

# Chapter 11

## Nuclear Magnetic Resonance: Methodologies and Applications

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### 11.1 Introduction

High-resolution nuclear magnetic resonance (NMR) spectroscopy has proven to be a powerful tool for the analysis of multicomponent systems such as olive oil (Sacchi et al. 1997; Vlahov 1999; Hidalgo and Zamora 2003; Mannina and Segre 2002;

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Mannina et al. 2003a; Dais and Spyros 2007; Brescia and Sacco 2008a, b; Mannina and Sobolev 2011). Recent advances in NMR methodology and instrumentation made this technique an alternative choice in olive oil analysis. With the exception of low detection limits (however, see below), the combined capabilities of multinuclear and multidimensional NMR methods made it feasible to obtain structural and quantitative information on a wide range of organic metabolites in plant extracts in a single experiment and, more importantly, with no (or minimal) sample pretreatment. These properties allow a substantial reduction in labor and time and the prospect of identification of unexpected or previously unknown components. NMR parameters, such as chemical shifts, spin multiplicities, coupling constants, and signal intensities, provide valuable pieces of structural information and ensure valid quantitative results.

Another facet of NMR spectroscopy involves relaxation time measurements and spin-echo methods in low magnetic fields. Low-resolution, time-domain NMR spectroscopy has found increasing use in the oil industry as a low-cost method of analysis of fats and oils (Gambhir 1992). New developments in relaxometry and diffusometry can be useful when the components of a mixture are chemically similar and have spectra that highly overlap (van Duynhoven et al. 2010). Discrimination of pure components in mixtures is based on differences in solution diffusivity. The implementation of pulsed-field gradients (PFGs) in NMR spectrometers advanced the analysis of mixtures in low- and high-field NMR. A brief note about a relatively old NMR technique based on deuterium NMR, Site-Specific Natural Isotope Fractionation Nuclear Magnetic Resonance (SNIF-NMR), will be mentioned briefly in relation to olive oil.

The aim of this chapter is to present the NMR methodologies that are being used in the analysis of olive oil and the progress they have made so far in olive oil quality control and authentication. Strategies will be suggested to overcome routine problems, for example, complex NMR data sets due to severe signal overlap in overcrowded  $^1\text{H}$  NMR spectra, or ambiguities in quantitative analysis using  $^{13}\text{C}$  NMR spectra. NMR instrumental and experimental hints to obtain high-quality spectra and useful information about sample preparation will be given as well. The potential of NMR spectroscopy for the analysis of olive oil will be presented, focusing on those components that can serve as potential markers of EVOO quality and authentication. A brief discussion will be made about the preprocessing of NMR data for chemometric analysis and about relevant applications to olive oil adulteration and classification studies according to geographical and varietal origin. The final section will be devoted to future trends and perspectives of the scientific aspects described in the chapter.

## 11.2 High-Resolution Nuclear Magnetic Resonance Spectroscopy

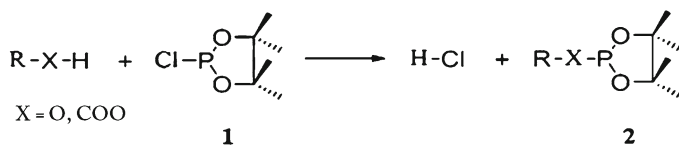
### 11.2.1 *Multinuclear Methodologies*

Three basic nuclear spins –  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{31}\text{P}$  – have been used extensively for the analysis of olive oil, giving complementary and sometimes unique information about the identity of major and minor constituents of olive oil. In contrast to  $^1\text{H}$  and

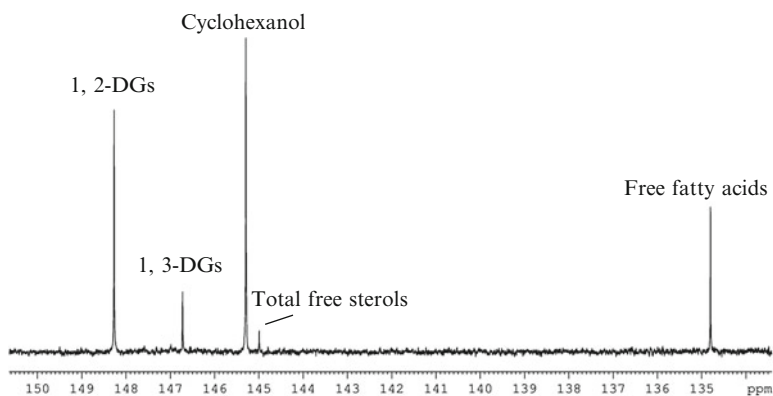
$^{13}\text{C}$  nuclei,  $^{31}\text{P}$  NMR spectroscopy is a distractive methodology, inasmuch as it requires derivatization of the olive oil components bearing hydroxyl and carboxyl groups (e.g., phenolic compounds) with a phosphorus reagent (see below). Recording high-resolution, one-dimensional (1D)  $^1\text{H}$ ,  $^{13}\text{C}$ , and  $^{31}\text{P}$  NMR spectra for qualitative analysis employing the one-pulse sequence is a trivial matter and the success of the analysis depends, among other things, on the available instrumentation and the skill of the NMR user.

In general,  $^1\text{H}$  NMR spectra of mixtures are expected to be more complicated than  $^{13}\text{C}$  and  $^{31}\text{P}$  spectra. The presence of scalar coupling among neighboring protons and the much smaller chemical shift range of protons (approximately 15 ppm) results in overcrowded spectra with severe signal overlap. The much higher paramagnetic contribution to the shielding of  $^{13}\text{C}$  and  $^{31}\text{P}$  nuclei increases dramatically their chemical shift range, approximately 250 ppm for  $^{13}\text{C}$  and approximately 1,000 ppm for  $^{31}\text{P}$ ; this capacity facilitates the wider distribution of signals. Moreover,  $^{13}\text{C}$  and  $^{31}\text{P}$  spectra are usually obtained under broadband proton decoupling to eliminate all couplings with protons of the molecule, resulting in single signals for nonequivalent carbon or phosphorus nuclei. This ensures further signal separation, while at the same time the intensity of the decoupled signals increases mainly due to a nuclear Overhauser enhancement (NOE) contribution. It is worth mentioning that proton decoupling greatly simplifies  $^{13}\text{C}$  ( $^{31}\text{P}$ ) spectra, but it removes valuable structural information inherent in the coupled spectra.

