falls between 1 and 10 μg/ml. When absorbed by lactating animals, the AFB1 is hydroxylated and its main metabolite, the aflatoxin M1 (AFM1), is excreted in the urine, faeces and milk. The International Agency for Research on Cancer (IARC 2002) classified AFB1, AFB2, AFG1 and AFG2 (class 1) and AFM1 (class 2B) as human and possible human carcinogens, respectively. Therefore, the presence of AFM1 in milk and milk products is considered undesirable (Reg. CE n. 165/2010) and the monitoring of contamination of feeds is needed to avoid the carry-over from feed to food. For this reason, the European Commission established both maximum levels of AFM1 in milk and maximum levels of AFB1 in feeds (animal materials, complementary and complete feeds) (Table 7.3).

In European countries the environmental conditions (temperature and humidity) were not favourable to the development of *Aspergillus* (Bailoni et al. 2003; Piva et al. 2006), but some problems could originate from the use of feeds (corn, peanut meal and cottonseed meal) imported from tropical and subtropical areas. However, during the years 2003 and, more recently, 2012, a prolonged drought in the field and summer temperatures over 30 °C caused a production of AFB1 contaminated corn and, consequently, critical levels of AFM1 in milk and derivates.

The carry-over of aflatoxins from feed to milk can vary from 0.1 to 6 %, depending on several factors (milk yield, days in milk, udder infections, etc.), and it is not always predictable or measurable with a high degree of precision. Using the Veldman et al. (1992) equation, it is possible to estimate the AFM1 concentration in milk from AFB1 intake: on this basis cows ingesting an amount of AFB1 higher

Table 7.3 Maximum levels of aflatoxins in animal feed (mg/kg relative to a feed with a moisture content of 12 %; Reg. CE n. 574/2011) and in milk (μg/kg; Reg. CE n. 165/2010)

	AFB1 (mg/kg)	AFM1 (μg/kg)
Complementary and complete feed with the exception of:	0.01	
– Compound feed for dairy cattle and calves, dairy sheep and lambs, dairy goats and kids, piglets and young poultry animals	0.005	
– Compound feed for cattle (except dairy cattle and calves), sheep (except dairy sheep and lambs), goats (except dairy goats and kids), pigs (except piglets) and poultry (except young animals)	0.02	
Raw milk, heat-treated milk and milk for the manufacture of milk-based products		0.050

than 40 μg/head/day produce milk with an AFM1 content higher than legal limits of 0.050 μg/kg. In order to minimise the aflatoxin contamination of feeds, a number of strategies in the field (pre-harvest) or in storage (post-harvest) may be suggested. Aflatoxin detoxification refers only to post-harvest treatments designed to remove, destroy and ultimately reduce the toxic effects of aflatoxins (Ryley and Norred 1999). Aflatoxins are quite stable, although some physical, chemical and microbiological methods have been developed for the detoxification of feeds. Another way to reduce the effect of contaminated feed in animals is the use of mycotoxin binders. These are added to the feed with the aim of "adsorbing" aflatoxin in the gastrointestinal tract and reducing the uptake and subsequent distribution to target organs. A variety of adsorbent materials have high affinity for mycotoxins by the formation of stable linkages (activated carbon, hydrated sodium calcium aluminosilicate and some polymers).

7.4 Conclusion and Perspectives

In conclusion, animal feeding can exert a great impact on the quality, traceability and safety of products of animal origin. Regarding the quality, future perspectives could envisage the production of "functional foods" obtained by improving the transfer of some nutrients with beneficial effects from feed to food and finalised towards satisfying specific needs e.g. omega 3 fatty acids for patients with cardiovascular diseases, antioxidants for athletes, etc. Regarding traceability, the development of sophisticated analytical techniques to quantify new markers in feeds and foods will make possible to identify the origin of animal products and to provide consumers with information on rearing methods (particularly with regard to the environment and animal welfare). Finally, the increasing demand for food safety requires a greater attention to all contaminants (natural or artificial) that can be moved from feeds to animal products.

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Chapter 8
Surveillance of Anabolic Abuse in Cattle: Suitability of Transcriptomic Technologies as Screening Tools

Sara Pegolo and Clara Montesissa

Abstract The European Council Directive 23/96/EC requires the EU member States to adopt National Monitoring Plans to control the illegal use of growth promoters in beef cattle due to the potential risk for the consumers derived from the presence of hormones and drug residues. To elude official analytical controls based on the analytical residue detection, often new effective anabolic compounds are developed and used at very low doses, or several less effective are combined in growth-promoting cocktails. Anabolic steroids act on multiple organs and metabolic pathways either through primary interaction or secondary effects. Thus indirect approaches, based on the evaluation of perturbations of different biological systems, have been proposed to identify growth promoter-treated animals. Target organ histology, transcriptomics, proteomics, and metabolomics have been explored as screening tools to address the confirmative analyses. Recently, the application of transcriptomics in toxicology has experienced an impressive growth leading to the foundation of a new discipline, toxicogenomics, increasingly applied to monitor the effects of xenobiotics in non-model species. This chapter reviews the application of transcriptomic technologies for the identification of gene markers for anabolic treatments not only in experimentally treated animals but also in beef cattle commercial samples collected at the slaughterhouse. Studies including the application of quantitative real-time PCR on selected candidate markers or more comprehensive approaches based on DNA microarray or RNA sequencing to obtain a whole transcriptome signature of the treatment were considered and their performances were discussed and compared.

Keywords Cattle • Residues • Anabolic steroids • Transcriptomics • Markers • Quantitative real-time PCR • DNA microarray • RNA sequencing

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8.1 Background

The European Council Directive 23/96/EC requires the adoption of National Monitoring Plans by EU member States to control the illegal use of growth promoters in food-producing animals due to the potential risk for the consumers derived from the presence of harmful residues in meat.

To enforce the prohibition on anabolic steroid abuse, the detection of steroid or any other illicit drug residues occurring in physiological fluids (urine or blood), collected from living animals at farms, or in tissue samples (liver, muscle, kidney), collected at the slaughterhouse, requires suitable identification and confirmation methods. The survey of illegal treatment by the detection of drug residues in urine and/or liver samples is based formerly on chemical, immunochemical, or biological screening methods; then the confirmation of residues is carried out unequivocally by gas chromatography–tandem mass spectrometry (GC–MS/MS) or liquid chromatography–tandem mass spectrometry (LC–MS/MS) (De Brabander et al. 2007; Nielen et al. 2007; Andersen et al. 2008; Dervilly-Pinel et al. 2011). The detection and quantification of undeclared drugs (even if authorized) in liver and urine represent a clear proof of illegal treatment, in Italy, and are sufficient to produce judiciary conviction of farmers. Thus to elude official controls based on the analytical residue detection, new compounds, effective at very low doses, are synthesized or combined in growth-promoting cocktails at dosage even lower.

To improve the surveillance of authorized drugs illegally used as anabolics, their elimination kinetics and metabolic transformation should be studied in deep to reveal perturbations of endogenous hormones profile (Capolongo et al. 2007; Pavlovic et al. 2013) and to provide useful chemical or morphological evidence that could discriminate legal from illegal drug administration (Cannizzo et al. 2011; Vascellari et al. 2012).

The abuse of natural hormones and pro-hormones is however hard to prove since most of these substances are metabolized in vivo; their metabolites not always known and occur at similar fluctuating levels as endogenous molecules (Rijk et al. 2010).

In Italy, since 2008, gross and microscopic evidences noticed during experimental studies in beef cattle, treated with synthetic corticosteroids and β-agonists, were confirmed and adopted in surveillance plans (PNR) as indicator to address screening and confirmative analytical detection. The efficacy of the histological method has been however recently challenged because of the lack of appropriate reference material considering the evolving nature of animal-rearing practices. In addition, administration of corticosteroids leads to thymus cortical atrophy and "starry sky" appearance; however, age-associated thymus involution was evidenced in cattle as in other mammals (Pegolo et al. 2012).

Many indirect approaches, based on the evaluation of perturbations in different biological systems, further to target organ histology, have been thus proposed to identify growth promoters (GPs)-treated animals, due to the action of anabolic

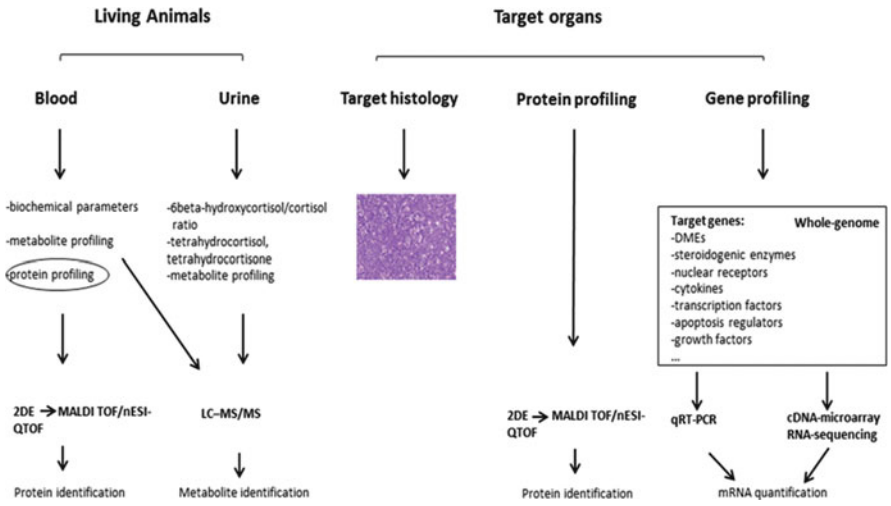

Fig. 8.1 Main strategies to trace for growth promoters administration to beef cattle

steroids on multiple organs and metabolic pathways through primary interaction and secondary effects.

Based on the physiological changes caused by these substances, transcriptomics, proteomics, and metabolomics were thus explored as screening tools to address the confirmative analyses. The application of "omic" technologies to the same samples (fluids and tissues) already adopted for chemical residue detection or histological examination will be a promising way to develop new screening methods for the surveillance of the anabolic steroid misuse (Fig. 8.1).

The term proteomics (or protein profiling) describes the study of the actual content of all proteins present in a cell, tissue, or organism at a specific physiological stage or as a reaction to a certain treatment. The use of proteomics for biomarker screening is already common in clinical diagnosis and research. Advanced methods for proteomic investigations include two-dimensional gel electrophoresis (2D gel), mass spectrometry, and protein microarrays which can be used for biomarker research. Although the proteomic approach is very promising, up to now very few publications are available in literature (Draisci et al. 2007; Della Donna et al. 2009; Stella et al. 2011).

On the other hand, metabolomics (or metabolite profiling) focuses, in an untargeted mode, on large scale and high-throughput measurement of small molecules (so-called metabolites) in biological matrices. Up to now, most of the work in metabolomics has focused primarily on clinical or pharmaceutical applications, such as drug discovery and assessment, clinical toxicology, clinical chemistry, cancer research, or food science and nutrition (Dervilly-Pinel et al. 2011).

The transcriptome is the set of all RNA molecules, including mRNA, microRNA (miRNA), ribosomal (rRNA), and transfer RNA (tRNA) produced in one cell or in a

cell population. Recently, the application of transcriptomics (or gene profiling) in toxicology has experienced an impressive growth leading to the foundation of a new discipline, toxicogenomics, increasingly applied to monitor the effects of xenobiotics in non-model species.

In this context, transcriptomics is starting to be applied to the identification of gene markers for anabolic treatments in beef meat production, thanks also to the decreasing costs of the genomic technologies.

Methods used nowadays for studying the transcriptome are quantitative real-time PCR (qRT-PCR), DNA microarrays, and RNA sequencing (RNA-seq).

Quantitative RT-PCR is used to analyze the expression of candidate diagnostic genes chosen on the basis of literature screening about the effects of GPs. It is clear however that good candidate genes are often difficult to identify and the use of single or few gene markers provides a limited and biased view of the biological response to xenobiotics. On the other hand, using either DNA microarrays or RNA-seq, it is possible to obtain whole-transcriptome expression profiles, which provide a broad and unbiased picture of the biological response to toxicants.

Microarrays, however, are not sensitive enough to measure minimal changes in gene expression, while qRT-PCR is more sensitive, its dynamic range of quantitation is much wider, it is better reproducible, and less expensive than microarray experiments. In addition, more biological samples can be measured by qRT-PCR in one experiment. On the other hand, the novel technology RNA-seq is able to detect "one single RNA molecule" and it is thereby closely as sensitive as qRT-PCR, has a higher dynamic range of expression levels respect to microarray, and almost no background signal.

In conclusion, the combination of both, identifying the most discriminant biomarker candidate genes using microarrays or RNA-seq technology and then verifying the differences in gene expression using qRT-PCR is a promising way to screen for illegal use of anabolics in beef cattle.

8.2 Transcriptomic Technologies

Transcriptomic technologies have been applied by several authors to evaluate the effects of GPs on gene expression profiles from candidate genes (qRT-PCR) or from the whole transcriptome (DNA microarrays, RNA-seq) in cattle (Table 8.1).

8.2.1 Quantitative Real-Time RT-PCR

In molecular biology, real-time polymerase chain reaction, also called quantitative real-time polymerase chain reaction (qRT-PCR) is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. For one or more specific sequences in a DNA sample, qRT-PCR enables both detection and quantification. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes.

Table 8.1 Studies evaluating the effects of different GPs in beef cattle tissues or matrices by transcriptomics technologies

Anabolic treatment	Target organ	References
• Intramuscular injections of DEX 30 mg/kg body weight twice daily for 4 days	Liver	Greger and Blum (2007)
• DEX sodium phosphate 0.4 mg/day per os for 23 days • DEX intramuscularly injected with 2 mg DEX isonicotinate 14 and 21 days far away from the onset of the oral administration	Liver	Cantiello et al. (2009)
• DEX administered either orally (a daily dose of 0.75 mg for 50 days) or intramuscularly (1.32 g, twice injected at 21-day intervals) • DEX administered orally (0.75 mg/animal for a total of 43 days) or in association with 17beta-E (20 mg, intramuscularly injected at 15-day intervals) • DHEA and ADD orally administered alone (50 mg, given once a week and for 28 days) or in combination (25 mg each, once a week for 28 days)	Testis	Lopparelli et al. (2011)
• DEX 0.75 mg/animal/day for 50 days per os • DEX 0.75 mg/animal/day for 43 days per os • DEX 0.75 mg/animal/day for 43 days per os in association with 17beta-E 20 mg/animal intramuscularly injected at 15-day intervals	Liver	Giantin et al. (2010)
• Incubation up to 24 h with 100 μM ADD, 100 μM BOLD, 10:90 μM ADD:BOLD and 100 μM DHEA (final concentrations)	Hepatocytes	Giantin et al. (2012)
• 10 mg/week of 17beta-E for 4 times • 35 mg/week of 17beta-E for 6 times • 175 mg/week testosterone for 6 times	Bulbourethral glands, prostate	De Maria et al. (2010)
• Five weekly intramuscular doses of 20 mg 17beta-E • 40 daily doses of 0.7 mg DEX per os • Revalor 200 (200 mg trenbolone acetate and 20 mg 17beta-E) for 89 days • Revalor 200 for 89 days plus 0.7 mg DEX daily per os for 40 days	Accessory glands	Divari et al. (2011a)

(continued)

Table 8.1 (continued)

Anabolic treatment	Target organ	References
• DEX 0.7 mg/day/animal for 40 days per os • PDN 15 mg/day/animal for 35 days per os	Adrenal and salivary glands, kidney, liver, lung, bulbourethral gland, prostate, testis, thoracic thymus, cervical thymus, subcutaneous fat, muscle	Divari et al. (2011b)
• DEX 0.75 mg/animal for 43 days	Neutrophils, lymphocytes	Lopparelli et al. (2012)
• Four subcutaneous injections estradiol benzoate (10 mg) and testosterone enanthate (200 mg) every 15 days for 2 months • Four subcutaneous injections estradiol benzoate (10 mg) and boldenone undecylenate (200 mg) every 15 days for 2 months	Prostate	Toffolatti et al. (2006)
• DEX administered either orally (a daily dose of 0.75 mg for 50 days) or intramuscularly (1.32 g, twice injected at 21-day intervals) • DEX administered orally (0.75 mg/animal for a total of 43 days) or in association with 17beta-E (20 mg, intramuscularly injected at 15-day intervals)	Testis	Lopparelli et al. (2010)
• 3 intramuscular injections of 10 mg 17beta-E at 17 days intervals plus 20 μg/kg CLEN per os for 40 days and 4 mg DEX per os for 6 days and 5 mg for further 6 days	Lymphocytes	Cantiello et al. (2007)
• Incubations with DEX 1, 10, 100, 1,000, 10,000 ng/ml • Incubations with chlorpromazine 0.5, 5, 50, 500, 5,000 ng/ml • Incubations with pentoxifylline 0.5, 5, 50, 500, 5,000 ng/ml	Peripheral blood mononuclear cells	Kiku et al. (2002)
• Revalor H (140 mg trenbolone acetate plus 14 mg estradiol) for 39 days	Blood	Riedmaier et al. (2009)
• Revalor H (140 mg trenbolone acetate plus 14 mg estradiol) for 39 days	Vaginal smear	Riedmaier et al. (2011)
• 0.5 mg MGA for 56 days • Finaplix H (200 mg trenbolone acetate) for 56 days • Ralgro (36 mg Zeranol) for 56 days	Uterus, liver, muscle	Reiter et al. (2007)
• Revalor H (140 mg trenbolone acetate and 20 mg estradiol) for 42 days	Liver	Becker et al. (2011)
• 0.75 mg/head DEX via feed daily for 43 days	Muscle	Carraro et al. (2009)

(continued)

Table 8.1 (continued)

Anabolic treatment	Target organ	References
• 0.75 mg/head DEX via feed daily for 43 days and intramuscular injection of 20 mg/head 17beta-E after 7, 21, and 35 days from the beginning of the treatment		
• Capsules of DHEA 1,000 mg dissolved in 10 ml Miglyol 812 for seven times at 24-h intervals • Intramuscular injections of DHEA 1,000 mg dissolved in 10 ml Miglyol 812 for seven times at 24-h intervals	Liver	Rijk et al. (2010)
• Revalor H (200 mg trenbolone acetate and 20 mg estradiol) for 68 ± 20 days	Muscle	De Jager et al. (2011)
• Unknown (putative corticosteroids to beef cattle)	Muscle	Pegolo et al. (2012)
• Revalor H (140 mg trenbolone acetate plus 14 mg estradiol) for 42 days	Liver	Riedmaier et al. (2012)

Studies have been performed to evaluate by qPCR the expression differences due to anabolic treatment in target genes chosen by screening the literature for the GPs effects in different target matrices (e.g., biotransformation enzymes, steroidogenic enzymes, nuclear receptors, cytokines, transcription factors, apoptosis regulators, and growth factors) or to analyze by a PCR array changes in the expression of regulatory RNA molecules such as the microRNA (miRNA).

8.2.1.1 Biotransformation Enzymes

Drug metabolizing enzymes (DMEs) represent a complex network of ubiquitous, oxidative, and conjugative enzymes and their main function is to detoxify xenobiotics (i.e., drugs, contaminants, and pollutants).

The finding of significant changes in the biotransformative capacity of target species could represent an important indirect effect of GPs, because of the likely influence on the efficacy of other drugs, that could be administered during the illicit anabolic treatments. In addition, a significant involvement of cytochrome P450 in the biotransformation of both steroid hormones and many commonly used drugs may result in the accumulation of potentially toxic residues in meat and tissues of illegally treated animals.

Several studies evaluated the effects of different GPs treatment on DMEs gene expression profiles in a large set of different matrices.

Greger and Blum (2007) showed that liver mRNA abundance of sulfotransferase A1 (SULT1A1) was significantly higher in dexamethasone (DEX)-treated calves compared with the control calves. In addition, mRNA abundance of cytochrome P450 (CYP) 2C8 tended also to be increased after DEX treatment. DEX,

administered per os or injected intramuscularly at growth promoting purposes, was also proved to decrease mRNA expression of CYP3A in veal calf liver (Cantiello et al. 2009). The effects of illicit protocols containing DEX and DEX plus 17beta-estradiol (17beta-E) on DME gene expression profiles were also evaluated in cattle testis and significant upregulation of cytochrome CYP1A1 and 2E1 was observed (Lopparelli et al. 2010). CYP2B6, CYP2E1, glutathione S-transferase (GSTA1A1), and SULT1A1 mRNAs were significantly modulated by two illicit protocols containing DEX (alone or together with 17beta-E) in beef cattle liver (Giantin et al. 2010). Finally, bovine hepatocytes incubated with boldenone (BOLD), its precursor boldione (ADD), dehydroepiandrosterone (DHEA), and an association of ADD with BOLD revealed significant regulation of several DMEs. In particular, DHEA-exposed cells showed an upregulation of CYP2B22 and CYP2C87. Likewise, ADD with BOLD increased mRNA levels of CYP4A11. In contrast, a reduction of CYP1A1 and CYP2E1 mRNAs was noticed in ADD- and DHEA-incubated cells. Among conjugative enzymes involved in steroid conjugation (You 2004), increasing amounts of GSTA1-like and SULT2A1-like mRNAs were only found in DHEA-exposed cells (Giantin et al. 2012).

8.2.1.2 Nuclear Receptors

Nuclear receptors (NRs) are ubiquitous transcription factors involved in the regulation of DME genes (Honkakoski and Negishi 2000). The ligand-stimulated steroid hormone receptors, such as the estrogen receptor (ER), androgen receptor (AR), glucocorticoid receptor (GR), progesterone receptor (PR), and mineralocorticoid receptor (MR), form complexes with coactivators and general transcription factors as well as recognize and bind hormone response elements in the regulatory regions of various hormone responsive genes. This prompts for modulating target gene transcription (Horie-Inoue et al. 2006).

Recently, De Maria et al. (2010) observed an upregulation of PR mRNA expression in bulbourethral glands and prostate from veal calves, after the administration of 17beta-E.

More recently, Divari et al. (2011a) verified the specificity and applicability of the PR to detect the illegal use of 17beta-E in accessory sex glands of sexually mature beef cattle. 17beta-E, either alone or in combination with other steroids, upregulated the PR gene expression, even in the absence of detectable histological changes in the accessory sex glands, confirming the high sensitivity of PR gene expression as an indirect diagnostic screening tool to detect illicit estrogen treatment in sexually mature male bovine. The same authors evaluated the effects of corticosteroids in several bovine tissues and observed a greater upregulation of the GR and MR genes followed DEX treatment in the bovine muscle tissues than in the kidney, liver, and salivary glands while upregulation of GR and MR expression following prednisolone (PDN) treatment was higher in adipose tissue than in the other tissues. The thymus seemed to respond to DEX treatment but not to the PDN one (Divari et al. 2011b). GR-alpha was also significantly upregulated both in veal

calves liver and in cattle neutrophils and lymphocytes, after DEX administration (Cantiello et al. 2007; Lopparelli et al. 2012). The combination of different GPs (consisting of boldenone undecylenate and estradiol benzoate, and of testosterone enantate and estradiol benzoate) significantly upregulated both ER and AR in prostate samples from veal calves (Toffolatti et al. 2006).

Illicit protocols containing DEX and 17beta-E showed upregulation of the peroxisome proliferator-activated receptor alpha (PPAR-alpha) and of ERα mRNA levels in cattle testis (Lopparelli et al. 2010). Some xenobiotics (i.e., fibrates and phthalates) seemed to activate PPAR-alpha which then induces the expression of enzymes involved in fatty acid oxidation (i.e., CYP4A1, Bhattacharya et al. 2005; Nakata et al. 2006). PPAR-alpha is known to play a role in the control of inflammation (Riccardi et al. 2009); furthermore, it has been shown that DEX (25 μg/kg administered once a day for 10 days) upregulates PPAR-alpha (Jalouli et al. 2003; Becker et al. 2008). Consequently, it has been hypothesized that PPAR-alpha might contribute to the anti-inflammatory activity of glucocorticoids (Cuzzocrea et al. 2008; Genovese et al. 2009; Riccardi et al. 2009).

Hepatic NRs, particularly constitutive androstane receptor (CAR), pregnane X receptor (PXR), and retinoid X receptor (RXR), are involved in the coordinated transcriptional control of genes that encode proteins involved in the metabolism and detoxification of xeno- and endobiotics. In particular, they contribute to DEX upregulation of human CYP2B, 2C, and 3A (Pascussi et al. 2000, 2003), and a dual dose-dependent mechanism of regulation (involving either GR or PXR) has been hypothesized to explain CYP3A induction (Pascussi et al. 2003; Lemaire et al. 2006; Luo et al. 2004).

A recent study examined the role of DEX on hepatic mRNA expression of CAR, PXR, and several NR target genes. For the NR examined, mRNA abundance of both CAR and PXR in DEX-treated calves was lower ($p < 0.05$) by 39 % and 40 %, respectively, than in control calves (Greger and Blum 2007). Upregulation of RXR-alpha and to a lower extent of CAR was also observed after DEX or DEX with 17beta-E administration even if only when the corticosteroid was administered per os. Moreover, a significant increase of ER-alpha and GR mRNA was pointed out in the DEX plus 17beta-E group (Giantin et al. 2010). Finally, an upregulation of AR, CAR, and PXR mRNAs was also observed in DHEA-exposed hepatocytes (Giantin et al. 2012).

8.2.1.3 Steroidogenic Enzymes

There is a consistent body of literature on comparative gene expression of steroidogenic CYPs and their modulation by xenobiotics (Fon and Li 2007; Murugesan et al. 2007; Martin and Tremblay 2008; Pogrmic et al. 2009).

It was recently demonstrated that some of the genes involved in testicular xenobiotic drug metabolism, such as genes coding for cytochrome P450, conjugative enzymes, and their related transcription factors (including GR and ER), were affected by illicit GPs in cattle testis (Lopparelli et al. 2010).

Consequently, the potential effects of GPs on steroidogenic enzyme gene expression have become a subject of interest and they have been evaluated by the same authors by qRT-PCR in cattle testis (the organ where steroidogenesis occurs and where steroids act and confer their effects). In particular, a GP-dependent effect on target gene mRNA levels was noticed for 3beta-hydroxysteroid dehydrogenase type 1 (HSD3beta 1, which leads to the androstenedione production) after DEX, DEX plus 17beta-E, and DHEA administration. The expression of cytochrome P450 side chain cleavage (P450scc, which converts cholesterol into pregnenolone) gene was also significantly affected in the DHEA plus ADD group. CYP17A1 (responsible for oxidation of pregnenolone into 17alpha-hydroxypregnenolone and its subsequent conversion into DHEA) was regulated after DEX administration even if with contrasting effects when administered per os or intramuscularly. Upregulation of HSD17beta3 and P450 aromatase (enzymes which catalyze the synthesis of testosterone and estradiol) was observed after DEX plus 17beta-E and DHEA administration, respectively (Lopparelli et al. 2011).

8.2.1.4 Cytokines

Cytokines are a large group of signaling proteins, produced and secreted by cells after cellular activation, and their production is regulated both at transcriptional and post-transcriptional levels. They can be considered as a useful indicator of the xenobiotic–target organ interaction. Nevertheless, their suitability as biomarkers has been challenged (e.g., lack of baseline data or the need of robust, standardized, and validated methods), despite the fact that many xenobiotics (i.e., paracetamol, dioxins, organotins, and cyclosporin A) were shown able to increase/decrease their expression levels (Colosio et al. 1998, 2005; Karol 1998; Foster 2001; Luster et al. 2003).

Several studies have considered the effects of 17beta-E, DEX, and clenbuterol (CLEN), given at pharmacological doses, on cytokine gene expression and/or concentration levels. Estrogens (including 17beta-E) were proved to increase IL-1alpha and IL-8, whereas their effects upon tumor necrosis factor (TNF)-alpha and interferon (IFN)-gamma depends on the dose used as well as the used cellular model (Matalka 2003; Dimayuga et al. 2005; Suzuki and Sullivan 2005). Interestingly, 17beta-E at preovulatory concentrations was shown to contribute (reverting) to the hydrocortisone-dependent depletion of TNF-alpha and IFN-gamma levels in leukocytes stimulated either with lipopolysaccharide or phytohemagglutinin (Matalka and Ali 2005).

On the other hand, DEX was proved able, even in cattle, to inhibit the IFN-gamma, TNF-alpha, and IL-8 production (Calcagni and Elenkov 2006; Djalilian et al. 2006; Elenkov 2004; Fitzgerald et al. 2007; Kiku et al. 2002; Joyce et al. 1997). In addition, Lopparelli et al. (2012) confirmed that DEX significantly upregulated TNF-alpha gene expression in neutrophils.

Also the beta-agonist CLEN is a potent suppressor of pro-inflammatory cytokines release, particularly of TNF-alpha, IL-1 alpha, and IL-6, both in vitro and in vivo (Izeboud et al. 1999a, b).

Cantiello and colleagues (2007) evaluated the effects of a cocktail of 17beta-E, CLEN, and DEX on cytokines gene expression in veal calves. A significant reduction in gene expression profiles only for IFN-gamma despite an overall reduction of cytokine gene expression profiles was recorded all throughout the experiment (IL-1beta, IL-8, and TNF-α).

These results, as a whole, confirmed that GPs concur to affect cattle immune system by modulating several immune mediators, even if a clear effect has not been yet demonstrated and needs further studies.

8.2.1.5 Gene Panel

Some studies evaluated the effects of GPs treatments on a wide panel of genes chosen by screening the respective literature for steroidal and inflammation-related effects on the specific target tissue.

Riedmaier et al. (2009) applied qRT-PCR for the evaluation of the effects of a combination of trenbolone acetate plus estradiol in blood samples from heifers at three time points. Authors proved that the GPs treatment significantly influenced mRNA expression of the steroid receptors (ER-alpha and GR-alpha), the apoptosis regulator Fas, the pro-inflammatory interleukins IL-1alpha, IL-1beta, and IL-6, and of major histocompatibility complex class II, creatine kinase, myotrophin, RNA-binding motif protein 5, and beta-actin and proposed these genes as biomarkers for this hormone combination in whole blood. These authors evaluated the effects of the same treatment on vaginal smear samples at four different time points (Riedmaier et al. 2011). Gene expression of 27 candidate markers was evaluated by qRT-PCR in vaginal epithelial cells (which are a primary steroid responsive organ). The applied anabolic combination significantly influenced the expression of the steroid receptor ER-alpha, the keratinization factor CK8, the pro-inflammatory interleukins IL1-alpha and IL1-beta, the growth factors fibroblast growth factor 7, epidermal growth factor, epidermal growth factor receptor, insulin-like growth factor receptor 1, transforming growth factor-alpha and lactotransferrin, the oncogen c-jun, and other factors, such as beta-actin and ubiquitin 3. Using biostatistical tools, such as principal components analysis or hierarchical cluster analysis, the application of a gene expression pattern was thus proposed for targeting the illegal use of GPs.

In another study (Reiter et al. 2007), uterus, liver, and muscle tissue from 24 cycling heifers were taken after the animals were treated either with Melengestrol Acetate (MGA), Finaplix-H® (200 mg Trenbolone Acetate), or Ralgro® (36 mg Zeranol) for 56 days. Gene expression was measured using qRT-PCR technology for 57 candidate genes, selected according to their action and composed to functional groups: angiogenesis, apoptosis, cell cycle, endocrine factors, energy metabolism, inflammatory factors, muscle function, oncogenes,

protein metabolism, and transcription factors. Significant differences were observed in the gene expression profiles of several target genes from the various tissues. However, even if the illicit treatments seemed to have a strong effect on the selected gene expression profiles, it is clear that using such a small number of genes it is not possible to draw conclusions about pathways and biological processes regulated by the GPs administration. More comprehensive approaches (DNA microarray, RNA seq) could be more suitable to obtain a broad signature of the biological response to xenobiotics and thus deepen the effect of illicit administration of anabolic compounds on the whole transcriptome.

Recently, a novel approach was applied by Becker et al. (2011) which analyzed the miRNA expression using PCR arrays (which allow to measure expression profiles of 730 different miRNAs) in the livers from heifers implanted with trenbolone acetate plus estradiol. miRNAs are noncoding small RNA molecules with a length of approximately 22 bp, which function as regulators of gene expression. In the liver, miRNAs have been demonstrated to be involved in several biochemical processes like proliferation, apoptosis, and glucose metabolism (Lee and Gorospe 2010; Song et al. 2010) and are also known to be targeted by anabolics (Becker et al. 2010). The influence of estradiol on liver miRNA such as miR-27b, miR-103, and miR-98 was also evidenced (Bhat-Nakshatri et al. 2009). The authors found a significant upregulation of miR-29c and miR-103 and a downregulation of miR-34a, miR-181c, miR-20a, and miR-15a. A trend toward proliferation and cell growth as well as a lower insulin responsivity of the liver could be demonstrated on the basis of transcriptional changes, confirming that the expression of miRNAs could be influenced by the application of exogenous hormones.

8.2.2 DNA Microarray

A DNA microarray (also commonly known as DNA chip) is a collection of microscopic DNA spots attached to a solid surface. DNA microarrays are applied to measure the expression levels of large numbers of genes simultaneously or to genotype multiple regions of a genome. Each DNA spot consist of a specific DNA sequence, known as probe (or reporters or oligo). These can be a short sequence of a gene or other DNA element that is used to hybridize a cRNA (also called anti-sense RNA) sample (called target) under high-stringency conditions. Probe–target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target.

Microarray analysis has been used so far to examine the effects of anabolic hormones in experimentally treated animals, as in the case of skeletal muscle samples from bulls administered with DEX and DEX plus 17β-estradiol (Carraro et al. 2009). Data analysis demonstrates that the expression profiles were strongly affected by DEX treatment with hundreds of genes upregulated with relevant fold change, whereas only seven genes were downregulated including the myostatin

gene. On the contrary, the number of differentially regulated genes was lower in response to the addition of estradiol to the DEX treatment. Differentially regulated genes were analyzed to describe the effects of these treatments on muscle physiology, highlighting the importance of specific pathways (e.g., Wnt or cytokine signaling) and cellular processes (e.g., cell shape and motility).

Rijk and colleagues (2010) analyzed by DNA microarray the livers from beef cattle after experimental treatment with DHEA. When comparing the gene expression profiles of per os and intramuscular treated animals to that of all control animals, the number of significantly regulated genes was 23 and 37, respectively. Among these, some genes encoded for proteins with poorly known or unknown function, while a consistent number were involved in immune response.

De Jager et al. (2011) evaluated the effects of the association of trenbolone and estradiol in bovine *longissimus dorsi* muscle. Gene expression profiles were determined by microarray and 121 differentially expressed were found. Among these, a decrease in expression of a number of fat metabolism-associated genes, likely reflecting the lipid storage activity of intramuscular adipocytes, was observed. The expression of insulin-like growth factor 1 (IGF1) and genes related to the extracellular matrix, slow twitch fibers, and cell cycle (sex determining region Y-box 8, a satellite cell marker) was increased in the treated muscle. Surprisingly, a very large 21- (microarray) to 97 (real-time quantitative PCR)-fold higher expression of the mRNA encoding the neuropeptide hormone oxytocin was observed in treated muscle.

Finally, Pegolo et al. (2012) successfully applied this technology for the first time to unknown samples collected at commercial slaughterhouse to screen for potential illicit treatment with corticosteroids. Unsupervised analysis of gene expression profiles showed a highly significant distinction between two groups, one including positive controls and a subset of commercial samples, the other comprising all negative controls and the remaining unknown individuals. The observed separation was confirmed by a two-class Significance Analyses of Microarray (SAM) test that identified over 3,900 differentially expressed genes and a class prediction approach that was able to discriminate between the two groups using just two genes, using as a training set positive and negative controls and as test set all unknown samples. Functional annotation of up- and downregulated transcripts showed several biological processes and molecular pathways that have been already reported in previous proteomic and transcriptomic studies to be altered upon controlled administration of low dosage corticosteroids (e.g., sarcomere proteins, myosin isoforms, and genes involved in ion channel activity). In addition, interesting regulation of several olfactory receptors (ORs) was observed and seemed to confirm what was recently reported by Griffin et al. (2009) who suggested that ORs might have a relevant role in myogenesis and muscle regeneration.

Despite a more accurate evaluation of the global effects of GPs on gene expression, the DNA microarray technology is sensitive to bias due to the experimental procedure that can affect the gene expression profiles and lead to draw erroneous conclusions about the differences observed. So, several control points

and filtering steps needed to be included to ensure correctness and accuracy of the results. High quality, unbiased, and reproducible gene expression data are always desirable in any DNA microarray experiment, but when the aim is to apply transcriptomics for the identification of illicit use of steroid hormones, data quality becomes essential for obvious reasons.

8.2.3 RNA-Seq

RNA-seq, also called "whole transcriptome shotgun sequencing" (WTSS), refers to the use of high-throughput sequencing technologies to sequence cDNA in order to get information about a sample's RNA content. The technique has been widely adopted in studies of diseases, such as cancer. With deep coverage and base-level resolution, next-generation sequencing provides information on differentially expressed genes, including gene alleles and differently spliced transcripts, noncoding RNAs, post-transcriptional mutations or editing, and gene fusions (Maher et al. 2009). Another advantage of this novel technology is the possibility of de novo detection for the identification of new mRNAs or new splice variants of expressed genes (Costa et al. 2010).

The potentiality of this novel technology for holistic gene expression analysis to discover illicit GPs administration in beef cattle was explored in a pioneer study by Riedmaier et al. (2012), which evaluated the effect of trenbolone acetate plus estradiol on gene expression in liver from Nguni heifers. The expression of 40 selected candidate genes was verified via RT-qPCR and significant regulation was found for 20 of these. Biostatistical tools for pattern recognition were applied and resulted in a clear separation of the treatment groups using these putative biomarkers, showing the potential of RNA-seq to screen for biomarker candidates to detect the abuse of anabolics.