Apart from the wide range of  $^{31}\text{P}$  chemical shifts, the 100 % natural abundance of the  $^{31}\text{P}$  nucleus and its high sensitivity, which is only approximately 15 times less than that of the proton nucleus, make  $^{31}\text{P}$  NMR experiments a reliable analytical tool to determine amounts on the order of micromoles, or lower, depending on the available instrumentation. These properties of the  $^{31}\text{P}$  nucleus should be contrasted with the low natural abundance (1.1%) and sensitivity of the  $^{13}\text{C}$  nucleus, which in addition is characterized by long relaxation times. Therefore,  $^{31}\text{P}$  NMR spectroscopy can be considered an alternative methodology to  $^1\text{H}$  and  $^{13}\text{C}$  NMR in cases where severe overlap is observed in  $^1\text{H}$  NMR spectra or quantitative  $^{13}\text{C}$  NMR experiments require lengthy accumulations and long relaxation delays to achieve a satisfactory signal-to-noise ratio (see below). Since no constituent of olive oil (besides phospholipids) contains phosphorus nuclei, detection of minor components bearing hydroxyl or carboxyl groups, such as diacylglycerols and phenolic compounds, has been accomplished with prior replacement of the labile hydrogens of functional groups of olive oil constituents with the reagent 2-chloro-4,4,5,5-tetramethyldioxaphospholane (labeled 1), according to the reaction scheme shown in Fig. 11.1, and the use of  $^{31}\text{P}$  chemical shifts of the phosphitylated compounds (labeled 2) to identify the labile centers (Spyros and Dais 2000). Compound 1 reacts rapidly (approximately 15 min) and quantitatively under mild conditions (within the NMR tube) with hydroxyl groups. As an example, Fig. 11.2 shows the 202.2 MHz  $^{31}\text{P}$  NMR spectrum of a phosphitylated olive oil sample in a region where the signals of diacylglycerol isomers, total free sterols, and free fatty acids appear. The signal at 145.15 belongs to the internal standard cyclohexanol used for quantitative purposes.



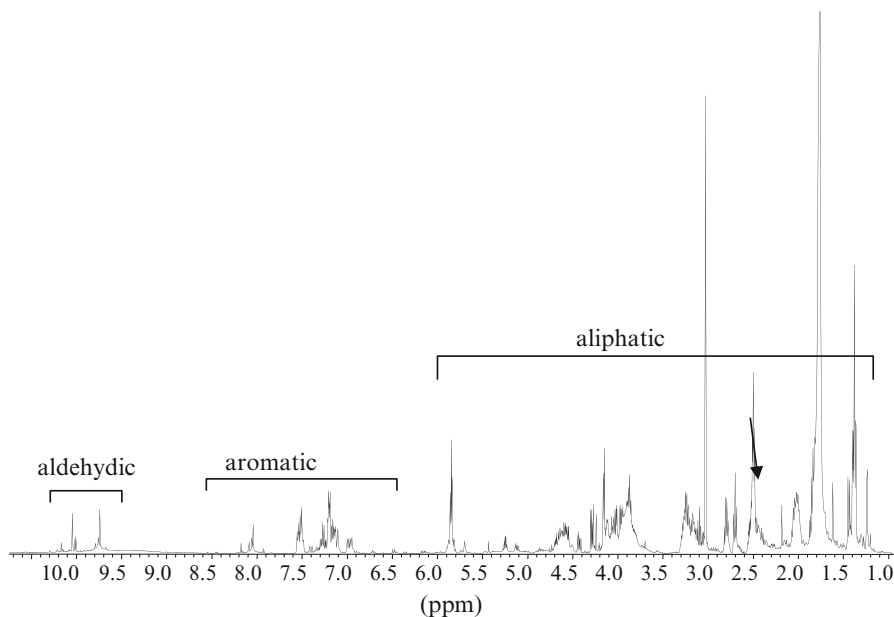
**Fig. 11.1** Reaction of hydroxyl and carboxyl groups of olive oil constituents with phosphorus reagent 2-chloro-4,4,5,5-tetramethyldioxaphospholane (**1**)



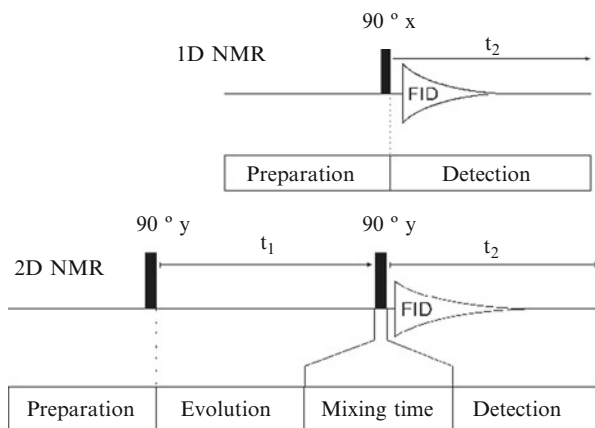
**Fig. 11.2** 202.2 MHz  $^{31}\text{P}$  NMR spectrum of virgin olive oil. The region where the phosphitylated total sterols, diacylglycerols, and free fatty acids resonate is illustrated. The phosphitylated cyclohexanol is used as internal standard. *1,2-DAD* 1, 2-diacylglycerols, *1,3-DAG* 1, 3-diacylglycerols (Source: Vigli et al. (2003), with permission of American Chemical Society)

### 11.2.2 Multidimensional Nuclear Magnetic Resonance Methodologies for Spectral Assignments

Depending on the extent of overlap in 1D  $^1\text{H}$  NMR spectra of mixtures, it is not always possible to extract spectroscopic parameters with certainty. This is exactly the case with the 500 MHz  $^1\text{H}$  NMR spectrum of the polar part of olive oil extracted with a mixture of methanol:water (80/20 v/v) in DMSO- $d_6$  solvent (Fig. 11.3). This spectrum, which can be divided into three frequency regions, contains signals of a large number of phenolic compounds (Christophoridou and Dais 2009). The development of multidimensional NMR techniques offers the possibility to unravel hidden resonances, either through bond (scalar spin-spin coupling) or through space (dipolar coupling) connectivity. The basic concept of 2D NMR is the introduction of an additional time interval, the evolution period, between the preparation and detection intervals of the 1D pulse sequence (Fig. 11.4). Depending on factors that influence the transverse magnetization of the nuclear spins during the evolution time  $t_1$  (e.g., Larmor precession or scalar coupling), different types of correlations are developed between homonuclear and heteronuclear spins. In some experiments