8.3 Concluding Remarks

The results obtained from the reported studies are promising, though restricted to the application of transcriptomic technologies as screening methods to complement the existing ones against the illegal use of GPs in meat production. It is clear, however, that the relative small number of animals used during experimental studies often do not allow to draw accurate conclusions, because the putative biomarkers could be influenced by various factors (e.g., breed, age, and diet).

The adoption of a different overall approach like meta-analysis to analyze all the data from transcriptomic studies carried out on the use of GPs in beef cattle could thus represent a further tool to identify biomarkers less influenced by intrinsic or extrinsic variables.

A more complex approach could be to combine the results from transcriptomic, metabolomic, and proteomic studies to relate differential gene expression to protein and/or metabolite variations. To our knowledge no studies dealing with that integrated approach were published, but it is likely that to find reliable biomarkers for the screening of different classes of GPs administered illegally to food-producing animals aiming to guarantee consumer safety, that is the right direction to follow.

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Chapter 9
The Dairy Products, Nutritional and Legal Value, an Opportunity for the Defense and Promotion of the Territory

Enrico Novelli

Abstract The dairy products are still food source of an undisputed nutritional value; in addition to calcium and phosphorus other so-called minor components from the point of view of quantity are the object of attention for their potential health benefits. Besides the traditional medium and long aging products, typical of the Italian cheese manufacture, other dairy products are now available with high service content or endowed with particular sensory traits. In the countries of the Mediterranean basin there is an ancient cheese tradition witnessed today by many cheeses that had a legal label whose significance is the protection of the origin and/or the production technology.

The traceability of these products is essentially a documentation process; however, currently available analytical advanced methods are capable, with reasonable certainty, of attributing the origin of the milk to a well-defined geographical area or to a specific mode of production. This is of practical importance not only to protect consumers but also to ensure fairness and transparency competition in the market among different brands. Food fraud, today strongly recurrent, finds also in the dairy sector several opportunities of application with not indifferent good volumes implicated and large economic values at stake. However, not infrequently an ordinary commercial fraud translates into a healthcare fraud with relapses also of considerable gravity for the consumer.

Of particular interest are the latest technological developments dedicated to the processing of milk in small production aimed at minimizing the environmental impact of the traditional large-scale production and provide income opportunities to those who work in marginal geographical areas.

Keywords Analytical methods • Consumer protection • Dairy products • Geographical indication • Protected designation

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9.1 Introduction

The dairy production in Italy has a tradition that goes back a long way. At an archaeological site near Piadena (CR) in Lombardy was found a perforated clay bowl dating back to 1500 B.C. the use of which is likely due to the draining of the curd. The Romans were capable manufacturers and consumers of cheese other than skilled breeders. The dissemination of this tradition by Romans also among the people of Central and Northern Europe is evidenced by the current wording of cheese and dairy art that in many European languages have common roots in the Latin words *caseus* and *formaticum* (Salvadori del Prato 2001).

The cheese-making tradition has been handed down over the centuries accompanied by a gradual adaptation of the technologies to consumer preferences, the quality of milk, and the climatic conditions. The uniqueness of the productive environment typical of many dairy products is now legally protected by a mark of origin (PDO, PGI, TSG). The Italian dairy production counts little less than 40 different types of cheese with PDO mark spread over almost the entire country.

The cheeses with PDO mark, due to their intrinsic characteristics and their link with the land, are the ones that convey most the tradition and local biodiversity. The mark of origin is applied only to those products for which the entire production process, including the supply of raw materials, takes place in a defined geographical area by which they result in a unique and specific link between cheese and territory. The geographical area includes both natural factors, such as climate and environment, and human factors, such as production techniques and craftsmanship, which allow to create an inimitable product outside a specific production area.

The DOP (Protected Designation of Origin) as well as PGI (Protected Geographical Indication) represents a guarantee for the consumer under the Reg. (EC) n. 510/2006. Its adhesion requires compliance with production rules and to be subject to control by independent certification bodies and supervision by the Regions and the Ministry.

There are in Italy nearly 40 different cheeses with PDO whose total production volume in 2011 amounted to just over 490,000 ton. Five cheeses over the total represent almost 86 % of production, among them Grana Padano (36 %) and Parmigiano-Reggiano (27 %) both cooked cheeses, Gorgonzola (10 %) veined soft cheese, Asiago (4.6 %) semi-cooked cheese, and Mozzarella di Bufala Campana (7.6 %) the so-called pasta filata cheese obtained with the exclusive use of buffalo milk (CLAL).

Compared with this PDO cheese production, we remember that in 2010 the production of cow's milk in Italy amounted to 110×10^6 hl, the production of buffalo milk was 2×10^6 hl, that of sheep was 3.6×10^6 hl, and finally goat's milk was 9×10^5 hl (Bozzetti 2011).

Each cheese with PDO mark has its own rules of production where it is stated a detailed description of the geographical area of origin, with specific reference to the location of the dairies but also for the farms where milk is produced, to guarantee the exclusive use of milk of local production. In the case of cheese made with milk

from animal species different from that of the cow, such as Buffalo mozzarella (exclusive use of buffalo milk), or Pecorino cheese that is obtained with only sheep's milk or even mixed cheeses such as "Casciotta of Urbino" obtained from sheep's milk (70–80 %) and cow's milk (20–30 %), it is clear that this aspect assumes also relevant legal value for the authenticity of the product.

9.2 The Authenticity of the Cheese

The Product authentication within the food sector is of interest not only for consumers protection, but also for producers and retailers. Indeed, regulatory authorities, food processors, retailers, and consumer groups are all interested in ensuring that foods are correctly labeled. With the European harmonization of the agricultural policy and the growing of the international markets, authentication of such foodstuffs attracts more attention. This trend is the result of efforts made by regional authorities, as well as producers to protect and support local productions (Karoui and De Baerdemaeker 2007). The quality of milk plays a very important role in the production of all types of cheeses, affecting both cheese yield and characteristics of the cheese (Summer et al. 2003). Among productive factors, few of them are considered very important by cheese-makers, among these animal feeding. In this regard, grass of natural highland pastures presents a highly diversified botanical composition, which may influence milk and then cheese quality. The relationships between the origin of cheeses and the type of pasture have been intensively highlighted using chromatographic techniques, isotope ratio mass spectrometry, and chemical analysis. Bugaud et al. (2001) have found that the proportion of mono- and polyunsaturated fatty acids determined by gas chromatography was higher in mountain milks than in valley milks. Analytical methods usually employed to discover fraud are aimed to determine one or more marker in a suspect cheese and a subsequent comparison of the data obtained with those established for equivalent material of known provenance (Downey 1996). This approach is getting always more complicated considering the increasing number of analytes that must be considered in any test procedure and the scarce knowledge of the range of each constituent.

It must be considered that the chemical methods require sophisticated analytical equipments and skilled operators; they are also time-consuming and, often, expensive. For all these reasons, there is a continuing demand for new, rapid, and relatively cheaper methods for direct quality measurement in food and food ingredients. Spectroscopic techniques, including the near-infrared (NIR), mid-infrared (MIR), front face fluorescence spectroscopy (FFFS), stable isotope, and nuclear magnetic resonance (NMR), could be profitably used for the determination of the quality and/or geographical origins of dairy products (Karoui and De Baerdemaeker 2007).

The authenticity of a complex food product such as the cheese involves a wide variety of topics like the geographical origin of forage resources used to feed livestock (that the specification rules generally lead back to the local area, at least

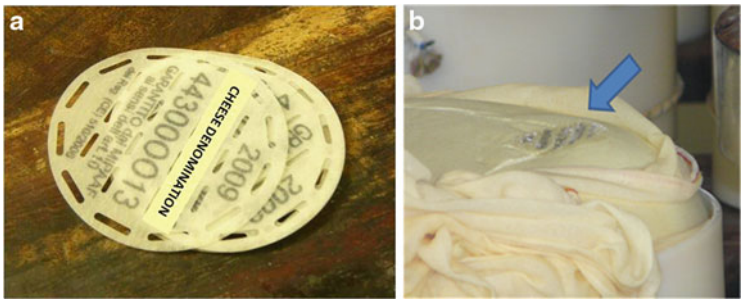

Fig. 9.1 (**a**) Example of casein plate for wheel identification and traceability; (**b**) casein plate already fixed on the cheese surface after 24 h from milk coagulation

for a large percentage) and the geographical location of the barn, but also the hygiene of the milk, the welfare of livestock, and the microbial biodiversity of the processing environments where milk is transformed in cheese. The Reg. (EU) N. 178/2002 has stated the traceability process to trace the origin of a food product or an ingredient of a food with the specific purpose of consumer's health protection in the case of recalling foods already in the market. Traceability is essentially a documental procedure well organized and codified conducted through the collection of data regarding the technological process, the raw materials, and ingredients used. For example, in the case of the cheese is now frequent the use of a casein plate (Fig. 9.1a, b) that is affixed to the surface of the cheese when curd is poured into the rind band, after that it becomes inextricably linked to the crust during maturation.

This plate carries the identification codes of the form and any other information about the production lot (origin of the milk, salt, rennet, cooking temperature, etc.). In addition, the rind band gives some brands or characters identifying the product to protect their origin even if it will be sold in portions (Fig. 9.2a, b).

More complicated is to determine the authenticity of a grated cheese where there is no longer any signs or identification code. These activities of portioning and grating therefore must be done under the supervision of the Consortium of Protection in order to prevent fraud of substitution or adulteration of the product.

9.3 The Age of the Product (the Ripening Period)

In certain cheeses the specification rules consider the duration of the period of ripening and therefore the cheese age as an element of quality. For example, the specifications for the Asiago cheese provide that the product ripened for 4/6 months can be labeled as "mezzano" and the ripening for more than 10 months has been labeled as "vecchio" whereas the indication of "stravecchio" is due to the cheese ripened for more than 15 months. Each of the three categories of ripening competes

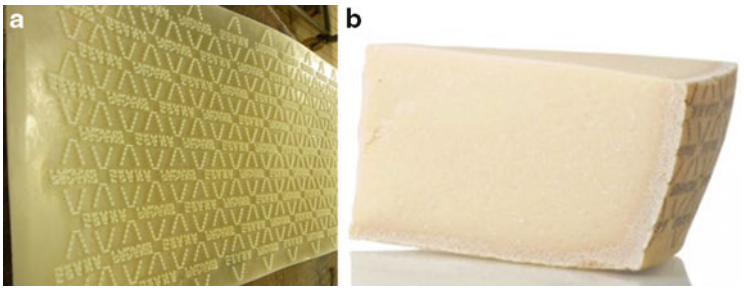

Fig. 9.2 (**a**) Inside face of the plastic ring band commonly used for fresh curd forming; (**b**) cheese after portioning where the Consortium mark is still visible on the external crust

its own commercial value as well as a specific sensory characteristic. From a chemical point of view the age of a cheese, within certain limits, can be established through the determination of the so-called maturation index which is nothing else that the percentage ratio between the soluble nitrogen and total nitrogen. This ratio generally increases with the duration of the maturation period, but must obviously be drawn a reference scale for each type of cheese. In any case, this analytical approach in the face of an high accuracy is time-consuming and its operating costs are not compatible with frequent controls. The chemometric approach of discriminant analysis (OPLS-DA) of the data obtained by ^{1}H NMR of a water-soluble extract of Parmigiano-Reggiano aged for 14, 24, and 30 months (Fig. 9.3) was efficiently employed for the correct identification of the samples as a function of the maturation period (Consonni and Cagliani 2008). The authors attributed the discriminant effect to the increase of threonine and the decrease of leucine during ripening.

A different approach to the study of cheese aging is that showed by Ottavian et al. (2012) for the Asiago d'allevo cheese using the NIR technique. Also for this approach the chemometric application results with a satisfying discriminant ability (Fig. 9.4), whereas Fig. 9.3 shows the NIR spectral data used for chemometrics analysis in the range between 1,000 and 2,500 nm. The authors found that the separation of samples of 6 and 36 month ripening's age was strongly related to the values of water activity, dry matter, moisture, ash (w.w.), fat (w.w.), protein (w.w.), and proteolysis index, together with some fatty acids (especially C14:1, C18:3 n-6, C18:2 c12-t10, C20:0, C20:1 n-9, and C20:3 n-6 and n-3 for the 6 months samples, C20:4 n-6 and EPA for the 36 months samples).

Similar results were reported also by Collomb et al. (2001). The fact that the water content influenced the classification of only 6 and 36 months samples can be seen also from the raw spectra of Fig. 9.5, which shows that the mean spectra of 12 and 18 months samples did not show major differences at the 1,900 nm wavelength. The most influential wavelength regions were those around 1,700 and 2,300 nm, together with peaks at 1,210, 1,400, and 1,900 nm. Absorption at 1,400 and 1,900 nm was ascribed to the water content (first overtone of the O–H stretch close to 1,400 nm and combination bands of the asymmetric and scissor stretch O–H vibrations close to 1,900 nm). The absorption bands at 1,220, 1,700, and

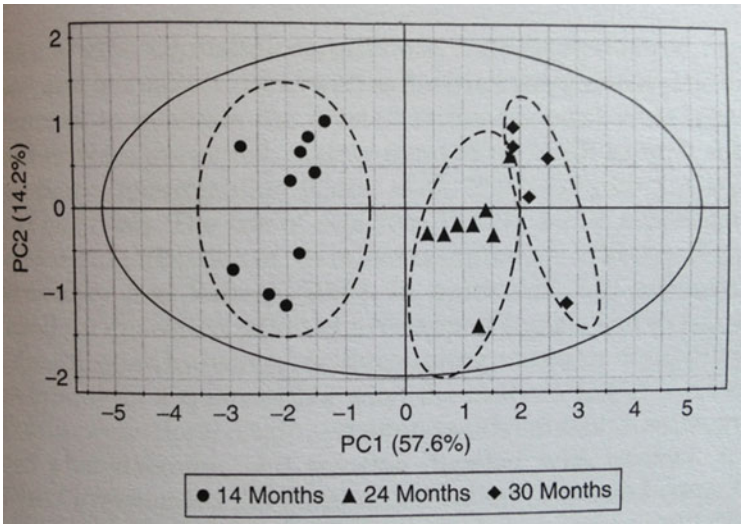

Fig. 9.3 O-PLS score plot performed by considering 23 Italian samples of different ripening stages of Parmigiano Reggiano (from Consonni and Cagliani 2008)

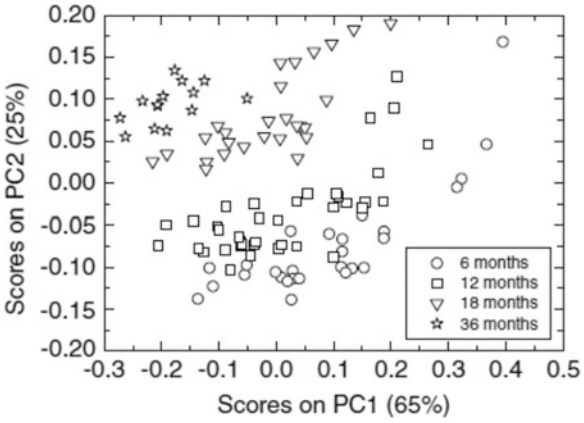

Fig. 9.4 Asiago d'allevo cheese. Discrimination according ripening age of 6, 12, 18, and 36 months. Exploratory PCA analysis on the NIR spectra pretreated with standard normal variate and first-order derivative (Ottavian et al. 2012)

2,300 nm were related to the lipid components and resulted from the second and first overtone of the C–H stretch, the combination bands of the C–H stretch, and the deformation of the CH_2 group, respectively. It should be noted that NIRS correctly captured the complex modification of the water–matrix interaction (due to water migration and to a lower extent of salt diffusion) that occurs during cheese maturation.

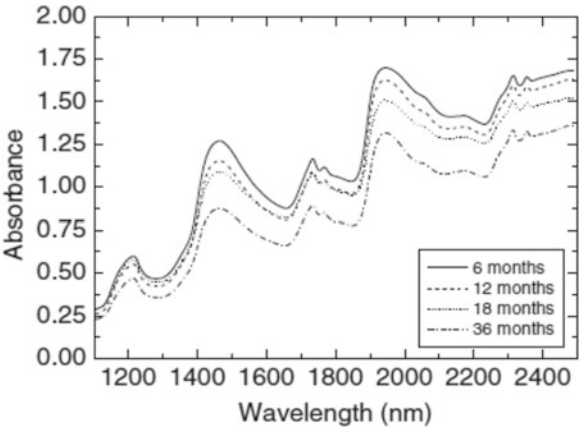

Fig. 9.5 Asiago d'allevo cheese. Discrimination according ripening age of 6, 12, 18, and 36 months. Raw mean spectra for the four ripening time (Ottavian et al. 2012)

9.4 The Geographical Area of Cheese Production

All specification rules for cheese with PDO or PGI report the accurate indication of geographical area of production. In some cases the geographical area of origin other than in terms of latitude and longitude is also specified in terms of altitude above sea level.

9.4.1 Altitude of Cheese Production

This specification has the clear objective of protecting, if not encouraging, the use of lands often in geographical contexts rather marginal that they would not have other perspectives other than the use as pasture for livestock production (milk and sometimes also meat production). Milk obtained with basic feeding of fresh forage has distinctive quality requirements such as being rich in unsaturated fatty acids, fat-soluble vitamins, and often much more pleasant even by a sensory point of view. Its use for cheese processing imparts a distinctive added quality to the end product, not only by sensory viewpoint but also in nutritional one. The specification rule of the Asiago d'allevo contemplates an additional mark called "Product of the mountain" reserved for the cheese whose production area is situated at an altitude not less than 600 m above sea level Schievano et al. (2008) applying chemometric analysis to NMR data (^{1}H and ^{13}C) observed a good discrimination of alpine cheese from lowland and mountain industrialized ones whereas the lowland and mountain industrialized cheeses were not distinguishable (Fig. 9.6). The reason for this bias is attributed by the authors to the fact that in both types the same feeding rationing is used (forage/concentrate ratio 60:40) where in the Alpine farm this ratio was 80:20. The discriminating chemical variables were the unsaturated fatty acids for the alpine cheeses and the saturated fatty acids for the industrialized farms.

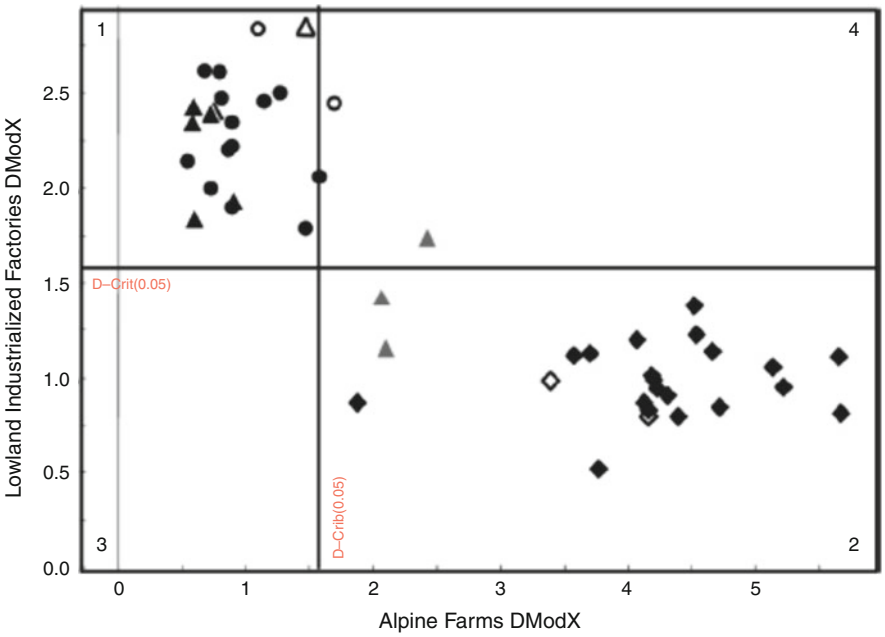

Fig. 9.6 Coomans' plot: *upper left corner* (*1*), lowland industrialized factories area; *lower right corner* (*2*), alpine farm area; *lower left corner* (*3*), common area of the two models; *upper right corner* (*4*), area of exclusion from either model. Training set: (*filled diamonds*) alpine farms; (*filled circles*) lowland industrialized factories; (*filled triangles*) mountain industrialized factories, winter production; (*gray triangles*) mountain industrialized factories, summer production. Test set: (*open diamonds*) alpine farms; (*open circles*) lowland industrialized factories; (*open triangles*) mountain industrialized factories (Schievano et al. 2008)

Instead, Ottavian et al. (2012) have sought to discriminate cheese production performed at different altitudes of pasture (from 1,000 up to 2,000 m a.s.l.), where in each case the same food system was adopted (more fresh forage and little concentrate). The application of chemometric (PLS-DA) to NIR spectra was just relatively able to differentiate cheeses made at different altitudes (in a few cases the sample attribution to the correct class failed). Obviously it was more reliably to distinguish the cheeses from farms in plain made according to methods of intensive feeding of dairy cattle from those obtained according to milk production on pasture (extensive).

9.4.2 Latitude of Cheese Production

The main elementary constituents of the organic matter are present in various stable isotope forms whereas the lightest ones are the most abundant (Table 9.1). The

Table 9.1 Percentage abundance of stable isotopes

Element	Stable isotope	Abundance (average %)
Hydrogen	^{1}H	99.99
	^{2}H	0.015
Carbon	^{12}C	98.89
	^{13}C	1.108
Nitrogen	^{14}N	99.63
	^{15}N	0.366
Oxygen	^{16}O	99.76
	^{17}O	0.038
	^{18}O	0.204

isotopic ratios are expressed in delta per thousand (‰δ) in relation to international chemical standards, according to the following general formula:

$$\delta(‰) = (R_s/R_r - 1) \times 1,000$$

where R_s and R_r are the ratios of the heavier isotope (less abundant) to the lighter one in the sample and in the reference, respectively (Matges et al. 1990).

The ratios of stable isotopes could be a useful analytical strategy for the authentication of dairy products as there are several region-specific patterns in environmental isotopic ratios (soil, water). Isotopes, like trace elements, are embedded in feeds used for animal feeding and then in the body of the animals. Therefore, these ratios may be specific for those geographical areas. The ratios of hydrogen ($^{2}H/^{1}H$) and oxygen ($^{18}O/^{16}O$) isotopes in the body are primarily influenced by beverage water. The isotopic ratio $^{2}H/^{1}H$ and $^{18}O/^{16}O$ of natural water depends on environmental factors that exert differential effects on the evaporation/condensation cycle. One consequence of these effects is that their ratio in meteoric water decreases with increasing latitude, the altitude, and, in the same region, with increasing distance from the sea (Figs. 9.7 and 9.8). Even isotope ratios $^{2}H/^{1}H$ in the various molecular sites of natural products depend on geographical and meteorological factors as well as specific metabolic processes responsible for their biosynthesis. The knowledge of the isotope ratio $^{2}H/^{1}H$ in the various molecular sites of a given species can therefore provide an information base for the study of territorial origins, the species, and a variety of manipulations carried out in the course of processing and storage of the product (Scano et al. 2000).

On the other hand, isotopic ratios of $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{34}S/^{32}S$, $^{87}Sr/^{86}Sr$ are more indicative of soil and feed origin (Pillonel et al. 2003). The isotopic ratios of H and O, mainly depending on the beverage water consumed, cannot be easily masked by feeding diet ingredients bought outside of the region. Nonetheless, a method based on the properties of beverage water is not influenced by the feeding method adopted. Conversely, the isotopic ratios $^{13}C/^{12}C$ and $^{15}N/^{14}N$ provide indication of the kind of the feedstuff, which becomes very informative when the diet differs in the proportions of C3 and C4 plants. Increasing maize proportions in the diet with intensive milk production system usually shifts the $^{13}C/^{12}C$ ratio. The proportion of maize in the diet could be helpful to ascertain the regional origin of milk, but only

Fig. 9.7 ^{1}H and ^{16}O distribution through meteoric water according to latitude

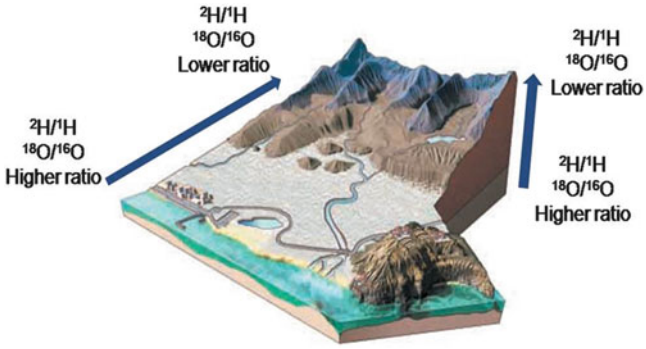

Fig. 9.8 $\delta^{2}H$ and $\delta^{18}O$ distribution trough meteoric water according to altitude and distance from the sea

when a certain type of feeding is very common in a certain geographical area. Manca et al. (2001) have applied PCA to the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ of casein and a good discrimination was found according to the place of origin of cheeses. Szul and Koziol (2003) have tested two traditional regional cheeses, Italian Montasio and Polish Oscypek. The results they obtained reflected the geographical and climatic conditions of the regions of the cheeses' origin, as well as the diet of the animals whose milk was used for cheese production. A clear differentiation between the examined cheeses was obtained in the case of carbon ^{13}C, while oxygen ^{18}O and nitrogen ^{15}N yielded fewer differences. Bontempo et al. (2012) using stable isotope ratios found a good discrimination level between the dairy products from two different types of pasture at two mountain sites.

A preliminary study on the potential application of stable isotopes of O, H, C, and N has been conducted to discriminate the geographical origin of the milk produced in the terroir of the provinces of Venice, Treviso, and Padua (North East of Italy). The milk samples were taken directly from farms geographically located as shown in Fig. 9.9 (Novelli et al. 2011).

The isotopic ratios obtained are shown in Table 9.2. Multivariate analysis PCA (Fig. 9.10) allowed to clearly discriminate the samples of milk produced in the Veneto plain than those produced beyond the Alps. Milk samples produced beyond

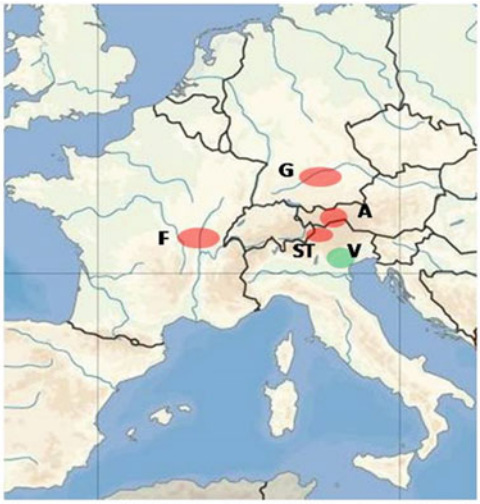

Fig. 9.9 Sampling sites of milk

Table 9.2 Isotopic abundance of milk samples collected in Veneto plain, South Tyrol (ST), Austria (A), Germany (G), and France (F). In bold type are indicated the abundance values significantly different and more useful for discriminating geographical origin

	Veneto (NE Italy)	ST + A + G + F	
	N° obs		
Stable isotopes	7	10	*P*
δ ^{13}C ‰ vs. V-PDB	**−17.79**	**−22.8**	<0.001
δ ^{15}N ‰ vs. AIR	5.10	5.83	0.001
δ ^{18}O ‰ vs. V-SMOW (milk water)	**−5.7**	**−7.21**	0.006
δ ^{18}O ‰ vs. V-SMOW (casein)	9.01	9.24	0.717
δ ^{2}H ‰ vs. V-SMOW	**−95.46**	**−111.3**	0.001
δ ^{34}S ‰ vs. V-CDT	2.56	2.68	0.688

the Alps scored an isotopic ratio for H to O that was lower than that produced in the Veneto plain. In addition, the traditional use of corn silage for feeding cows in intensive systems in Northern Italy highlighted a $^{13}C/^{12}C$ ratio greater than that detected in milk samples produced in the farms located at higher latitudes where presumably the feeding management for cow is mainly carried out with forage and variable supplements of concentrate.

Using $^{18}O/^{16}O$ and $^{2}H/^{1}H$ isotopic ratios, Renou et al. (2004) were able to differentiate milks produced in lowland (<200 m) from those produced in mountain (altitude 1,100 m). Their research showed that milk enrichments differed significantly between sites for both ^{18}O and ^{2}H. On the plain, the ^{18}O enrichments were significantly higher for grazing cows than those fed on maize silage or hay.

However, the stable isotope approach also has some important constraints. Draw conclusions using isotope analysis should be done with caution and always taking into account the great variability that factors such as climate, altitude, latitude, the

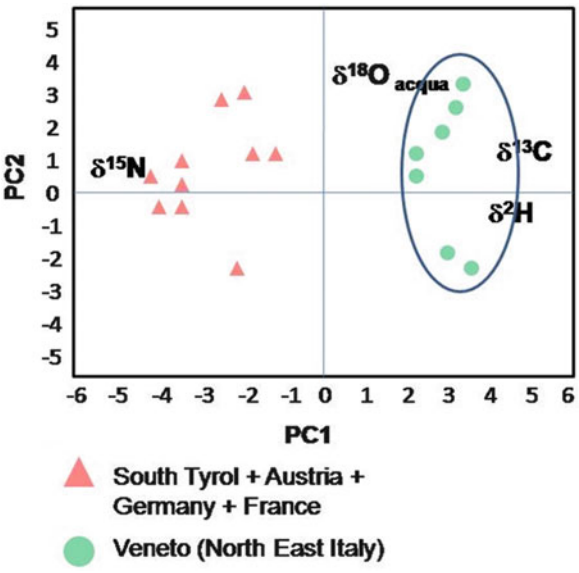

Fig. 9.10 PCA model score of milk samples collected in Veneto plain, South Tyrol, Austria, Germany, and France. The cluster separation was mainly due to $\delta\ ^{2}H$ and $\delta\ ^{13}C$ abundance on PC1

diet of dairy cattle can make to the isotopic profile of the milk before and to the cheese after. Therefore, dairy products made with milk from animals originating from different areas, but climatically or geologically similar, might have relatively similar isotopic abundance profiles. In addition, Ritz et al. (2005) demonstrated that the breed of cows can influence the isotopic enrichment of milk, even in circumstances where the feed and water consumed were similar. Another disadvantage of analyzing stable isotopes is the time-consuming and expensive preparation of samples for some elements and the high costs of the analytical equipment.

9.5 Hygiene of the Rooms of Cheese Ripening: Molds and Yeast Control

Among the various contaminants that can affect the cheese ripening rooms, molds and yeasts are the most common and at the same time they are particularly insidious for the damages that may cause to the product specially in the case of fresh cheeses. Certain types of cheese (gorgonzola and camembert among those most known) due their sensory characteristics to the mold proliferation. However, in dairy products the presence of fungi invariably accompanies an increase in the pH (lower acidity) due to the production of amines and free amino acids and the consumption of lactic acid which can achieve more or less serious defects (Fig. 9.11).

The mold genera occurring most frequently in the air of the cheese ripening room are *Penicillium*, *Cladosporium*, and *Aspergillus* (Serra et al. 2003).

Fig. 9.11 Fungal proliferation (*black* and *gray* colonies) on the crust of semi-hard cheese

Not less important is the fact that some fungal species can produce toxins also in food whose composition is predominantly of lipids and proteins such as cheese (mainly Ochratoxin A). Therefore, except when mold growth plays a specific technological significance, fungi inhibition is carried out by addition of additives on the crust to develop an antifungal action (Natamycin, E235). Among the various techniques in use to contain the fungal contamination in the ripening rooms (use of chemicals with bactericidal action), the use of ozone in gaseous form offers an interesting perspective of application. Ozone is a gas spontaneously formed from oxygen by the action of energy fields (lightning, electric shock) that quickly decomposes itself to oxygen. It is a strong oxidant as well as an excellent antimicrobial through the destabilizing of the membrane of Gram+ and Gram− bacteria. Bacterial spores are also destroyed by ozone.

Ozone was defined as GRAS by the US-FDA and employed as direct food additive. In Italy the Ministry of Health in 1996 recognized the ozone for the treatment of water and air as a tool for the sterilization of confined environments contaminated by viruses, bacteria, spores, molds, and mites. Treatments with gaseous ozone could inactivate 3 log CFU of most of the fungi on various surfaces, both in the laboratory and in simulated field conditions (Hudson and Sharma 2009). Fumigation with ozone can control postharvest pathogenic fungi on commodities like table grapes (Ozkan et al. 2011), whereas Serra et al. (2003) after the ozone fumigation of cheese ripening room they noted that the treatment reduced the viable airborne mold load but did not affect viable mold on surfaces. A trial was conducted to test the effect of gaseous ozone on molds and yeast contaminating the cheese maturing rooms (Balzan et al. 2012). Using two adjacent rooms (treatment and control), the cheeses were settled on plastic shelves located in the opposite side of the door (Fig. 9.12).

In one room were placed four ozone generators (two near the door and two in the opposite side) operating according to corona discharge with a production capacity of 5 g ozone/h each one. The emission of ozone was continued for 28 consecutive

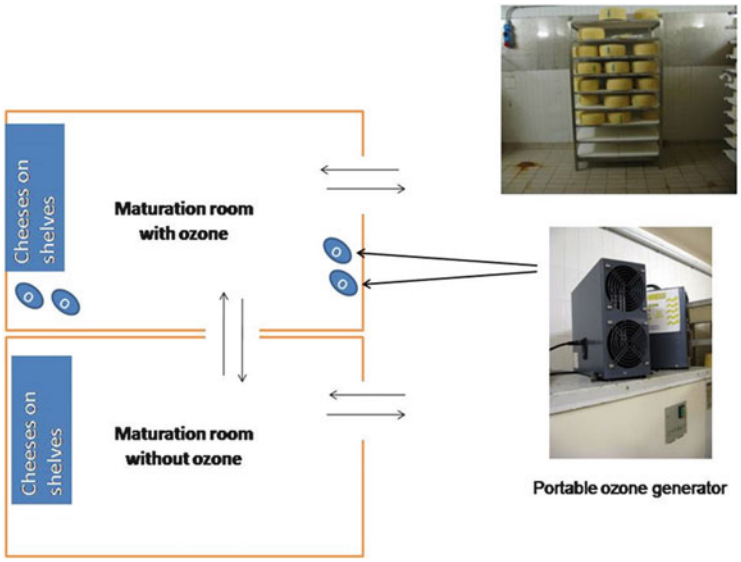

Fig. 9.12 Scheme of the experimental trial for cheese ripening room treatment with gaseous ozone for molds and yeast eradication. Treatment conducted with cheese inside the room

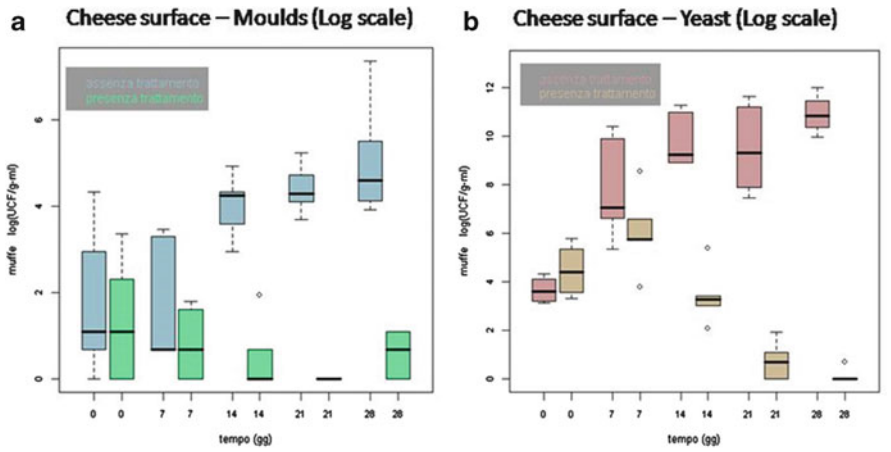

Fig. 9.13 Box-plot of molds (**a**) and yeast (**b**) trend of growth on the cheese surface during 28 days of ozone treatments. Box-plot *blue* and *pink* room without ozone, *green* and *brown* room with ozone

days from 6:00 PM to 4:00 AM. In the room submitted to ozone treatment the microbial behavior was completely different from the control one. The molds were almost absent from all the surfaces tested already at the end of the first week of maturing time and this situation persisted until the end. The yeasts showed a linear decrease until disappearing at the end of the third week (Fig. 9.13).

Fig. 9.14 Cheese at the end of 28 days ripening. On the *left* cheese from the room without ozone and on the *right* with ozone. On the *left* cheese there is a thin layer of molds on the surface that can spread during the portioning of the wheel (courtesy of Lattebusche S.c.a. Sandrigo, Vicenza—Italy)

At the end of ripening the wheels in the room with ozone appeared perfectly clean without visible mold or yeast colonies (Fig. 9.14).