**Fig. 11.3** 500 MHz  $^1\text{H}$  NMR spectrum of polar fraction of olive oil in  $\text{DMSO-d}_6$  solvent. Three separate frequency regions for aldehydic, aromatic, and aliphatic protons of phenolic compounds were observed



**Fig. 11.4** Schematic representation of one-dimensional (1D) and two-dimensional (2D) pulse sequences with mixing time. FID = free induction decay

an additional time interval, a single pulse, a time period, or a combination of both is added between the evolution and the detection interval, the so-called mixing period (Fig. 11.4), to guarantee the transfer of magnetization from one type of spin to the other. The evolution time is kept variable. Actually, it is made stepwise longer (incremented) in analogy to the detection time  $t_2$ . For each  $t_1$  increment, a separate

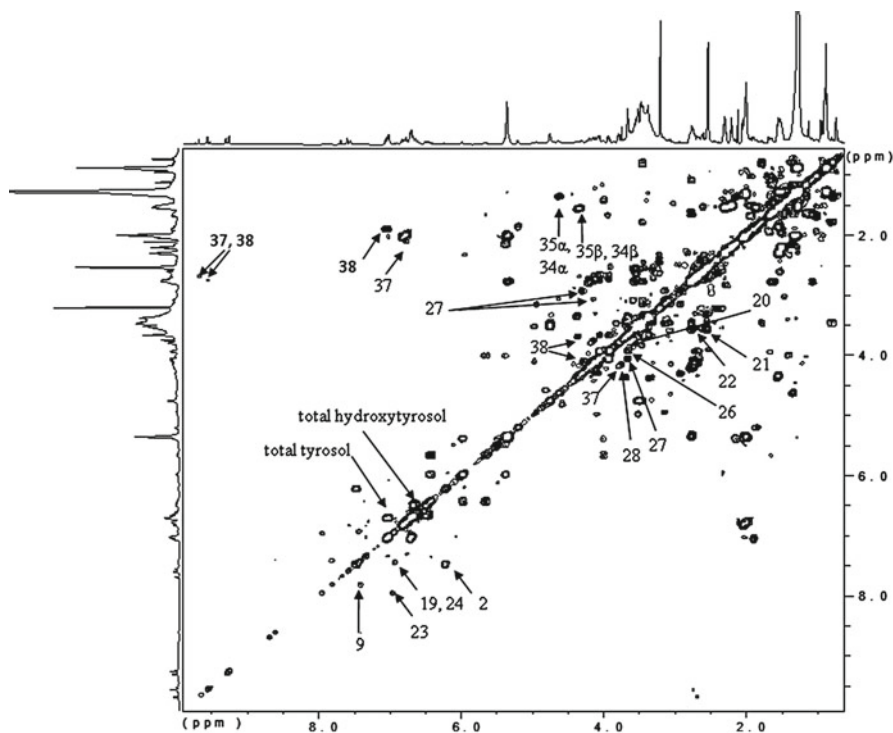
free induction decay (FID) is detected in  $t_2$ . Thus an NMR signal is obtained in the time domain,  $S(t_1, t_2)$ , which is a function of two time variables. Double Fourier transformation gives a 2D spectrum (stacked or contour plot), whose axes describe different spectroscopic parameters (e.g., chemical shifts, coupling constants, NOEs) evolved during the evolution period. Correlations of different spectroscopic parameters in homonuclear or heteronuclear 2D spectra are visualized by the so-called cross peaks. Moreover, the removal of unwanted signals by the selection of pertinent coherence pathways by means of pulsed field gradients make unnecessary the time-consuming and relatively inefficient phase-cycling procedure. NMR pulse sequences of different dimensionalities (2D, 3D, and 4D) have been developed in recent years and implemented in modern NMR spectrometers. However, no more than ten basic 2D NMR methods have found widespread application in food analysis. These methods are summarized in Table 11.1. Pulse sequences are arranged according to the type of correlated nuclei, the nature of the correlation, and pertinent applications. A short description and appropriate references for each type of the pulse sequence in Table 11.1 can be found in Berger and Braun (2004).

The gradient-selected 500 MHz COSY spectrum in Fig. 11.5 has greatly facilitated the profiling of phenolic compounds contained in the polar part of olive oil depicted in Fig. 11.3. This spectrum, recorded in less than 15 min, allowed the identification of most phenolic compounds and confirmed previous lengthy assignments based on model compounds (Christophoridou and Dais 2009). Figure 11.6 shows the 600 MHz gradient-selected TOCSY spectrum of an olive oil sample, which shows correlations between all protons of olive oil, even distant ones, as long as there are couplings between all intervening protons. Table 11.2 shows the identity of protons and the numbering system adopted by most researchers. The observed connectivities between the glycerol backbone protons and between the acyl-chain protons provides solid proof of previous assignments based on triacylglycerol model compounds. It is interesting to note that the very good resolution of the TOCSY spectrum permits the observation of the allylic protons ( $H6'$ ) of the linolenyl chain, which are connected with the methyl protons ( $H9$ ) of the same chain ( $\delta$  0.96). TOCSY and COSY experiments provide a particularly powerful combination, which could be sufficient to identify most metabolites present in mixtures.

The assignment of carbon resonances of glycerol and fatty acids is of crucial importance for the analytical characterization of olive oil. The 600 MHz  $^{13}\text{C}$  NMR spectrum of olive oil in  $\text{CDCl}_3$  illustrated in Fig. 11.7 shows a large number of signals spread over a wide range of chemical shifts. This made the spectrum appear complicated but nevertheless much more informative than the  $^1\text{H}$  NMR spectrum, which extends to a narrow region of a few parts per million (Fig. 11.6). The resonances of the glycerol backbone carbons, as well as those of the fatty acids, were assigned in previous studies by several investigators. Most of these studies utilized triacylglycerol model compounds to assign carbon signals. However, this procedure raised doubts about the correctness of the assignments, especially for the interior carbons of acyl chains, and by the fact that carbon chemical shifts were found to be concentration dependent, particularly for those in the carbonyl region of the spectrum (Mannina et al. 2002). Two-dimensional NMR spectroscopy is capable of