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Part III
Food Technology

Chapter 10
Exploration of Microorganisms Producing Bioactive Molecules of Industrial Interest by Solid State Fermentation

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Abstract The prospect of biomolecules using microorganisms in fermentation processes is widely used, in this context to solid state fermentation (SSF) has advantages such as the possibility of using agricultural and industrial waste and reduction of water waste. Studies show that different microorganisms can be used in SSF; actinomyces and fungi are the most used due to growth in media with low water activity. Among the highlight biomolecules produced are antibiotics, anticarcinogenic agents, anticoccidians, antiviral, neuroactive, antioxidants, and enzymes. The enzymes are produced in greater scale among the different classes; hydrolases have gained importance because of cellulases, hemicellulases, proteases, chitinases, lipases, and phytases. Cellulases are a complex capable of acting on cellulosic materials, promoting its hydrolysis to release sugars, of which glucose is the one with largest industrial interest. Xylanolytic enzymes act on xylan, hemicellulose components, which may be attached to the cellulose and lignin in the plant cell wall. The study of chitinase has been stimulated by their possible involvement as agents of defense against pathogenic organisms that contain chitin,

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such as insects, nematodes, and fungi. Proteases catalyze the hydrolysis of peptide bonds of proteins and may have activity on ester and amide bonds. Lipases allow catalysis of the hydrolysis and synthesis, often in chemo, regal, or enantioselective reactions. Furthermore, phytase catalyzes the hydrolysis of phytate to phosphate and inorganic phosphorus, increasing the bioavailability of phosphorus for monogastric animals.

Keywords Enzymes • Solid state fermentation • Bioprospecting • Fungus • Actinomycetes • Biomolecules

10.1 Introduction

The exploitation of biodiversity rises as a new exploitation method of biological natural resources, generating bioprospecting, that is defined as the method to determinate, evaluate, and explore legally and systematic life diversity in particular location, whose main goal is seek for genetic and biochemical resources for commercial purposes. Therefore, microorganisms are versatile for molecules production with biological activities by fermentation processes, such as the solid state fermentation (SSF).

The SSF is widely used for obtaining biotechnological products, and it has become an interesting alternative to reduce the processes cost. This type of fermentation can use agricultural and agro industrial waste as substrates, which present low value, are nutrient rich, and have restricted water availability that helps to select contaminants, especially bacteria and yeasts. The obtainment of the final product by SSF is easier and the amount of waste is minimized (Lima et al. 2003). The use of surplus/waste as substrate for SSF allows the reduction of the final product cost and the implementation of a closed, sustainable, and environmentally friendly product chain. Among these substrates we can mention sawdust, bagasse from sugarcane of the sugar and alcohol industry and straw, bark and bran from cereal and fruit production. For biotechnological processes, the microorganisms are widely required because they are, in most cases, unicellular; when they are multicellular, they are poorly differentiated; they simplify cultivation in fermenter; they have rapid absorption of nutrients, fast metabolism, and high versatility, transforming different compounds and producing a wide variety of products. Microorganism is considered viable for a process when it is able to grow on cheap substrates; it is genetically stable, but liable for genetic manipulation; it provides high production yields on large scale, and, also, recovered at low cost; it does not produce incompatible substances with the target final product and it is not pathogenic. Actinomyces and fungi are the most used by SSF, since they grow under low water activity conditions.

Actinomyces were originally classified as fungi, as they present aerial hyphae, however, detailed studies of the cell wall composition, particularly the lipid membrane and the composition of its peptidoglycan, classified them as true aerobic bacteria. Molecular taxonomy studies created the class *Actinobacteria*, which includes all gram-positive bacteria with guanine and cytosine content greater than

55 %. Within this new class, actinomyces with capacity to produce mycelium are classified as *Actinomycetales* and include 10 subclasses and 34 families. Each year, new proposals are presented in literature of new species, genera, or families, and so, the classification of these organisms is constantly renewed (Stackebrandt et al. 1997; Stackebrandt 2000). Actinomyces of genus *Streptomyces*, the most commonly isolated and studied, are considered important microorganisms for industrial production and they have been described as the main antibiotics producers. Species of this genus are noted for producing more than half of the 10,000 bioactive compounds documented until 2001 (Anderson and Wellington 2001). Due to its high metabolic diversity, actinomyces have also been explored as major producers of many bioactive substances (Korn-Wendisch and Scheider 1992). The Kingdom Fungi consists of about 1.5 million species of which 77,000 species are known. These microorganisms have important ecological functions in nature, such as decomposition of organic material and reduction of mineral discharge to environment, immobilization and nutrient release, association with plants that can vary from beneficial to pathogenic, release of organic acids for the soil, among others. They are capable of degrading various substances with aid of exoenzymes to achieve required solubility and shape to be transported and incorporated by the cells. These enzymes are amylases, pectinases, xylanases, lipases, cellulases, and proteases, which are important for many applications in promising industrial processes (Silva et al. 2008). Furthermore, they produce other metabolites as antibiotics, chelating agents, and others (Hawksworth et al. 1996; Fransson et al. 2004; Klein and Paschke 2004).

10.2 Production of Bioactive Substances by Microorganisms

The production of bioactive compounds by SSF may be conducted as shown in Fig. 10.1.

Actinomyces are producers of antibiotics (Bull 2004; Berdy 2005; Strohl 2004), antitumor agents (Olano et al. 2009), and immunosuppressive agents (Mann 2001). The *Streptomyces* have the ability to produce many bioactive compounds. Around 23,000 antibiotics have been discovered from microorganisms. It is estimated that about 10,000 of them have been isolated from actinomyces (Okami and Hotta 1988).

Regardless of its chemical structure, these bioactive substances can be classified as peptides, quinones, macrolides, terpenes, polyketides, among others (Li and Piel 2002; Salmon et al. 2003). Most peptides derived from *Streptomyces* species are cyclical and contain elements such as chromophores or amino acids in its structure. Peptides include ciclomarine A, which can be obtained from *Streptomyces* with great anti-inflammatory and antiviral activity (Renner et al. 1999), and piperazimicines A-C, which are cytotoxins isolated from *Streptomyces*

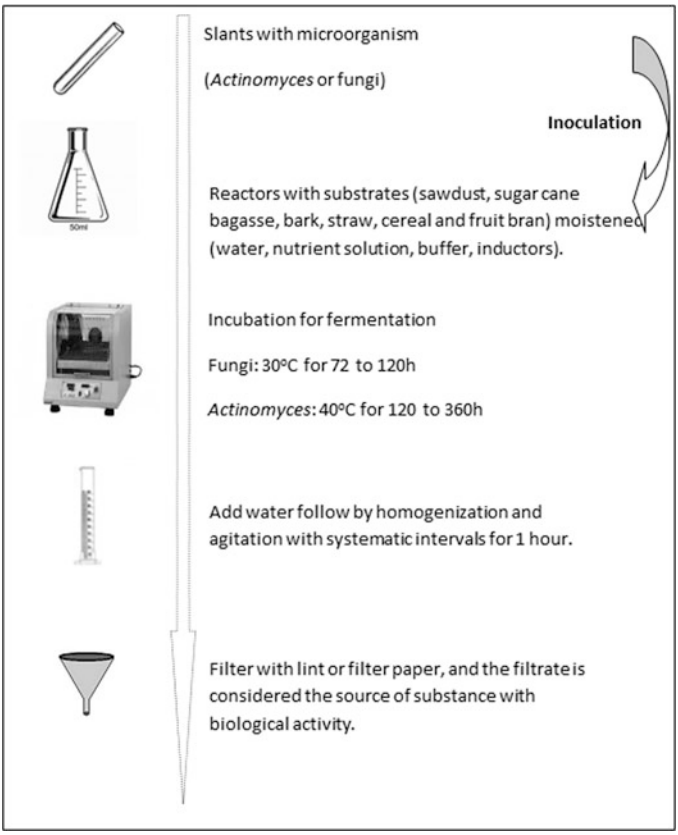

Fig. 10.1 Solid state fermentation process. Microorganisms kept in slants are sterile inoculated in reactors containing substrate previously sterilized. Afterwards they are incubated for fermentation, followed by water addition, homogenization and filtration to obtain the substances with biological activity

sp. Though, piperazimicine A showed high cytotoxicity against tumor cells in vitro (Miller et al. 2007) and salinamides A and B, produced by *Streptomyces* sp. CNB-091, showed anti-inflammatory activity (Moore et al. 1999). Quinones are compounds with conjugated dione cyclic in its structure and they are common constituents and biologically relevant molecules. As an example: C-glycoside himalomicines A and B complex and tetracenomycin D. The first is the anthraquinone with fridamicine E chromophore, precursor of anthracycline antibiotic obtained from *Streptomyces* sp. 6921, that show great antibacterial activity (Maskey et al. 2004), while the second, the anthraquinone antibiotic produced by *Streptomyces corchorusii* AUBN (Adinarayana et al. 2006) showed cytotoxic activity against hepatic carcinoma cells. Terpenes (large and diverse class of hydrocarbons) are biosynthetically derived from units of isoprene, with molecular formula C_5H_8. *Streptomyces* sp. NPS008187, isolated in Alaska, synthesized three

new pyroles sesquiterpenes which showed antibacterial activity (Macherla et al. 2005). Carotenoids are tetraterpenos mostly know, and they can be obtained from strains such as *Streptomyces griseus* (Lee et al. 2001).

The polysaccharides produced by basidiomycetes fungi are extensively studied in China and Japan, due to its medicinal and tonics attributes. Examples include *Agaricus blazei* which produces substances with anticarcinogenic activity (Mizuno et al. 1990); *Flammulina velutipes* that produces elements which helps to reduce cholesterol (Miles and Chang 1997); extracts of *Ganoderma lucidum* which are immune system boosters and promoters of tonic effects for cardiac system (Hikino et al. 1985); *Lentinula edodes* biomolecules with anti-HIV effect (Chihara 1992); and others. Filamentous fungi (ascomycetes) of *Penicillium* genus are fairly flexible for antibiotics production by fermentation processes alone or associated with chemical modification, such as penicillin G produced by *P. chrysogenum*; griseofulvin of *P. griseofulvin* used for infections treatment of skin, hair, and nails; cyclosporin, used as an immunosuppressant in transplant surgery; and fusidic acid, used to help control the infection by *Staphylococcus aureus* resistant to methicillin. Duarte et al. (2012) described the achievement of marine fungi molecules from different genus such as *Penicillium*, *Fusarium*, *Trichoderma*, *Hupocrea*, *Phoma*, and *Scopulariopsis*, among others with cytotoxic, antifungal anticoccidial, antiviral, and neuroactive activity. Whereas Xiong et al. (2009) studied the production of antibacterial compounds by *Cladosporium* sp., Meenupriya and Thangaraj (2011) describe the bioactive molecules obtained from marine organisms present anticancer, antimicrobial, and anti-inflammatory activity. Thus, these researchers obtained molecules from *Aspergillus ochraceus* with activity against microorganisms that cause humans diseases.

10.3 Production of Enzymes by Microorganismis

Enzymes include a abundant class of substances produced by actinomyces and fungi. The advantages of using microorganisms for enzyme production in replacement of the traditional animal and vegetable sources are relatively high performance, low cost, and susceptibility to genetic manipulation. Currently, microorganism enzymes are used in food processing, manufacture of detergents, textile and pharmaceutical industries, medical therapy, molecular biology, biofuels industry, wastewater treatment, environmental preservation, bioremediation, and biological control. These microorganisms have a wide ecological and biochemical diversity, and furthermore, they have a high capacity for production of secondary metabolites. Therefore, they can be considered an excellent source for finding new enzymes with new specificities and different biochemical characteristics. They are capable of producing several enzymes that can be considered promising for biotechnological applications, including oxidoreductases, transferases, hydrolases, lyases, isomerases, and synthases. Hydrolases are noteworthy, because these are cellulases, hemicellulases, proteases, chitinases, phytases, and lipases, whose features and applications are described below.

10.3.1 Cellulases

Cellulases are enzymes consisting of complex capable of acting on cellulosic materials, promoting its hydrolysis. These biocatalysts enzymes are highly specific, acting synergistically to release sugars, where glucose is the one with greater industrial interest due to the possibility of its conversion to ethanol, sweeteners, phytohormones, organic acids, etc. The steps involved in cellulose degradation by cellulase are not fully understood, but it is formed as a multienzyme system including three enzymes that act together for hydrolysis of cellulose: endoglucanases (EC 3.2.1.4), which cleave randomly cellulose polymer by changing the degree of polymerization; cellobiohydrolases (EC 3.2.1.91), which hydrolyze the polymer at its nonreducing end, releasing cellobiose; and cellobiases (β-glucosidase, EC 3.2.1.21), which are responsible for cleavage of small chain, both celloligosaccharides and cellobiose, until glucose (Fleuri and Lima 2013). The prospect of cellulose degradation (most abundant polymer in nature present in vegetable cells) is linked to program implemented in Brazil in 1970 that meant to replace gasoline with ethanol from sugarcane. Consequently, research for agriculture and new technologies have been greatly intensified, leading Brazil in a favorable position in terms of secure energy sources. However, only a part of the biomass produced is used for bioenergy production, as one-third of the sugarcane is used for sugar production, one-third is residue, which is burned to produce electricity, and the other third of the remaining residue is left in the field and decomposed by microorganisms (Zanin et al. 2000; Soccol et al. 2010). However, a significant increase in ethanol production may be possible if new technologies converting the polysaccharides of the two-thirds of the remaining biomass of the entire process in bioethanol. For the last four decades, much effort is being made to development of second-generation bioethanol, through abundant and renewable lignocellulose biomass by physical, chemical, and enzymatic treatments, isolation and/or combined (Hahn-Hägerdal et al. 2006; D'Souza-Ticlo et al. 2010; Soni et al. 2010). The raw lignocellulose materials include agribusiness, municipal waste, and wood from angiosperms and gymnosperms. The agro industrial materials are important for its residue character, after processing raw materials with high value, and the natural capacity that Brazil has for generation of these products, that is: sugarcane bagasse and straw, soybean straw, rice straw, and corncobs. Among the mentioned biomass, bagasse from sugarcane is predominant in Brazil, producing, in 2007, 147 million tons of wet mass (Chandra et al. 2010). Furthermore, these materials may also be used for solid state fermentation (SSF), since they are inexpensive materials and they have shown effective results for biocatalysts and bioactive compound production (Lever et al. 2010; Sukumaran et al. 2009; Bhattacharya and Banerjee 2008; Lin and Tanaka 2006; Mishima et al. 2006). The polysaccharides present in lignocellulose biomass must be hydrolyzed with acid (in the presence of high temperature and pressure) and/or cellulases and other enzymes to release fermentable sugar in a high yield. Pre-treatment help to hydrolyse the lignin and to solubilize the cellulose partly, so the enzyme can act on the molecule and available

all the remaining hexoses and pentoses. The process of enzymatic conversion of lignocellulose into ethanol is affected by the use and purchase of cellulases preparation, since they are marketed by a small number of suppliers and have high cost. For this process to become economically viable, large-scale production of cellulases at low cost, using agro-industrial residues as substrate, is necessary (Maclean and Spatari 2009; Chandra et al. 2009).

Actinomyces produce cellulase with high activity and stability in extreme temperature and pH conditions (Lima et al. 2005; Jang and Chen 2003). Such cellulases exhibit great activity in a wide range of pH, between 4.0 and 8.0, which it is also promising (Lima et al. 2005; Jang and Chen 2003; George et al. 2001; Bhat 2000). The proportion of current total production of cellulases as additives for detergents for laundry industry market exceeds 30 %. Due the increase of environmental pressure on paper and textile industries, it is assumed that cellulases should play an important role in the development of clean technology, both for denim processing and for discoloration of paper for recycling purposes (George et al. 2001). Currently, one of the main applications for cellulases are textile industry, where the need of high temperatures (50–65 °C) and alkaline pH requires the use of thermostable enzymes for efficient jeans treatment (Bhat 2000).

The main commercial cellulase preparations are obtained from filamentous fungi, such as *Aspergillus niger* (Cellulocast of Novozyme) and *Trichoderma reesei* (Megazyme). Among cellulases producing fungi, we can name genus *Aspergillus*, *Trichoderma*, *Penicillium pinophilum*, *Sporotrichum*, *Fusarium*, *Talaromyces*, *Thermoascus*, *Chaetomium*, *Humicola*, *Neocallimastix*, *Piromonas*, and *Sphaeromonas* (Fleuri and Lima 2013).

10.3.2 Xylanases

Xylanases enzymes act on xylan, hemicellulose components, which may be associated to cellulose and lignin in the plant cell wall. Xylan is formed by xylose units linked with β-1,4 glycosidic bonds; they, also, may have arabinose, glucuronic acid or 4-methyl ether, and acetic, *p*-cumaric, and ferulic acids (Brienzo et al. 2008). Among the xylanases enzymes, there are β-1,4 endoxylanases (β-1,4-D-xilanil-xylan hydrolase, EC 3.2.1.8), which depolymerize xylan by random hydrolysis of main skeleton, and β-xylosidases (β-1,4-D-xilosidic-xylo hydrolase, EC 3.2.1.37), which hydrolyze small oligosaccharides (Collins et al. 2005). Xylanase's most important application is in the pulp and paper industry where high temperatures (55–70 °C) and alkaline pH of the pulp substrate requires utilization of thermostable enzymes for efficient bleaching (Beg et al. 2001; Collins et al. 2005; Saha 2003). However, other applications, such as food industry can be mentioned like: dough preparation (Collins et al. 2005), for clarification of beer and juices, and partial hydrolysis of xylan in animal feeds. Nascimento et al. (2003) found that the xylanase extract obtained from *Streptomyces malaysiensis* showed biochemical characteristics (temperature 50–65 °C and pH 6.0–8.0) with great potential for

pulp and paper industry. Beg et al. (2000) showed optimal values of temperature, range between 50 and 75 °C and pH from 6.0 to 9.0 for strain *Streptomyces* QG-11-3. Most known thermostable xylanases are produced by strains of *Thermatoga*, with half-life of 90 min at 95 °C (Sunna and Antranikian 1997). However, very significant thermostability of xylanases has been studied in many *Streptomyces* strains, including *Streptomyces* sp. T7 with stability at 50 °C, at pH 6.0 for 6 days (Deng et al. 2005). Costa et al. (2000) described the production of xylanase complex using *Penicillium janthinellum* with sugarcane bagasse hydrolyzed as substrate.

10.3.3 Proteases

Proteases (EC 3.4.21.12) catalyze hydrolysis of peptide bonds of proteins, and they may have activity on ester and amide bonds. The proteolytic enzymes synthesized by microorganisms have become significant for research because of its wide application at different industries and medicine, as well as its involvement in microbial metabolism. They are used in leather industry, pharmaceutical and food industries, in hydrolysis of substrates used for microbiological growth and parenteral nutrition preparation, detergents, and cosmetics. Proteases enzyme preparations are particularly important in medicine for burns cleaning and removal of necrotic tissue and blood clots lysis (Landau and Egorov 1996). Proteases can also be applied for monogastric animals feed aimed at reduction of anti-nutritional agents of vegetable ingredients, increased digestibility, increasing endogenous enzymes activity, and reduction of environmental pollution (García et al. 2000). Actinomyces and fungi produce a variety of extracellular peptidases, including endopeptidases (serine and metallo-peptidases, specially) and exopeptidases (amino- and carboxypeptidases) with specificity for many substrates. Peptidases obtained from actinomyces, such as serine-peptidases from *Streptomyces exfoliates*; serine and metallo-peptidases from *Streptomyces lactamdurans*; and serine-peptidase from *Streptomyces pactum* are involved in the nitrogen protein sources assimilation, in degradation of aerial mycelium, in sporulation processes, and in production of antibiotics (Kim and Lee 1996). Peptidases and other enzymes used in detergent formulations may have high activity and stability in a wide range of pH and temperature. Serine and metallo-peptidases have been described for the genus *Streptomyces*, as observed with strains *Streptomyces* sp. 594, *Streptomyces malaysiensis* AMT-3, and *Streptomyces alboniger* (Born 1952). However, the nature and characteristics of each component of the peptidase complex from *Streptomyces* has not been extensively studied. Likewise, thermostable actinomyces produce peptidase with major role in degradation of keratin components, such as chicken feathers present in the poultry industry waste (De Azeredo et al. 2004). Specifically, keratinase produced by actinomyces can have great biochemical characteristics with pH ranging between 6.0 and 9.0 and optimal temperature between 50 and 70 °C, as observed for some species (Gushterova et al. 2005). Fungi are, also, capable for protease production. Zanphorlin et al. (2011) used

wheat bran moistened with casein and nutrient for protease production using fungus *Myceliophthora* sp. The enzyme showed optimum pH and temperature of 9.0 and 40–45 °C, respectively. Rojas et al. (2009) studied fungal proteases obtained from *Eladia sacculum* in biodeterioration processes. Cabaleiro et al. (2002) studied protease production by fungi *Phanerochaete chrysosporium* and *Phlebia radiata* in SSF using nylon sponge and corncob. Proteases obtained from this process were distinguished by microbial growth time and activity, and they are of different classes.

10.3.4 Chitinases

Chitin is linear polymer of β-1,4-*N*-acetylglucosamine, which is the most abundant natural amino polysaccharide. Moreover, it is present in cell wall of most fungi and it is the main constituent of insects and crustaceans exoskeleton (Fleuri et al. 2009a). The hydrolysis of chitin occurs by action of enzyme complex involving two enzymes: chitinase or poly (1,4-*N*-acetyl-β-D-glicosaminida) glucan hydrolase (EC 3.2.14), which breaks randomly internal bonds in the chitin chain, generating oligomers and disaccharides, and β-*N*-acetyl-glucosaminidase or β-*N*-acetyl-β-D-hexosaminide-*N*-acetyl-hexosamino hydrolase (EC 3.2.1.52), which cleaves nonreducing terminal unit, releasing *N*-acetylglucosamine. The first ones have higher affinity for larger molecules, while the others prefer small oligomers, including quitobiose (Merzendorfer and Zimoch 2003). Study of chitinase has been increasing because its contribution as defense agents against pathogenic organisms that have chitin, such as insects, nematodes, and fungi (Sahai and Manocha 1993). Besides, chitinases can be used as a protective agent against pathogenic fungi, in protoplast preparation, and production of biologically active substances as aminoglucanooligossacarides (Fleuri et al. 2009a, b). Han et al. (2008) observed application of chitinase in medicine (hypocholesterolemic action and antihypertensive), in agriculture (anti-phytopathogenic), in bioremediation, and in maintenance of food quality. It is estimated that there are between 10 and 25 different chitinases. Tikhonov et al. (1998) produced and purified chitinases from *Streptomyces kurssanovii*. Brzezinska et al. (2012) studied the degradation of chitin substances with chitinase from *Streptomyces rimosus*, which was isolated from soil. Many fungi genus (*Beauveria* sp., *Aspergillus* sp., *Thermoascus* sp., *Chaetomium* sp. *Trichoderma* sp.) are able to produce chitinases by SSF.

10.3.5 Lipases

Lipases are enzymes that are increasing at the biotechnological enzymes scenario. They are very versatile, allow catalysis of hydrolysis and synthesis of chemical reactions; often in chemo, regal, or enantioselective, lipases are applied in many industries such as food industry, pharmaceuticals, fine chemicals, oil chemistry,

detergents, and biodiesel (Barros et al. 2010). The participation of lipases in the world market of industrial enzymes has grown significantly; it is estimated that in the future they will have world significance comparable to peptidases today which count for 25–40 % of industrial enzymes sales (Hasan et al. 2006). Lipases act in the organic–aqueous interface; they catalyze hydrolysis of carboxylic-ester bonds and liberate fatty acids and organic alcohols. However, the reverse reaction (esterification) and also various transesterification reactions can occur in environments with restricted water (Freire and Castilho 2008). The transesterification term refers to radical change between an ester and an acid (acidolysis), or ester and alcohol (alcoholysis), or between two esters (interesterification). Their ability to catalyze these reactions with high efficiency, stability, and versatility make these enzymes very commercially important. Lipases are enzymes of the group of serine hydrolases (EC 3.1.1.3). Their natural substrates are triglycerides; however, its activity is increased when located at the interface polar/nonpolar, and they have higher affinity for long-chain fatty acids (Hasan et al. 2006). Among lipases obtained from actinomyces, there is *Streptomyces rimosus*, *S. coelicolor* (Côte and Shareck 2008), *S. fradiae*, *S. coelicolor* (Sharma et al. 2001), *S. exfoliatus*, *S. albus*, and *S. cinnamomeus* (Abramic et al. 1999). Bielen et al. (2009) and Abramic et al. (1999) reported that lipases have been traced from different microorganisms for different kinds of applications, and that streptomycetes have a large number of genes encoding different enzymes with many lipolysis activities. Among these actinomyces are cited *S. exfoliates*, *S. albus*, *S. coelicolor*, *S. rimosus*, and *S. exfoliatus*. Mander et al. (2012) studied the transesterification with the lipase obtained from *Streptomyces* sp. CS133 for production of biodiesel. Even with a wide variety of microbial lipases, use of these enzymes on industrial scale is still limited due to high production costs, low activity, and limited biochemical characteristics, which facilitates searching of other microbial lipases sources. Extracellular lipases from fungi *Rhizopus homothallicus* (thermostable) were obtained by SSF with sugarcane bagasse as substrate. Moreover, these authors mention that the yield of enzyme production by SSF is higher than liquid fermentation due to increased rate of biomass growth. There are lower protease production that can degrade other enzymes, as well as higher stability for pH and temperature of the enzyme obtained by this type of fermentation (Mateos Diaz et al. 2006). The main commercial lipase preparation is from *Aspergillus oryzae*, created from lipase clones derived from *Thermomyces lanuginosa* (Lipolase from Novo Nordisk) and lipase clones from *Rhizomucor miehei* (Lipozyme, Novo Nordisk S/A). They are especially applied as detergents and production of analogues of cocoa butter from cheap oil sources (Romdhane et al. 2010).

10.3.6 Phytases

Phosphorus is an important ingredient for various biochemical pathways, biological processes, and skeletal integrity. Vegetable ingredients are important sources of

phosphorus, and phytate (inositol hexaphosphate or IP6) is the mineral storage for plants. The amount can differ between plant species. However, phytic acid is not a suitable source of phosphorus for nonruminants animals, since 85 % of the phosphorus is bound to inositol making phytic acid or inositol hexametaphosphate, kept it chelated and unavailable. Diets fed to animals are supplemented with inorganic sources of phosphorus such as calcium phosphate or animal sources like meat and bone flour, due to the lack of availability of phosphorus and a possible deficiency of this mineral for animals in diets with vegetable ingredients. As result, diets for nonruminant animals have amount of phosphorus addition to nutritional requirements, with elimination of excess not absorbed by the animal. Furthermore, phytate acts as anti-nutrient associated to proteins, amino acids, lipids, and minerals, while it interacts with their digestive enzymes reducing activity, influencing digestion, and impairing nutrients utilization. In this sense, phytase catalyzes the hydrolysis of phosphate and phytic acid to phosphorus inorganic, increasing the bioavailability of phosphorus for monogastric animals. Phytase classification is based on first position of the phosphate to be hydrolyzed; named 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26). Supplementation of phytase in diet benefits animal nutrition and improves digestibility of protein, gross energy, and increases the availability of calcium, phosphorus, zinc, manganese, and magnesium. Furthermore, these enzymes improve phosphorus availability in 50 %, and it is important toll to reduce environmental excretion, because of better utilization of phytic phosphorus from vegetable sources, reducing utilization of inorganic sources. Main phytases are classified as their activity on determined pH. Acid phytases show better dephosphorylating between pH 5.0 and alkaline phytase in pH 8.0. All phytases show great pH between 4.0 and 6.0 (Kies et al. 2001; Lei and Stahl 2000). Phytase is produced in large commercial scale by recombination DNA techniques, from fungi of genus *Aspergillus niger*. Enzymes that blend with phytase from *Peniophora lycii*, *Schizosaccharomyces pombe*, and *Escherichia coli* are also found on the market.

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Chapter 11
Recent Biosensors for Food Analysis in Brazil and Italy

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Abstract The importance of safe and high-quality food products is doubtless and consumer demand for increased food quality and safety assurances moves down the chain with retailers and service providers asking suppliers and producers to provide verifiable proof that robust food quality and safety control systems have been effectively implemented. Food analysis needs rapid and reliable methods to ensure the quality of products and process control. Food quality control is essential both for consumer protection and also for food industry. Nowadays, the convergence of new technologies, including biotechnology, nanotechnology, and electronic technology, has opened new horizons in development of biosensors. These devices offer advantages as alternatives to conventional methods because they enable real-time detection, portability, and fast laboratory or in-field analysis. This contribution presents a review about the development and application of biosensor technology in foods, and future trends in Brazil and Italy.

Keywords Biosensors • Food quality • Food safety • Food analysis • Food contamination

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11.1 Background

In recent years, food-safety emergencies have shaken consumer confidence in the food production chains, focusing attention on how food is produced, processed, and marketed. National Health Agencies, around the world, have recognized food safety and food quality as a top priority. They have established new policies, modernizing legislation into a coherent and transparent set of rules, which can guarantee high level of consumer protection and, thus, human health. An effective food safety policy requires the assessment and monitoring of the risks to consumer health, associated with the presence of contaminants in raw materials, farming practices, and food processing activities.

In the course of the third Spring School organized by the Brazilian State University of Sao Paulo (UNESP) and the Italian University of Padova (Italy) in the Botucatu Campus (Bioscience Institute) in September 18–20th, 2012, these themes were faced and in this chapter recent biosensors developed in the two countries are reviewed.

In fact, the rigorous control of food quality and safety is of growing interest for both consumers and food industries. In the food industry, the quality of a product is evaluated by periodic chemical and microbiological analysis. It has become important to periodically measure a variety of contaminants in food, such as bacteria, viruses, natural toxins, chemical compounds (pesticides, toxic metals, veterinary drugs residues, undesirable fermentation products), and packaging materials. Most of the toxic agents found in foods are natural contaminants from environmental sources, but some of them are chemical compounds deliberately added during food processing (Codex 2009). Consumers are concerned about long-term impacts of mixtures of these chemical additives (pesticides, toxic metals, flavorings, and colors) and about their chronic, as well as, acute effects, especially on children (Jackson 2009).

While the knowledge of phytochemical effects on human health and risks from chemical residues in food has led to a growing interest and attention towards the fast growth of functional and enriched food on the market, only little emphasis has been placed on the analytical aspects (Lavecchia et al. 2013). In this regard, specific, new technologies have recently been developed to examine food components and different analytical procedures were developed to assess food quality and to determine food contaminants. Normally, these procedures are based on various instrumental techniques, such as chromatography, spectrophotometry, electrophoresis, titration etc. These analytical procedures do not easily allow continuous monitoring, mainly because they are based on expensive instrumentations and they need time-consuming multistep sample extraction and pretreatments and well-trained operators, which increase the time and cost of the analysis. Meanwhile, National Health agencies and food industry request affordable methods to determine compounds of interest in foods.

The demand for fast and real-time analyses aimed at the detection of substance related to food quality and safety led to rapid advancements in biosensors technology (Mozaz et al. 2004). These compact analytical devices incorporate a biological

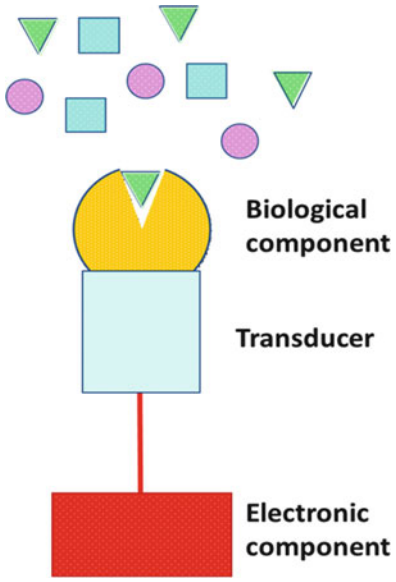

Fig. 11.1 Schematic drawing of a biosensor

sensing element, either closely connected to or integrated within a transducer system able to convert a biological event in an electrical signal (see Fig. 11.1). The recognition mechanism is based on the interaction of biological recognition component with the analyte of interest, by different recognition mechanisms. Information, which is produced in the recognition event, is transformed by means of the transducer into a signal. The Physical Chemistry and Analytical Chemistry Divisions of IUPAC (Thevenot et al. 1999) state a *definition applicable to electrochemical biosensors*: "A biosensor is a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is in direct spatial contact with a transduction element."

Four major types of transducers can be found: electrochemical, mass, optical, and thermal (Thevenot et al. 1999). They have been used to develop biosensor aimed at the detection of a broad spectrum of analytes present in complex sample matrices. Great promises in different areas, such as clinical diagnostics, food analysis, and environmental monitoring (Table 11.1), have been proposed and the sensitivity of a particular sensor system varies depending on the transducer's properties and the biological recognizing element.

In general, biosensors consist of three main components as shown in Fig. 11.1: a recognition element, a transducer unit, and a controlling electronic unit, including an input/output interface. The recognition element, which binds to the analyte of interest, is the component producing the primary signal. The transducer represents the biosensor component, which is responsible for the transformation of the primary signal, coming from the recognition element, to a form that can be filtered, amplified, and transferred to the electronic component, which finally processes and displays, and even stores, the analytical result. The choice of the biological

Table 11.1 Most common transducers used in biosensor development

Transducer	Advantages	Disadvantages	Application
Ion-selective electrode	Simple, reliable, easy to transport	Sluggish response, requires a stable reference electrode, susceptible to electronic noise	Amino acids, carbohydrates, alcohols, and inorganic ions
Electrodes	Simple, extensive variety of redox reaction for construction of the biosensors, facility for miniaturize	Low sensitivity, multiple membranes, or enzyme can be necessary for selectivity and adequate sensitivity	Glucose, galactose, lactate, sucrose, aspartame, acetic acid, glycerides, biological oxygen demand, cadaverine, histamine, etc.
FET	Low cost, mass production, stable output, requires very small amount of biological material, monitors several analytes simultaneously	Temperature sensitive, fabrication of different layer on the gate has not been perfected	Carbohydrates, carboxylic acids, alcohols, and herbicide
Optical	Remote sensing, low cost, miniaturizable, multiple modes: absorbance, reflectance, fluorescence, extensive electromagnetic range can be used	Interference from ambient light, requires high-energy sources, only applicable to a narrow concentration range, miniaturization con affect the magnitude of the signal	Carbohydrates, alcohols, pesticide, monitoring process, bacteria, and others
Thermal	Versatility, free from optical interferences such as color and turbidity	No selectivity with the exception of when used in arrangement	Carbohydrates, sucrose, alcohols, lipids, amines
Piezoelectric	Fast response, simple, stable, output, low-cost, or readout device, no special sample handling, good for gas analysis	Low sensitivity in liquid, interference due to nonspecific binding	Carbohydrates, pathogenic microorganisms, contaminants (e.g., antibiotics, fungicides, pesticides), toxic recognition as bacterial toxins

element and proper transducer depends on the properties of the sample of interest and the physical magnitude to be measured. The recognition element, that is, the biocomponent, determines the degree of selectivity or specificity of the final biosensor.

In this chapter, a brief commentary on some aspects of biosensor construction is reported. Current situation, recent development, and applications of biosensors for food technology in Brazil and Italy are reviewed.

11.2 Biosensor for Small Molecule Determination in Food Analysis

Among proposed biosensors, electrochemical transduction systems were the most used. Among them, amperometric and potentiometric transduction have found the widest applications, even if other transducers are available. The combination of oxidoreductases, as recognition bioelements, and amperometric electrodes, as trasducers, gave good results for food analysis, mainly because enzymatic activity, depending on substrate concentration in food samples, can be easily measured with reasonable sensitivity. This combination constitutes one of the most successful classes of biosensors.

Recently, Ferreira et al. (2004) immobilized two enzymes, namely, β-galactosidase and glucose oxidase, in order to determine lactose in cheese whey. The biosensor was based on the determination of oxygen consumption, which occurs during the enzymatic reaction. Authors studied the influence of temperature on the biosensor signal, observing a nonlinear relationship between the biosensor electric response and lactose concentrations a function of temperature and analyte concentration. This was due to differences in temperature dependencies of enzyme activities. Nonlinear correlations were proposed to automatically compensate the effects of temperature. Mello et al. (2003) developed a biosensor based on horseradish peroxidase (HRP) and immobilized DNA onto silica–titania and applied the novel system to the measurement of polyphenol compounds in vegetables samples. Various analytical parameters influencing the biosensor performances were studied as a function of chlorogenic acid, as reference polyphenol compound. The effect of working potential, type and concentration of the buffer, pH, response time, and interferences was investigated. The biosensor showed a linear response in the range from 1 to 50 μM chlorogenic acid, applying a potential of −50 mV versus Ag/AgCl, with a sensitivity of 181 nA/μM/cm^2 and a detection limit of 0.7 μM. The biosensor was tested for polyphenol determination in vegetable extract and the results were compared with the Folin–Ciocalteau traditional method. The biosensor showed suitability to the quantification of the total polyphenol in the tested samples.

Other authors described a highly selective and stable electrochemical biosensor for the determination of glucose in soluble coffee (de Mattos and Areias 2005) The biosensor electrode consisted of a thin film of ferric hexacyanoferrate, electrodeposited on the glassy carbon electrode and *glucose oxidase* immobilized on top of the electrode surface. Stability of the thin film was evaluated by injecting standard solution of H_2O_2 and glucose during 4 h in a flow-injection system, with the electrode polarized at −50 mV versus Ag/AgCl. The system was able to handle about 60 samples per hour, with high stability, and was proposed for industrial process control. A linear calibration in the range of 0.15 and 2.50 mM glucose and a detection limit of 0.03 mM were obtained. Another biosensor for glucose determination in food samples was developed by one of the authors (Baratella et al. 2013). The experimental work demonstrated the peculiar electro-catalytic behavior of a new generation of iron oxide nanoparticles (surface-active magnetite nanoparticles,

SAMNs), which were used for the development of a cheap carbon paste electrode aimed at hydrogen peroxide detection, containing an ionic liquid, namely, 1-butyl-3-methylimidazolium hexafluorophosphate (BMIM-PF6), and characterized by a sensitivity of 206.51 μA/mM/cm^2, a detection limit of 0.8 μM H_2O_2, and a noise of 1.01 nA. Furthermore, these metal oxide nanoparticles were used to form stable conjugates with rhodamine isothiocyanate, acting as a bridge, permitting the covalent binding of glucose oxidase. The resulting bio-conjugate was used to develop a nanocomposite, carbon paste-BMIM-PF6-based biosensor, characterized by a sensitivity of 48 μA/mM/cm^2, in the 0–1.5 mM glucose concentration range, and a detection limit of 0.9 μM glucose. The system was tested on fruit juices as real samples, without any sample preparation procedure and results suggest that BMIM-PF6–(SAMN@RITC–GOx)/CPE biosensor could be a promising, low-cost option for the development of GOx-based biosensors for glucose determination.