**Table 11.1** Two-dimensional (2D) NMR techniques used mostly in food science

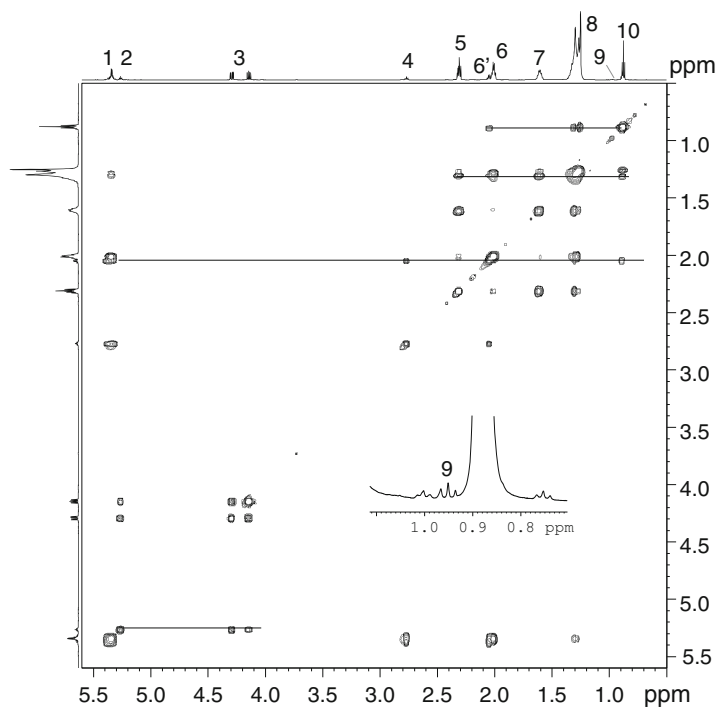
Name	Acronym	Application	Comments
<i>Homonuclear (H-H) techniques</i>			
Correlation Spectroscopy	H,H-COSY	Signal assignment	Replaced by the more powerful TOCSY
(a) Normal		Detection of small couplings	Diagonal signals with mixed phases
(b) With delay		Determination of $J$ values	Phase sensitive; low-intensity diagonal signals
Double quantum filtered correlation spectroscopy	DQF-COSY		
Total correlation spectroscopy	TOCSY	Signal assignment within a scalar-coupled proton system	Extent of magnetization transfer depends on length of spin-lock period
Nuclear Overhauser enhancement spectroscopy	NOESY	Distance information, 3D structure of large molecules, molecular dynamics information	Major disadvantage is its dependence on molar mass and viscosity, which can change its sign; this may cause its disappearance under certain conditions
Rotating frame Overhauser enhancement spectroscopy	ROESY	Separation of chemical exchange and NOE in small and medium-sized molecules; dynamic information	Sign is always positive; applicable to small and medium-sized molecules; shorter mixing times
<i>Heteronuclear (H-X) techniques</i>			
Heteronuclear correlation spectroscopy	HETCOR	Heteronuclear shift correlation through one-bond coupling	Used for carbon signal assignment; no field gradients required; short setup; good resolution at carbon dimension, but low sensitivity
Heteronuclear multiple quantum coherence	HMQC	Heteronuclear shift correlation through one-bond coupling	Two to four times more sensitive than HETCOR depending on molecular size; good resolution in proton dimension; broader cross peaks than HETCOR due to unsuppressed H-H couplings
Heteronuclear single quantum coherence	HSQC	Heteronuclear shift correlation through one-bond coupling	Better resolution than HMQC; no signal broadening by H-H coupling
Heteronuclear multiple bond coherence	HMBC	Heteronuclear shift correlation through two- and three-bond coupling	Suppression of unwanted one-bond coupling does not work equally well for all protons
Heteronuclear Overhauser effect spectroscopy	HOESY	Through space H-X correlations; distance information between quaternary carbons and nearby protons	Good resolution at X-nucleus dimension
Incredible natural abundance double quantum transfer experiment	2D-INADE QUATE	Structure elucidation; provides unequivocal C-C connectivity	Poor sensitivity



**Fig. 11.5** 500 MHz gradient-selected COSY spectrum of polar fraction of olive oil in DMSO- $d_6$  solvent. Codes correspond to phenolic compounds: (2) *p*-coumaric acid, (9) vanillic acid, (19) vanillin, (20) homovanillyl alcohol, (21) free tyrosol, (22) free hydroxytyrosol, (23) apigenin, (24) luteolin, (26) (+) pinoresinol, (27) (+) 1-acetoxypinoresinol, (28) syringaresinol, (34 $\alpha$ ) aldehydic form of oleuropein; isomer 5*S*, 8*R*, 9*S*, (34 $\beta$ ) aldehydic form of oleuropein; isomer 5*S*, 8*S*, 9*S*, (35 $\alpha$ ) aldehydic form of ligstroside; isomer 5*S*, 8*R*, 9*S*, (35 $\beta$ ) aldehydic form of ligstroside; isomer 5*S*, 8*S*, 9, (37) dialdehydic form of oleuropein lacking a carboxymethyl group, (38) dialdehyde form of ligstroside lacking a carboxymethyl group

confirming and, in some cases, correcting earlier assignments. The combination of the gradient-selected HSQC and HMBC experiments can be used to observe correlations between heteronuclei. The former experiment correlates the chemical shifts of carbons with those of the directly attached protons, whereas the latter experiment connects the carbon with protons two or three bonds away. A recent modification of the HSQC pulse sequence combines the usual C-H bond correlation with carbon multiplicity selection similar to that obtained using the DEPT-135 experiment. The edited gradient-selected HSQC spectrum (Fig. 11.8) shows correlations between the glyceridic protons and carbons of olive oil. Correlations phased negatively (unframed cross peaks) and positively (framed cross-peaks) represent methylene and methine protons, respectively. This spectrum is of major help for the assignment of the glyceridic carbons and several of the acyl-chain carbons. In addition, it achieves a partial separation of the olefinic proton signals through their correlation with the well-resolved olefinic carbon resonances in the region 127–131 ppm (Fig. 11.8).