Amati et al. (2008) reported on a biosensor for the determination of the total antioxidant capacity and the total polyphenol concentration in extra virgin oil (EVO), as well as the main kinetic parameters of the process during the thermal oxidation of EVO. They also evaluated the increase of radical concentration during the thermal oxidation process, using a superoxide dismutase biosensor. The investigation concerning this important food product was of high interest, as it referred to the state of alteration of the EVO, used for cooking or frying, as a function of temperature.

Currently, the food industry is very receptive to biosensor technology, while a new market will probably be developed in the long run. A method for the rapid detection of common compounds will probably offer the best opportunity for biosensors in this industrial field, but several key issues also need to be resolved before biosensors find widespread applications. Newly, a new biosensor for the direct determination of lactic acid and malic acid in wines was developed (Mazzei et al. 2007). This multi-enzymatic biosensor was realized for the selective determination of three analytes: D(−) and L(+)-lactic acid and L(−)-malic acid. The measurement was based on a multi-enzymatic biosensor employing the catalytic activities of L(+)-lactate oxidase, D(−)-lactate dehydrogenase, and horseradish peroxidase for the determination of total D(−)- and L(+)-lactic acid and a bienzymatic electrode for L(−)-malic acid determination, realized by coupling the L(−)-malic dehydrogenase and horseradish peroxidase. For both electrodes, enzymes were immobilized on an oxygen-selective Clark electrode and the simultaneous determination of the two organic acids was accomplished either in batch or in a flow injection analysis apparatus, using the same biosensors as detectors. The analytical performances were tested in standard aqueous solutions and on real wine samples, showing high repeatability, short response times, and reduced cost of analysis, suggesting that the experimental approach here described could be convenient to monitor the progress of malo-lactic fermentation in wines.

A paper by Centonze and coworkers described a biosensor for simultaneous monitoring of glucose and ethanol content in drinks and alcoholic fermented media (Mentana et. al 2013). The methodology was based on the immobilization of glucose oxidase and alcohol oxidase by co-cross-linking with bovine serum

albumin and glutaraldehyde onto a dual gold electrode, modified with a perm selective over oxidized polypyrrole film. The biosensor was integrated in a flow injection system, coupled with an online microdialysis fiber as sampling tool. Flow rates inside and outside the fiber were optimized in terms of linear responses (0.01–1 and 0.01–1.5 M) and sensitivities (27.6 ± 0.4 and 31.0 ± 0.6 μA/mM/cm^2) for glucose and ethanol, respectively. Excellent anti-interference characteristics, with total absence of "cross talk," and good response stability, under the operational conditions, allowed the application of the dual biosensor to the accurate real-time monitoring of alcoholic drinks and white grape musts.

Arecchi et al. (2010) described an amperometric biosensor for the detection of phenolic compounds in food, based on tyrosinase as bioelement. The enzyme was immobilized by drop-coating on a glassy carbon electrode, covered by a polyamidic nanofibrous membrane, prepared by electrospinning. With respect to others examples in the literature, the selectivity of the tyrosinase biosensor resulted in modification by the presence of the nanostructured coating, which seemed to affect the permeability of polyphenols as a function of solution pH, depending on polyphenol dissociation constants. The biosensor exhibited a response time of 16 s, a detection limit of 0.05 μM, and a linearity up to 100 mM. This biosensor was successfully used for real-time monitoring of the release kinetics of phenols, encapsulated in polymeric microcapsules.

Moreover, innovative detection methods for toxic compound detection in foods were proposed by different groups in Brazil and Italy. An example dealt with the development of a biosensor as an analytical device for the detection of beta-lactam residues in milk (Ferrini et al. 2008). This indirect method was based on the measurements of carbon dioxide (CO_2), produced by the microbial growth of a reference microorganism, namely, *Bacillus stearothermophilus var. calidolactis*. The addition of milk samples to the cultivation medium led to microbial growth inhibition, if beta-lactams are present, and, consequently, a decrease of CO_2 production rate. The analysis was based on the differences of CO_2 production between a milk sample, spiked with beta-lactams, and a twin milk sample, containing beta-lactams plus a broad spectrum beta-lactamases, using an electrochemical device. Moreover, the ability to sense all of the beta-lactams speeds the total time of analysis, when chemical identification and quantification are not required. The analytical method was adequate for milk control for qualitative screening purposes, complying with the requirements stated in Europe by the Decision 2002/657/EC. Campanella et al. (2009) described a new biosensor for rapid determination of nonsteroidal anti-inflammatory drugs (NSAIDs), based on the inhibition of cyclooxygenase by NSAIDs in fresh milk. The results showed the full validity of the method, which was optimized by comparing the inhibition of two enzyme isoforms, COX-1 and COX-2, in the presence of different tested pharmaceutical drugs (diclofenac, naproxen, ibuprofen, tolmetin). Recovery trials were performed in adulterated milk and fresh cheese samples with known concentrations of NSAIDs, always obtaining recovery values >88 %.

To date control strategies in detecting anabolic agents for promoting growth of food producing animals are mainly related to screening techniques based on immunochemical and physiochemical methods, whose major limit is represented

by relative low analytical sensitivity. As a consequence, consumers are currently exposed to molecules with potential carcinogenic effects, such as 17β-estradiol, the most powerful substance with estrogenic effect. Therefore, high analytical sensitivity screening and confirmatory methods are required, coupling easiness of use and efficiency. Ricciardi et al. (2013a, b) reported on the immunodetection of 17-β-estradiol in serum by antibody-immobilized microcantilever resonators, an innovative biosensing platform able to quantify an adsorbed target mass (such as cells, nucleic acids, biomolecules, etc.) thanks to a shift in resonance frequency. The analytical tool showed to be capable of discriminating treated and untreated animals, showing the ability of detecting traces of 17β-estradiol in serum at concentrations lower than the present accepted physiological serum concentration threshold value (40 ng/kg) and commercial ELISA tests (25 ng/kg). The method exhibits a limit of detection of 20 ng/kg and a limited cross-reactivity with high concentrations (10 μg/kg) of similar molecules (testosterone).

11.3 Biosensors for Bacteria and Bacteria Toxins Detection

The presence and diffusion of pathogenic bacteria in foodstuff represent the main concern for food safety, and innovative determination methods for microorganism and biological toxin detection, based on biosensor technology, were proposed. Recently, Rejeb et al. (2009) reported on a biosensor based on acetylcholinesterase (AChE) inhibition by mycotoxins, namely, aflatoxin B-1 (AFB-1). AChE was present in solution and an amperometric choline oxidase biosensor was used for monitoring AChE residual activity by determining choline produced from acetylcholine hydrolysis. To create the biosensor, choline oxidase was immobilized by cross-linking onto a screen-printed electrode modified with Prussian Blue and this was used to detect the H_2O_2 produced by choline oxidation at low applied potential (−0.05 V versus a screen-printed internal silver pseudo-reference electrode). For the development of the AFB-1 assay, several parameters, such as AChE and substrate concentrations, the effect of methanol, and pH, were evaluated and optimized. Authors found a linear working range of 10–60 ppb for AFB-1, and concentrations as low as 2 ppb, corresponding to the legal limit of AFB-1 in food for humans, were detected, after a pre-concentration step. The suitability of the method was evaluated using commercial olive oil samples.

Reis group's reported a new methodology based on the colorimetric response induced by pathogenic bacteria (*Staphylococcus aureus* and *Escherichia coli*) (Pires et al. 2011). The addition of bacterial supernatants caused a colorimetric modification in the presence of 10,12-pentacosadyinoic acid (PCDA) and *N*-[(2-tetradecanamide)-ethyl]-ribonamide (TDER) vesicles, even in diluted concentrations, indicating that chemical interactions occur between the vesicles and bacteria. It was observed that bacterial substrates released from *S. aureus* induced a more intense color change, compared to the optical response induced by *E. coli*. *S. aureus* metabolites also induced a more pronounced color change when TDER/PCDA vesicles were incorporated into cellulose strips. Authors analyzed the colorimetric response in the presence of interferent molecules, using

apple juice as food matrix. Both apple juice samples, sterile and inoculated with bacteria, induced a TDER/PCDA color change; however, the *S. aureus* supernatants induced a slightly greater color response both in the suspensions and in the cellulose strips. TDER/PCDA vesicles showed a great potential for the development of biosensors to detect food pathogens in intelligent food packaging.

A simple and fast response biosensor for screening nisin, directly identifying nisinogenic bacteria, by bioluminescence detection of *Lactococcus lactis* was proposed (Virolainen et al. 2012). The method is based on the nisin-controlled gene expression system which facilitates efficient overexpression of heterologous genes. The overlay of putative nisinogenic colonies with the biosensor strain gives identification results within 1 h. Functionality and specificity of the method were verified by screening for the presence of nisin producing bacteria among 144 raw milk colonies and a panel of 91 lactococcal strains. Studies performed on strains and colonies, which did not induce bioluminescence but inhibited *Lactococcus lactis NZ9800lux* growth, demonstrated that only nisinogenic bacteria can cause bioluminescence induction. Bacteria known to produce bacteriocins, other than nisin, failed to induce bioluminescence, further confirming the specificity of the assay. Moreover, authors discovered a new non-inducing, but inhibitory, lactococcal strain harboring a modified nisin Z gene and demonstrated that the source of the inhibitory action is not a non-inducing variant of nisin, but a bacteriocin of lower molecular weight. The concentration of nisin producing bacteria in a raw milk sample was 1.3×10^2 CFU/mL. A total of seven nisin Z producing colonies of *L lactis subsp. lactis*, which were shown to belong to three different groups by genetic fingerprinting, were also identified in raw milk samples. The presented biosensor is robust, cost-effective, and simple to use, avoiding the pitfalls of traditional screening methods by directly specifying the identity of the toxic substance.

Zamolo et al. (2012) developed an ultrasensitive electrochemiluminescence-based sensor for the detection of palitoxin (PlTX), one of the most potent marine toxins, frequently detected in seafood, taking advantage of the specificity provided by anti-PlTX antibodies, the good conductive properties of carbon nanotubes, and the excellent sensitivity achieved by a luminescence-based transducer. The sensor was able to produce a concentration-dependent light signal, allowing PlTX quantification in mussels, with a limit of detection of 2.2 μg/kg of mussel meat), more than two orders of magnitude more sensitive than that of the commonly used detection techniques, such as LC-MS/MS.

An antibody-immobilized microcantilever resonator system was proposed for the detection of mycotoxins, such as aflatoxins and ochratoxin A, which are considered as the most important chronic dietary risk factor, more than food additives or pesticide residues (Ricciardi et al. 2013a, b). The feasibility of using microcantilever resonator arrays to effectively identify total aflatoxins and ochratoxin A, at low concentrations (3 ng/mL and less than 6 ng/mL, respectively), with relatively low uncertainty (about 10 %) and good reproducibility for the same target concentration, was shown. Furthermore, the developed immunosensing method shows a limited cross-reactivity to different mycotoxins, paving the way to a highly specific technique, able to identify different mycotoxins in the sample.

Surface plasmon resonance (SPR)-based DNA biosensors were shown to be rapid, label-free, and selective tools for the detection of PCR products (Pascale et al. 2013). An SPR sensor based on DNA hybridization for the detection of *Fusarium culmorum*, a fungal pathogen of wheat, was described. A 0.57 kb DNA fragment of *F. culmorum* was amplified by specific primers, and a 25-mer oligonucleotide probe was selected within the sequence of the PCR amplified. The biotin-labeled probe was immobilized on a streptavidin sensor chip and tested for biospecific interaction with PCR products of *F. culmorum*. The SPR biosensor was applied to the detection of *F. culmorum* in fungal cultures and in naturally infected wheat samples.

Another example from Italy dealt with an electrochemical immunoassay, developed using magnetic beads as solid phase and carbon screen-printed arrays as transducers, was developed for the detection of sulfonamides in food matrices (Centi et al. 2010). Magnetic beads, coated with protein A, were modified by immobilization of specific antibodies and a competition between the target analyte and the corresponding labeled analyte was carried out. Analyte labeling was performed with alkaline phosphatase. After the immunosensing step, beads were captured by a magnet onto the working electrode surface of a screen-printed eight-electrode array for a multiple electrochemical detection. Screen-printed eight-electrode arrays were chosen as transducers due to the possibility to repeat multiple analysis and to test simultaneously different samples. The determination was performed by differential pulse voltammetry, as fast electrochemical technique. Calibration curves demonstrated that the developed electrochemical immunoassay was able to detect concentrations as low as ng/mL. The short incubation times and the fast electrochemical measurement make this system a possible alternative to classic ELISA tests.

Nucleic acid aptamers have been presented as a new way to detect pathogenic compound. These macromolecules have attracted intense interest and found wide applications in a range of areas (Palchetti and Mascini 2012; Tombelli and Mascini 2009). Aptamers exhibit many advantages as recognition elements in biosensing when compared to traditional antibodies. They are small in size, chemically stable, and cost effective. More importantly, aptamers offer remarkable flexibility and convenience in the design of their structures, which has led to novel biosensors that have exhibited high sensitivity and selectivity. Recently, Castillo et al. (2012) development a biosensor based on DNA aptamers for detection of ochratoxin A (OTA). The thiolated DNA aptamers specific to OTA of different configurations have been immobilized by chemisorption to the surface of a gold electrode. Electrochemical impedance spectroscopy in the presence of a redox probe, such as $[Fe(CN)6]^{-3/-4}$, has been used for the determination of the charge transfer resistance, following the addition of OTA containing samples. Charge transfer resistance increased with increasing OTA concentration in the range 100–0.1 nM. The limit of detection (0.12 nM) depended on aptamer configuration. The sensor was renewable and validated on food samples with satisfactory recovery.

Silva et al. (2012) described a new DNA biosensor for the detection of toxigenic *Penicillium sclerotigenum* in pure culture or infected yams. The *P. sclerotigenum* detection takes place on a self-assembled monolayer of a (magnetite)/(poly

(allylamine hydrochloride) (Fe_3O_4–PAH) composite that serves as an anchoring layer for the DNA hybridization interaction. Electrical impedance spectroscopy (EIS) was used to evaluate and quantify the hybridization degree. The Fe_3O_4–PAH composite represents a good platform for the immobilization of biomolecules, due to the presence of many possible binding sites for nucleotides and to its large surface-to-volume ratio, and good biocompatibility. The biosensor was capable of not only qualitatively detecting the presence of the fungus genome at low concentrations, but also showed a good quantitative impedimetric response. A Fe_3O_4–PAH-probe biosensor would require only small volumes and low concentrations of the analyte when used, for instance, in detecting *P. sclerotigenum* contamination of food, besides presenting many competitive advantages, such as selectivity, specificity, and reproducibility, relative to alternative techniques.

The development of a novel electrochemical immunosensor for the sensitive detection of staphylococcal enterotoxin A (SEA) based on self-assembly monolayer (SAM) and protein A immobilization on gold electrode was reported (Pimenta-Martins et al. 2012). Three different methods of protein A immobilization were tested: physical adsorption, cross-linking using glutaraldehyde, and covalent binding after activation with *N*-hydroxysuccinimide/*N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride on cysteamine-modified gold electrode. The EDC/NHS method for protein A immobilization was selected to lead development of the biosensor. The coating steps of the surface modification were characterized by cyclic voltammetry and the biosensor response by chronoamperometry. The advantages of the immunosensor were exposed in its high sensitivity and specificity. The proposed amperometric immunosensor was successfully used for determination of SEA in contaminated and non-contaminated cheese samples.

11.4 Pesticide Biosensors

Pesticides represent another class of compounds that have been studied in food analysis by different research groups in Brazil and Italy. Pesticides have been associated with many health hazards and rapid and reliable monitoring of these contaminants is mandatory.

Traditional chromatographic methods, such as high-performance liquid chromatography and capillary electrophoresis, equipped with mass spectrometry detectors, are effective for the analysis of pesticides in the environment, but present previously cited limitations. Thus, the use of biosensors to replace classical analytical methods by simplifying or eliminating sample preparation and making field-testing easier and faster with significant decrease in cost per analysis is attractive (Kantiani et al. 2010; McGrath et al. 2012).

A sensitive electrochemical acetylcholinesterase (AChE) biosensor was successfully developed with polyaniline (PANI) and multi-walled carbon nanotubes (MWCNTs) core–shell modified glassy carbon electrode (GC) and used to detect carbamate pesticides in fruit and vegetables (apple, broccoli, and cabbage).

The pesticide biosensors were applied in the detection of carbaryl and methomyl pesticides in food samples using chronoamperometry. The GC/MWCNT/PANI/AChE biosensor exhibited detection limits of 1.4 and 0.95 μM, respectively, for carbaryl and methomyl. These detection limits were below the allowable concentrations set by Brazilian regulation standards for the samples in which these pesticides were analyzed. Reproducibility and repeatability values of 2.6 % and 3.2 %, respectively, were obtained with the procedure. The proposed biosensor was successfully applied for the determination of carbamate pesticides in cabbage, broccoli, and apple samples, without any spiking procedure. The obtained results were in full agreement with those from HPLC analysis (Cesarino et al. 2012).

Pedrosa et al. (2007) reported on an acetylcholinesterase (AchE)-based amperometric biosensor developed by immobilization onto a self-assembled modified (SAM) gold electrode. Cyclic voltammetric experiments performed with the SAM-AchE biosensor in phosphate buffer solutions, containing acetylthiocholine, confirmed the formation of thiocholine and its electrochemical oxidation at +0.28 V vs Ag/AgCl. An indirect methodology involving the inhibition effect of parathion and carbaryl on the enzymatic reaction was developed and employed to measure pesticides in food samples without pretreatment or pre-concentration steps. Values higher than 91–98.0 % recovery indicated the feasibility of the proposed electroanalytical methodology to determine pesticides in food samples. The results obtained by the biosensor were compared with HPLC measurements, confirming the amperometrically measured values. The same research group reported on the detection of carbamates (a common class of pesticides in Brazil) in different vegetables (Cesarino et al. 2012). An electrochemical acetylcholinesterase biosensor was successfully developed on polyaniline and multi-walled carbon nanotubes core–shell modified glassy carbon electrode. The pesticide biosensor was applied to the detection of carbaryl and methomyl pesticides in food samples, by chronoamperometry. The biosensor exhibited detection limits of 1.4 and 0.95 μM for carbaryl and methomyl, respectively, which were below the allowable concentrations set by Brazilian regulation standards. Reproducibility and repeatability values of 2.6 % and 3.2 %, respectively, were obtained, and the proposed biosensor was successfully applied on cabbage, broccoli, and apple samples, without any spiking procedure. The obtained results were in full agreement with those obtained by HPLC.

Other paper describes the development of methodology for carbaryl determination in tomatoes (Caetano and Machado 2008). The measurements were carried out using an amperometric biosensor based on the inhibition of acetylcholinesterase activity due to carbaryl adsorption. The analytical curve obtained in buffered solutions showed excellent linearity in the 5.0×10^{-5} to 75×10^{-5} M range, with a limit of detection of 0.4×10^{-3} g/L. The application of the developed methodology on tomato samples involved a simple sample solubilization, followed by carbaryl spiking at different concentrations. Recovery values were in the 92.4–99.0 % range. For comparison, HPLC experiments were also carried out under similar conditions. However, with this analytical procedure, tomato samples have to be manipulated by an extraction procedure, which yielded much lower recovery values (78.3–84.8 %). Finally, the biosensor was employed to analyze

carbaryl directly inside the tomato fruit, without any previous manipulation. In this case, the biosensor was immersed in the tomato pulp, which was previously spiked with the pesticide for 8 min, and removed and inserted in the electrochemical cell. A recovery of 83.4 % was obtained, showing a very low interferent effect of matrix constituents.

11.5 Conclusions and Perspectives

Advances were recently made in biosensor applications for the determination of food composition and contaminants in Brazil and in Italy. Innovative devices were developed, aimed at the determination of food quality and at the presence of toxic substances, microorganisms, and residues. The proposed biosensors, using electrochemical, optical, and piezoelectric transducers, have the potential to achieve the low limits of detection imposed by legislation, and, at the moment, some of them could be transferred for further industrialization.

It is clear from the reviewed literature that common needs are more often being addressed. Nevertheless, it appears that further research into biosensor technologies and sample preparation techniques must be performed to create new systems that should be truly portable from laboratory to field. Anyway, biosensor technologies allow to provide simple to use, inexpensive, and portable systems that can be used to ensure the health and safety of consumers around the world.

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Chapter 12
Natural Ingredients as Additive for Active Antioxidant Food Packaging

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Abstract Active packaging materials have been developed to interact with the packed products and to extend their shelf life. When regarding antioxidant active packaging, it also has the advantage of allowing lower antioxidant addition to the food product. Although many synthetic antioxidant have already been used as antioxidant additives for polymers, recent studies have demonstrated that natural antioxidants are promising sources of additives. In this chapter, some aspects related to natural antioxidants have been reviewed and a few natural antioxidant sources, used as additive for active antioxidant food packaging materials, have been discussed.

Keywords Antioxidant • Active packaging • Natural additives

12.1 Introduction

Packaging materials are traditionally used to hold, protect, and sell food products. The protecting layer has the aim of preserving quality in order to minimize physical, chemical, and biochemical alterations that might contribute to the product degradation. The food industry, seeking for competitive advantages, search for safe and high-quality products. In this matter, packaging systems have been studied with the objective to interact with the packaged food, helping to extend shelf life (Azeredo et al. 2000). Such packaging materials are called "active packagings." The term "active" has been applied for the first time to food by Ted Labuza, in 1987, in a Scottish Conference about the nutritional impact of processed foods

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(Rooney 2005) and the article that literally applied the term "active packaging" was first published in December of 1986, entitled "Alcan Micro Match: an active packaging system" by Smith, J.D. (Mendes 2010).

Among the most important active packaging materials is the antioxidant packaging, which has a protective effect against the oxidation of the packed product (Vermeiren et al. 1999; López-de-Dicastillo et al. 2012). Its utilization in food can also allow the production of food with lower antioxidant addition (Rooney 1995; Soares and Hotchkiss 1998; Hayashi et al. 2006; Souza et al. 2011), helping to avoid allergies related to food preservative ingestion (Ahvenainen 2003).

Among the many existing antioxidant additives, those from natural sources have been cited as promising substitutes for synthetic additives in packaging materials (Hayashi et al. 2006; Grisi et al. 2008; Souza et al. 2011). In this work, some aspects related to natural antioxidants have been considered and a few natural antioxidant sources, used as additive for active antioxidant packaging of food materials, have been discussed.

12.2 Natural Antioxidants

Halliwell and Gutteridge (1995) defined antioxidants as "any substance that, when present at low concentrations compared with that of a substrate that can be oxidized, significantly delays or inhibits oxidation of that substrate," but later defined them as "any substance that delays, prevents or removes oxidative damage to a target molecule" (Halliwell 2007).

The antioxidant activity can be effective through various ways: as inhibitors of free radical oxidation reactions (preventive oxidants), by inhibiting formation of free lipid radicals; by interrupting the propagation of the autoxidation chain reaction (chain breaking antioxidants); as singlet oxygen quenchers; by synergism with other antioxidants; as reducing agents, converting hydroperoxides into stable compounds; as metal chelators, transforming metal pro-oxidants (iron and copper derivatives) into stable products; and finally as inhibitors of pro-oxidative enzymes (lipo-oxygenases) (Kancheva 2009; Carocho and Ferreira 2012).

The oxidative deterioration of fats and oils in foods is responsible for rancid odors and flavors, with a consequent decrease in nutritional quality and safety, caused by the formation of secondary, potentially toxic compounds. The addition of antioxidants is required to preserve food flavor and color, and to avoid vitamin destruction. Among synthetic antioxidants, the most frequently used to preserve food are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butyl hydroquinone (TBHQ). Reports reveal that BHA and BHT could be toxic, and their high manufacturing costs, along with the increasing consciousness of consumers with regard to food additive safety, created a need for identifying a more natural, and probably safer, antioxidant alternative (Moure et al. 2001; Bonilla et al. 2012).

The replacement of synthetic antioxidants by natural ones may have benefits due to health implications and functionality of food systems, such as solubility in both

oil and water, which is of interest for application in emulsions (Moure et al. 2001). Vegetable materials contain many compounds with antioxidant activity. Several plants (seeds, fruits, leaves, and roots) and derivatives have been studied as sources of potentially safe natural antioxidants for the food industry. Among the antioxidant components most thoroughly investigated from vegetables sources, polyphenols, flavonoids, carotenoids, vitamins, organic acids, and tocopherols are the most studied (Kaur and Harish 2001; Oliveira et al. 2011).

Polyphenols are secondary plant metabolites and confer both desirable and undesirable food qualities to fruits and vegetables. They are ubiquitous in plant material and sometimes present as esters and glycosides, possessing antioxidant activity as chelators and free radical scavengers, with special impact over hydroxyl and peroxyl radicals, superoxide anions, and peroxynitrites. One of the most studied and promising compounds, belonging to the hydroxybenzoic group, is gallic acid, which is also the precursor of many other tannins, while cinnamic acid is the precursor of all the hydroxycinnamic acids (Krimmel et al. 2010).

Flavonoids and related compounds occur in many plant and fruits. They belong to an antioxidant group of compounds, composed of flavonols, anthocyanins, isoflavonoids, flavanones, and flavones. Their antioxidant properties are conferred on the phenolic hydroxyl groups, attached to aromatic ring structures, and they can act as reducing agents, hydrogen donators, singlet oxygen quenchers, superoxide radical scavengers, and even as metal chelators. They are also able to activate antioxidant enzymes, reduce α-tocopherol radicals (tocopheroxyls), inhibit oxidases, mitigate nitrosative stress, and increase levels of uric acid and other low molecular weight molecules. Some of the most important flavonoids are catechin, catechin-gallate, quercetin, and kaempferol (Prochazkova et al. 2011).

Carotenoids are a group of natural pigments that are synthesized by plants and microorganisms, but not by animals. They are frequently used as natural coloring materials, but they also possess antioxidant activity, especially in the presence of light. The main antioxidant property of carotenoids is against singlet oxygen. They can be separated into two vast groups: the carotenoid hydrocarbons, known as the carotenes, which contain specific end groups, such as lycopene and β-carotene, and oxygenated carotenoids, known as xanthophylls, such as zeaxanthin and lutein (Carocho and Ferreira 2013).

Many studies have evidenced the antioxidant potential of natural plants, vegetables, oils, fruits, teas, etc., and in Table 12.1 a list of some natural compounds with antioxidant activity is presented.

Studies about the use of these natural antioxidants, as food additives, have increased in last years, and results are encouraging.

Table 12.1 Antioxidants from natural sources

Sources of active compounds	References
Mango/acerola	Investigation on the antioxidant activity of leaves, peels, stems bark, and kernel of mango (*Mangifera indica* L.) (Sultana et al. 2012)
	Agronomic characterization and antioxidant potential of fruit from clones of the acerola plant (Cunha et al. 2012)
Palm oil/açaí	Phenolic acid analysis and antioxidant activity assessment of oil palm (*E. guineensis*) fruit extracts (Neo et al. 2010)
	Açaí (*Euterpe oleraceae*) "BRS Pará": A tropical fruit source of antioxidant dietary fiber and high antioxidant capacity oil (Rufino et al. 2011)
Oregano oil/yerba mate tea	Sensory attribute preservation in extra virgin olive oil with addition of oregano essential oil as natural antioxidant (Asensio et al. 2012)
	Antioxidant activity of yerba mate extracts: Interactions between the individual polyphenols (Valerga et al. 2013)
Wine/jabuticaba	Prediction of total antioxidant capacity of red wine by Fourier transform infrared spectroscopy (Versari et al. 2010)
	Jaboticaba peel: Antioxidant compounds, antiproliferative and antimutagenic activities (Leite-Legatti et al. 2012)
Cocoa/coffee	Comparison of the antioxidant activity of commonly consumed polyphenolic beverages (coffee, cocoa, and tea) prepared per cup serving (Richelle et al. 2001)

12.3 Antioxidant Food Packaging

Incorporation of antioxidants into packaging films has become very popular, since oxidation was recognized as one of the main causes of food spoilage. Oxidation alters the taste (rancidity) and nutritional quality (loss of vitamins and essential fatty acids) of foods, and generates reactive and toxic compounds, which may represent a danger to consumers (Laguerre et al. 2007).

Synthetic antioxidants are the most used antioxidant additives to prevent/retard the oxidation process. Such additives recently received a great deal of interest due to toxicological concerns, prompting an increased interest in natural antioxidants, such as those derived from fruits, vegetables, plants, and others (Bonilla et al. 2012).

Recently, researches about the applications of natural antioxidants in active packaging have being cited in the literature, as reported in Table 12.2.

Antioxidants properties of protein-based films from fish skin gelatin, incorporated with different citrus essential oils, including bergamot, kaffir lime, lemon, and lime, were investigated. Films incorporated with lemon essential oil showed the highest ABTS radical scavenging activity and ferric reducing antioxidant power, among other modified films (Tongnuanchan et al. 2012).

Souza et al. 2011 developed several active films from starch cassava, containing mango and acerola pulps as antioxidant additives, using a response surface methodology for film characterization. The films were used to pack palm oil (maintained for 45 days of storage) under accelerated oxidation conditions (63 % relative

Table 12.2 Natural antioxidant additives for active packaging materials

Antioxidante natural	Based films	Packaged product	Results	References
Green tea extract	Chitosan Gelatin		The incorporation of aqueous green tea extract into chitosan films improved mechanical and water vapor barrier properties and enhanced polyphenolic content and antioxidant activity of the films The incorporation of GTE into gelatin films enhanced the total phenolic content, DPPH radical scavenging activity, and reducing power. However, DPPH radical scavenging activity and reducing power decreased during storage	Siripatrawan and Harte (2010) Wu et al. (2013)
Essential oils of pimento and oregano	Milk protein	Beef muscle	Oregano-based films stabilized lipid oxidation in beef muscle samples, whereas pimento-based films presented the highest antioxidant activity	Oussalah et al. (2004)
Marigold extract	Low-density polyethylene	Soybean oil	Spectroscopic, optical, and mechanical properties of the films were affected by the addition of the marigold extract. However, bags made of the films showed a positive effect on soybean oil stability when used as packaging	Colín-Chávez et al. (2012)
Barley husks	Low-density polyethylene	Blue shark muscle	The results confirm the efficacy of active packaging with a natural antioxidant derived from barley husks to slow the progress of lipid hydrolysis and increase oxidative stability in blue shark muscle	Pereira de Abreu et al. (2011)
Mango and acerola pulps	Starch	Palm oil	Although the film-forming procedure affected the	Souza et al. (2011)

(continued)

Table 12.2 (continued)

Antioxidante natural	Based films	Packaged product	Results	References
			antioxidant compounds, the results indicated that antioxidants were effective additives for protecting the packaged product	
Palm fruit	Cassava starch	Soybean oil	Results have indicated that the palm antioxidant compounds in the packaging material were preferentially oxidized, lowering the oxidation of the packed product	Grisi et al. (2008)
Cocoa/coffee	Cassava starch	Palm oil	Results have indicated efficacy when protecting the product in accelerated storage conditions. Besides the antioxidant effect, the resulting films also were colored by the cocoa and coffee pigments and flavored by their pleasant natural flavors	Silva (2009)
Barley husks	Low-density polyethylene	Blue shark muscle	The results confirm the efficacy of active packaging with a natural antioxidant derived from barley husks to slow the progress of lipid hydrolysis and increase oxidative stability in blue shark muscle	Pereira de Abreu et al. (2011)
Mustard meal	Xanthan gum	Smoked salmon	The composite coating improved the stability of smoked salmon against lipid oxidation without imparting a negative sensory quality to the salmon	Kim et al. (2012)

humidity and 30 °C) to simulate a storage experiment. Bio-based films were prepared (casting) by dispersing cassava starch (4 %), sucrose (1.4 %), inverted sugar (0.7 %), and mango and/or acerola fruit pulp (0–20 %) in distilled water,

according to a (2^2) second-order experiment design (for a total of 11 experiments). It was noted that palm oil packed in bio-based films containing mango and acerola pulps showed a low peroxide index, when compared to the product packed in control films. The results indicated the efficacy of fruit pulps as antioxidant additives, acting to protect the packaged product. This effect can be considered concentration-dependent; palm oil packed in films with low pulp concentrations presented a higher oxidation value (IP = 64.27 %), when compared to packed in films with higher pulp concentrations, showing a lower PI value (IP = 31.62 %), during the same storage period. The increase of carotenoid content in the film showed a higher correlation with peroxide index (98.39 %) than polyphenols (56.99 %), confirming the efficacy of carotenoids incorporated into films in comparison to polyphenols. As mango pulp possesses a higher amount of carotenoids than acerola pulp, the product packed in films containing mango pulp showed less oxidation. It was observed by authors that the packaging material, rather than the packaged product, was oxidized, due to the active compound loss during storage. Figure 12.1 shows the mango and acerola antioxidant film and the surface response of the increase in the peroxide value of the packed oil after 45 days storage (63 % relative humidity and 30 °C), influenced by the mango and acerola pulp concentration as additive.

A similar behavior was observed in films containing palm fruit pulp and its oil, as added antioxidants, which were used to pack soybean oil. In this case, a decrease in TC ranging from 79.90 to 99.60 % was observed during 90 days of storage (Grisi et al. 2008). Active polymers added to palm oil had presented the best antioxidant effect in packed oil (64 % RH, 30 °C). Authors noticed that increasing the palm fruit derivates' (extract and oil) addition decreased the final film total carotenoid content. However, when observing the total carotenoid content in the packed product, the higher was the antioxidant addition to the film, especially palm oil, the higher was the total carotenoid content. Results have indicated that, also in this study, the packaging material was oxidized, rather than the packaged product. Besides the presence of fibers, resulting from palm fruit addition, and oil incorporation, the polymer appeared with good transparency. Figure 12.2 shows the film appearance and transparency after palm fruit addition, and the surface response surface of the palm fruit extract vs. palm oil effect on the total carotenoid content (TC) of the packed product (soy bean oil) after 90 days of storage (63 % relative humidity and 30 °C).

12.4 Conclusions

Literature results have demonstrated that natural antioxidants can be incorporated, as additives, in conventional or biodegradable active packaging materials. Unfortunately, food and pharmaceutical industries have taken little advantage of such an innovation opportunity. A possible reason can be the lack of studies about large-scale production of such antioxidant materials.

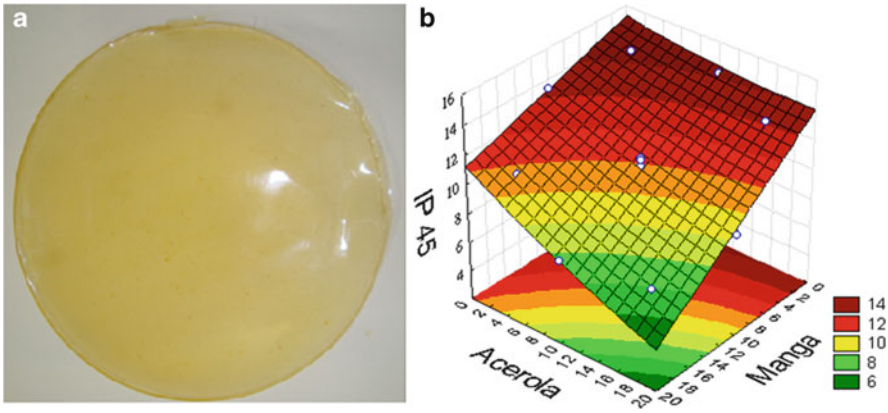

Fig. 12.1 (**a**) The film appearance and (**b**) the effect of mango and acerola addition as additive on the peroxide value increase of the packed oil after 45 days at storage (63 % relative humidity and 30 °C), Souza et al. (2011)

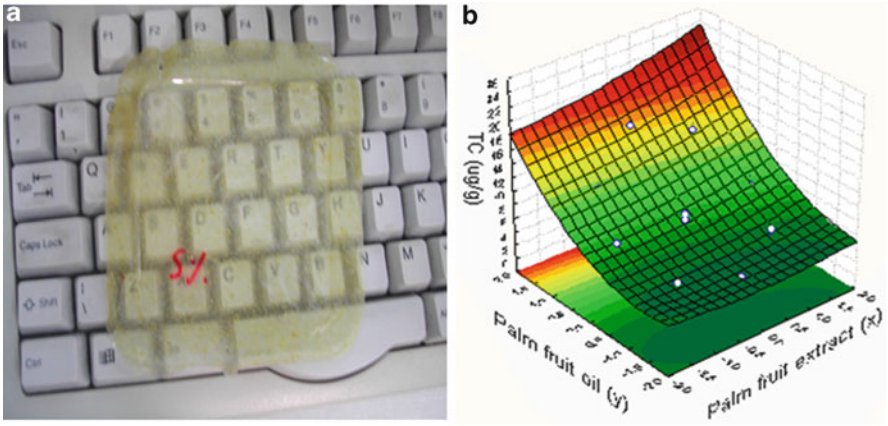

Fig. 12.2 (**a**) The palm fruit film appearance and (**b**) the response surface of the total carotenoid content in packed oil after 90 days at storage (63 % relative humidity and 30 °C), Grisi et al. (2008)

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