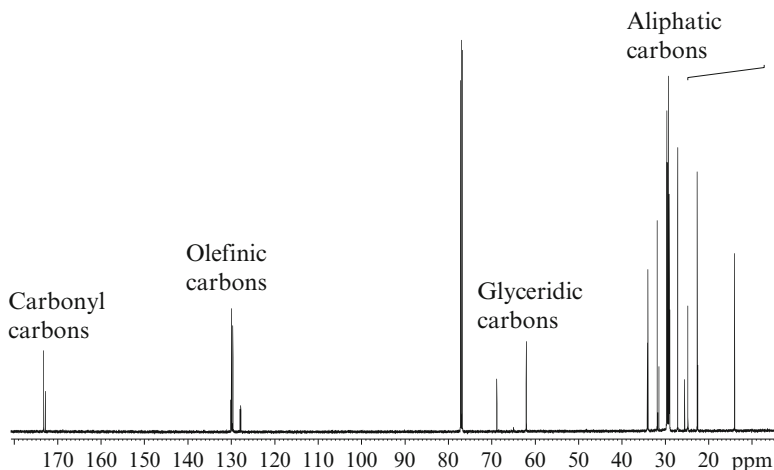




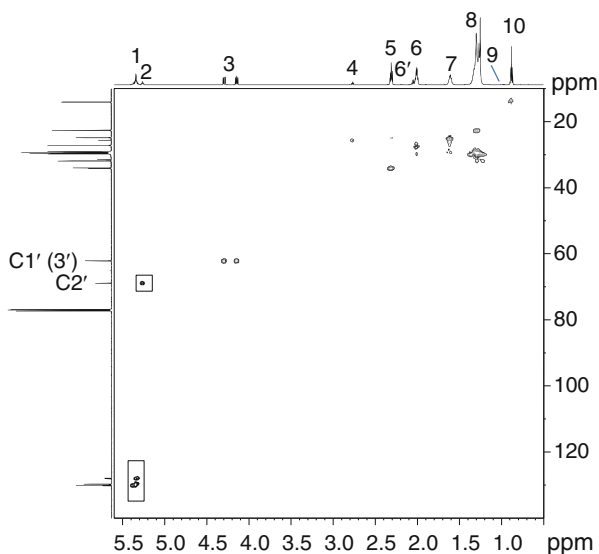
**Fig. 11.6** 600 MHz gradient-selected TOCSY spectrum of olive oil sample in  $\text{CDCl}_3$  solvent. The numbering system for protons was explained in Table 11.2. The *inset* magnifies the triplet of the methyl protons of the alpha-linolenyl chain

**Table 11.2** Chemical shifts and assignment of signals in  $^1\text{H}$  NMR spectrum of virgin olive oil in chloroform-d solvent

Signal	$\delta$ (ppm)	Proton	Attribution
1	5.30	$\text{CH}=\text{CH}$	All unsaturated fatty acids
2	5.16	$\text{CH-OCOR}$	Triacylglycerols
3	4.19	$\text{CH}_2\text{-OCOR}$	Triacylglycerols
4	2.77	$\text{CH}=\text{CH-CH}_2\text{-CH}=\text{CH}$	Linoleyl and alpha-linolenyl chains
5	2.22	$\text{CH}_2\text{-COOH}$	All acyl chains
6	2.03	$\text{CH}_2\text{-CH}=\text{CH}$	Oleyl and linoleyl chains
6'	2.04	$\text{CH}_2\text{-CH}=\text{CH}$	Linolenyl chain
7	1.62	$\text{CH}_2\text{-CH}_2\text{-COOH}$	All acyl chains
8	1.21	$(\text{CH}_2)_n$	All acyl chains
9	0.96	$\text{CH}=\text{CH-CH}_2\text{-CH}_3$	Alpha-linolenyl chain
10	0.85	$\text{CH}_2\text{CH}_2\text{CH}_2\text{-CH}_3$	All acyl chains except alpha-linolenyl

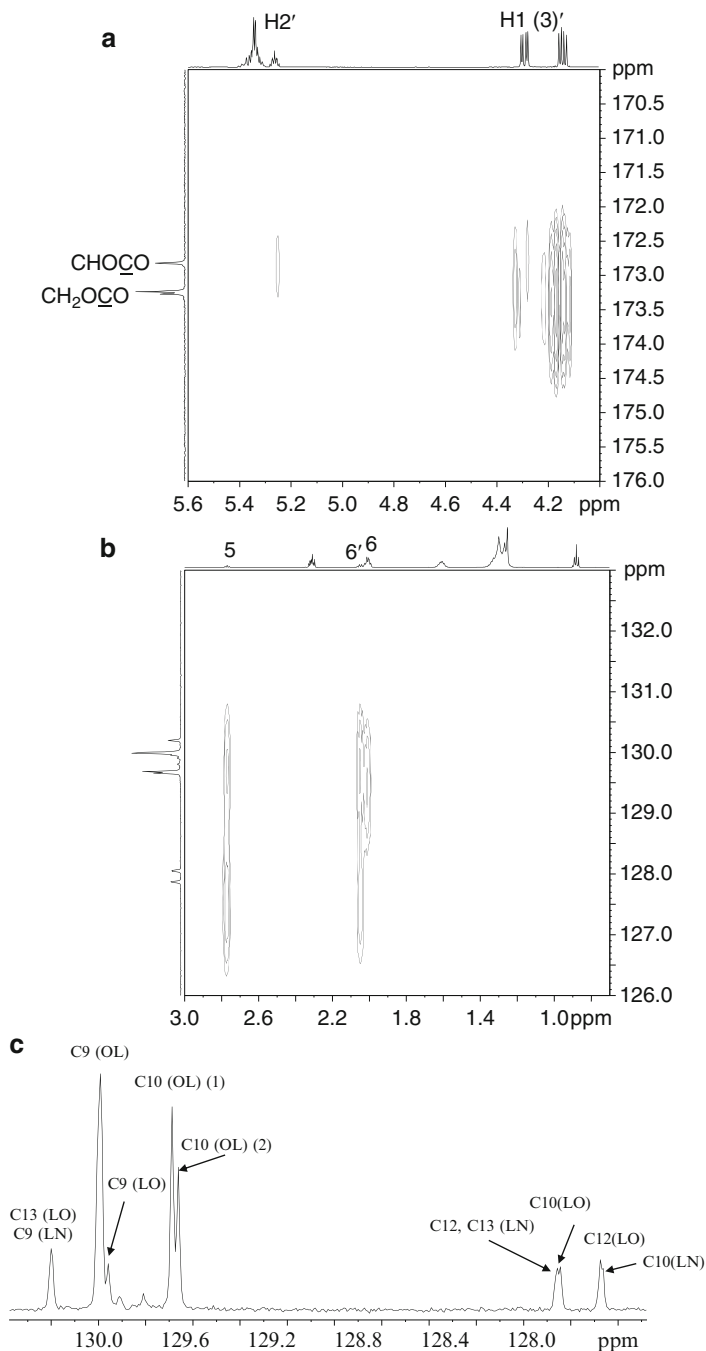


**Fig. 11.7** 500 MHz  $^{13}\text{C}$  NMR spectrum of an olive oil sample in  $\text{CDCl}_3$  solvent. Four separate frequency regions for the carbonyl, olefinic, glyceridic, and aliphatic carbon nuclei are observed (Source: Vlahov (1999), with permission of Elsevier)



**Fig. 11.8** 600 MHz edited HSQC (E.HSQC) spectrum of olive oil sample in  $\text{CDCl}_3$  solvent showing one-bond correlations between carbons and directly attached protons. Correlations phased negatively (*unframed*) and positively (*framed*) represent the methylene and methine protons, respectively

The HMBC spectrum of the olive oil sample in Fig. 11.9a illustrates cross peaks correlating the protons of the glycerol segment with the carboxyl carbons of the attached fatty acid chains. Earlier assignments of these carbon signals for vegetable oils were explained by taking into consideration the double bond inductive effect on the carbonyl groups (Vlahov 1999) or the use of model compounds (Mannina et al. 1999a).



**Fig. 11.9** Portions of 600 MHz gradient-selected HMBC spectrum of olive oil sample in  $\text{CDCl}_3$  solvent showing connectivities (a) between glycerol backbone protons and corresponding carbonyl carbons; (b) between allylic and bis-allylic protons and olefinic carbons of the fatty chains; (c) detailed assignment of olefinic carbons. Numbers 1 and 2 in parentheses denote the position of the attached acyl chains to the glycerol moiety

Another portion of the same spectrum (Fig. 11.9b) depicts the connectivity between the olefinic carbons and the corresponding allylic and bis-allylic protons of oleyl, linoleyl, and linolenyl chains. The assignment of the olefinic carbons for an olive oil sample is shown in Fig. 11.9c. The positional distribution of the acyl chains on the glycerol backbone and the complete assignment of the  $^{13}\text{C}$  NMR signals in tripalmitin were achieved using the hybrid pulse sequence HSQC-TOCSY (Simonova et al. 2003). This technique, which exploits the resolving power of two powerful pulse sequences, is based on the magnetization transfer between a carbon atom and all remote hydrogen atoms that belong to a common coupling pathway with the carbon atom's directly bonded hydrogen.

### 11.2.3 Quantitative Nuclear Magnetic Resonance

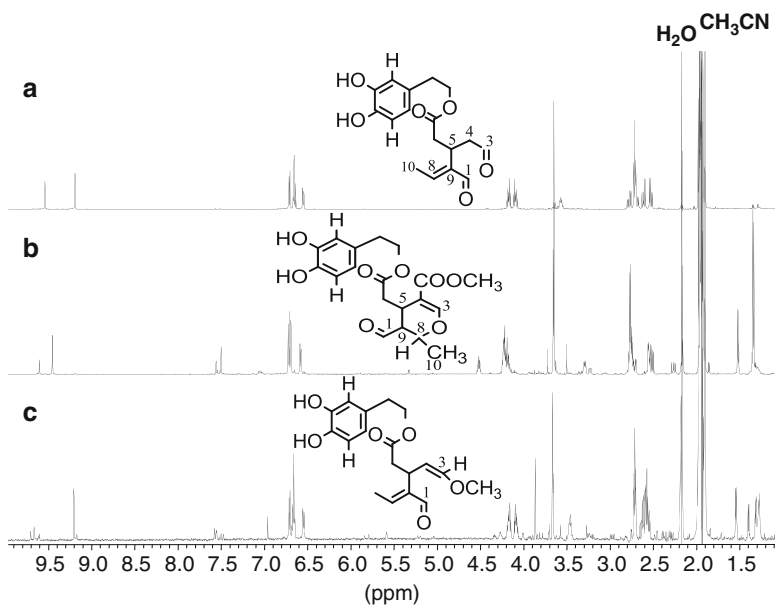
Quantitative analysis using  $^1\text{H}$  NMR is not a difficult task since signal intensities are directly proportional to the number of protons in each functional group. Moreover, proton nuclei are characterized by relatively small spin lattice relaxation times ( $T_1$ ) because they constitute the exterior of the molecule, and thus they relax effectively by intra- and intermolecular  $^1\text{H}$ - $^1\text{H}$  dipolar interactions. For this reason, no long relaxation delays are necessary to run quantitative  $^1\text{H}$  NMR spectra. However, certain precautions should be taken into consideration regarding  $^{13}\text{C}$  and  $^{31}\text{P}$  nuclei. As mentioned earlier,  $^{13}\text{C}$  is a very insensitive nucleus, and therefore more scans are required to obtain a satisfactory signal-to-noise ratio (S/N). In addition, spin-lattice relaxation times ( $T_1$ ) of carbon nuclei, which are located in the interior of the molecule, are much longer than those of protons, and therefore a large delay time ( $5 \times T_1$ ) is required between successive pulse sequences to guarantee full relaxation of the excited nuclei (return of the nuclear magnetization to thermal equilibrium). A long relaxation delay is a prerequisite for quantitative analysis in order to obtain strong and reproducible signals amenable to accurate integration. A remedy for this problem could be the addition of paramagnetic agents, e.g.,  $\text{Cr}(\text{acac})_3$ , whose lone electrons interact strongly with nuclear spins, decreasing significantly the  $T_1$  values. Another factor that plays an important role in  $^{13}\text{C}$  quantitative analysis is the NOE. For protonated carbons, where  $^1\text{H}$ - $^{13}\text{C}$  dipolar interactions are the dominant relaxation mechanism, NOE is close to its highest value (approximately 1.987) and can be ignored. In contrast, nonprotonated carbons will give signals that are highly dependent on NOE. Consequently, these carbons should not be used as a basis for analytical measurements, unless depression of the NOE is accomplished using the so-called inverse gated decoupling technique. In this experiment, the decoupler of the spectrometer is on only during acquisition and off during the rest of the experiment, thus prohibiting the buildup of NOE while acquiring decoupled spectra. Inverse gated decoupling is vital for the  $^{31}\text{P}$  nucleus, whose relaxation is stimulated by the chemical shift anisotropy mechanism in addition to  $^1\text{H}$ - $^{31}\text{P}$  dipole-dipole interactions. Needless to say, when taking up all the

aforementioned safety measures for quantitative analysis based on  $^{13}\text{C}$  and  $^{31}\text{P}$  nuclei, the duration of the experiment is prolonged significantly.

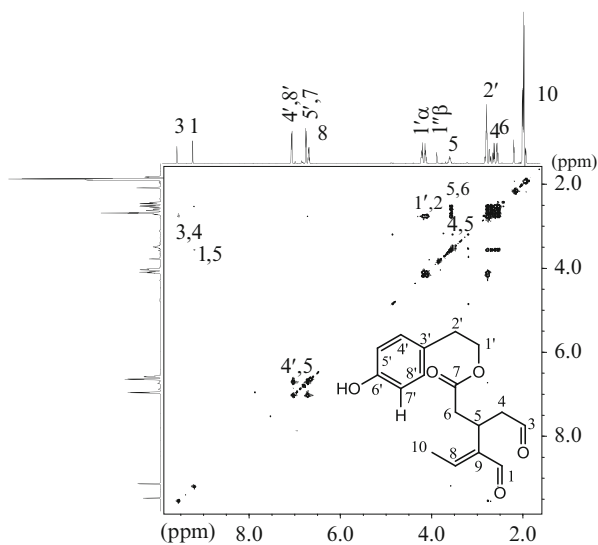
Another way to obtain quantitative  $^{13}\text{C}$  NMR spectra for protonated carbons is the use of the distortionless enhancement by polarization transfer (DEPT) pulse sequence, which transfers the polarization (bulk magnetization) from the abundant  $^1\text{H}$  nuclei to the dilute carbon  $^{13}\text{C}$  nuclei (Vlahov 1997; Vlahov et al. 2001). The drawback of this technique is that resonances of quaternary carbons (e.g., carbonyl carbons of the fatty acyl chains) cannot be detected.

### 11.2.4 *Hyphenated Nuclear Magnetic Resonance*

The combination of liquid chromatography (LC) with NMR has proven to be a powerful and time-saving method for the separation and structural characterization of unknown compounds in complex mixtures (Exarchou et al. 2005). In addition, the low detection limits attainable by chromatographic techniques help to increase the sensitivity of NMR. This was further improved by introducing a postcolumn solid-phase extraction (SPE) unit (Corcoran et al. 2002), which allowed significant enrichment of the analyte concentration and, hence, the performance of 1D and 2D experiments, even for the less sensitive  $^{13}\text{C}$  nucleus. The LC-SPE-NMR technique has been used in two instances: for the analysis of phenolic compounds in olive oil (Christophoridou et al. 2005) and the identification of phytochemicals in olive-leaf extracts (Goulas et al. 2009). Figure 11.10 illustrates selections of 600 MHz LC-SPE-NMR spectra of various phenolic metabolites resulting from the hydrolysis of oleuropein (Christophoridou et al. 2005). These spectra were recorded for HPLC fractions transferred to a peak-trapping unit equipped with solid-phase cartridges after UV detection and water addition for temporary storage, dried with nitrogen gas, and transferred to the NMR flow probe with  $\text{CD}_3\text{CN}$ . The spectrum in Fig. 11.10a is consistent with the dialdehydic form of oleuropein lacking a carboxymethyl group. Figure 11.10b is more interesting because it reveals for the first time the existence of two coeluted isomers of the aldehydic form of oleuropein in olive oil, namely 5*S*, 8*R*, 9*S* and 5*S*, 8*S*, 9*S* at C8 (the ring of elenolic acid linked to the hydroxytyrosyl moiety contains three chiral centers at C5, C8, and C9). Figure 11.10c illustrates the spectrum of the hemiacetal at C-3 of the dialdehydic form of oleuropein lacking a carbomethoxy group detected by Montedoro (Montedoro et al. 1992) in olive oil. Another example indicating the potential of this technique is shown in Fig. 11.11, which depicts the 600 MHz LC-SPE-TOCSY spectrum resulting from a fraction of the UV chromatogram corresponding to the dialdehydic form of ligstroside lacking the carbomethoxy group (Christophoridou et al. 2005). Overall, the hyphenated NMR allowed the detection of 27 phenolic compounds, including a large number of secoiridoid derivatives and several new phenolic components, which had not been reported previously as constituents in the polar part of olive oil.



**Fig. 11.10** 600 MHz LC-SPE <sup>1</sup>H NMR spectra of oleuropein derivatives: (a) dialdehydic form of oleuropein lacking a carboxymethyl group; (b) two coeluted isomers of aldehydic form of oleuropein; (c) hemiacetal of dialdehydic form of oleuropein. The suppressed signals of H<sub>2</sub>O and CH<sub>3</sub>CN solvents give spikes at  $\delta$  1.95 and  $\delta$  2.18 (Source: Christophoridou et al. (2005), with permission of American Chemical Society)



**Fig. 11.11** 600 MHz LC-SPE-NMR-TOCSY spectrum of dialdehydic form of ligstroside lacking a carboxymethyl group

### ***11.2.5 Site-Specific Natural Isotope Fractionation Nuclear Magnetic Resonance***

SNIF-NMR is a sophisticated NMR technique that exploits the nonrandom distribution of deuterium nuclei at specific sites of organic molecules physically present in food products (Martin and Martin 1990). The deuterium nonrandom distribution, which occurs in the course of physical, chemical, and biochemical transformations, is expected to be different for natural and synthetic molecules of the same kind, affording thus a means for their discrimination. A quantitative measure of the deuterium fractionation is the deuterium isotopic ratio ( ${}^2\text{H}/{}^1\text{H}$ )<sub>*i*</sub> for specific molecular sites *i*. Isotopic fractionation in plants occurs during photosynthesis following different pathways (e.g., Calvin or C3 plant photosynthesis and Hatch-Slack or C4 plant photosynthetic mode). Therefore, the isotopic ratio ( ${}^2\text{H}/{}^1\text{H}$ )<sub>*i*</sub> for some common compounds of plant extracts is expected to be different for different plants. This methodology has been used extensively to detect adulteration of wine, honey, and fruit juices (Martin and Martin 1995; Cotte et al. 2007). Application of SNIF-NMR to olive oil is rare, focusing mainly on investigating the intramolecular distribution of deuterium in fatty acids and triacylglycerols (Lui et al. 1995; Royer et al. 1999). Nevertheless, these preliminary studies have shown that the deuterium isotopic ratio may be used as an index for botanical characterization of olive oils and differentiation according to their regional origin. The main limitations of this technique are due to the rather poor sensitivity and chemical shift resolution of  ${}^2\text{H}$ -NMR, which preclude observation of complex metabolites present in diluted media or available only at the submilligram level. Also, SNIF-NMR requires a rather tedious sample preparation.

### **11.3 Nuclear Magnetic Resonance Diffusometry and Relaxometry**

Low-resolution pulsed NMR (time-domain NMR) has been used for many years for the determination of oil content and moisture in oilseeds and the solid fat content in fat blends (Gambhir 1992). Methodologies that have been employed for this type of analysis relied on free induction decay (FID) analysis and the spin-echo technique. The introduction of pulsed-field gradients (PFG) combined with spin-echo pulse sequences (Stilbs 1987) enabled the separation of the various components of mixtures on the basis of differences in their self-diffusion coefficients, the latter being dependent on the hydrodynamic size of each component. Discrimination according to hydrodynamic size was accomplished through differences in signal decay, which is the same for all proton resonances associated with a pure component. A typical PFG spin-echo sequence consists of two short magnetic field gradient pulses and a diffusion delay time,  $\Delta$ , between pulses. The Hahn spin-echo sequence or the stimulated echo sequence can be used, depending on the range of diffusion time  $\Delta$  to be

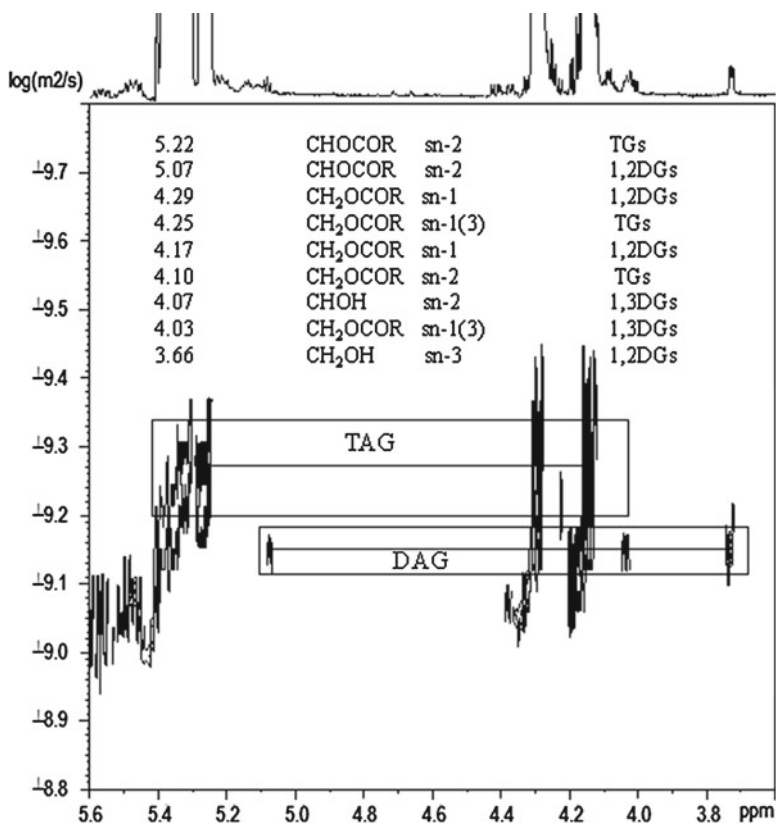
explored. In restrictive media, such as seeds, the self-diffusion coefficient,  $D$ , can be derived from the attenuation of the signal in a field gradient as a function of the parameters of the product  $G\delta$  and the delay time  $\Delta$ ;  $G$  and  $\delta$  are the amplitude and the duration of the magnetic field gradient pulses, respectively. The order of the magnitude of the measurable self-diffusion coefficient ranges from  $10^{-5}$  to  $10^{-10}$  m<sup>2</sup>/s.

Recently, high-power PFG spin echo yielding a field strength of 350 G/cm was applied for the rapid screening of extra virgin olive oil (EVOO) adulteration with seed oils (Šmejkalová and Piccolo 2010). Application of a spin-echo pulse sequence allowed the determination of the diffusion coefficients of all vegetable oils for each signal in the low-resolution spectra. These parameters were used subsequently to classify the various vegetable oils and to detect adulteration of olive oil. An interesting application of this method was found in the analysis of liquid mixtures at high magnetic fields. This is the so-called diffusion-ordered spectroscopy (DOSY) NMR experiment, which was proposed more than 10 years ago (Morris and Johnson 1992). DOSY is a 2D NMR experiment, in which the signal decays exponentially according to the self-diffusion behavior of individual molecules. Because the diffusion behavior is related to properties of an individual molecule – size, shape, mass, and charge – as well as its surrounding environment (solution, temperature aggregation state), each component in a mixture can be separated based on its own diffusion coefficient. The value of DOSY is that it can be used as a noninvasive method to obtain both physical and chemical information. The easy and cheap implementation is another advantage of DOSY. It could be said that DOSY can be an alternative to LC-NMR. Figure 11.12 shows the 600 MHz <sup>1</sup>H DOSY spectrum of an olive oil sample in the region spanned by the glycerol backbone protons of di- and triacylglycerols. The horizontal (F1) axis encodes the proton chemical shifts, whereas the vertical (F2) axis describes self-diffusion coefficients. The cross peaks align themselves along horizontal lines, each corresponding to one sample component (molecule). The acylglycerols are separated by diffusion coefficient (the higher the molecular weight, the lower the diffusion coefficient).

## 11.4 Instrumentation-Practical Aspects

The major drawback of NMR as an analytical tool is its inferior sensitivity compared to other analytical methods (e.g., chromatography, mass spectrometry). Therefore, efforts have been made in recent years to augment NMR sensitivity. Fundamentally, there have been three principal ways in the pursuit of improved NMR sensitivity: the development of superconducting NMR solenoids capable of producing higher magnetic fields; improvement or invention of novel experimental methods, such as the inverse-detected heteronuclear shift correlation methods; and considerable efforts in the area of NMR probe development. Magnetic fields as high as 23.5 Tesla (1,000 MHz for the proton nucleus) are currently manufactured. However, a magnet of 14.1 Tesla (600 MHz) with a homogeneity of tens of Hertz and resolution as high as 0.05 Hz is adequate and satisfies almost all the research needs of food scientists.





**Fig. 11.12** 600 MHz DOSY  $^1\text{H}$  NMR spectrum of olive oil sample in region where di- and triacylglycerol resonances appear. The acylglycerols are separated by diffusion coefficient

Inserted into the magnet is the detector system or probe. The probe contains tunable radio-frequency coils for excitation of the nuclear spins and detection of the resultant signals as the induced magnetization decays away. There are several types of probes, which fulfill almost all needs for sample analysis in the liquid state (and solid state). For 1D and, particularly, 2D NMR experiments, including experiments requiring solvent suppression, broadband (BB) inverse probes with z-gradient facilitate observation/irradiation of all NMR nuclei in the range  $^{31}\text{P}$  to  $^{15}\text{N}$  in addition to  $^1\text{H}$  and  $^{19}\text{F}$ . The inner coil of these multipurpose probes is optimized for  $^1\text{H}$  observation, while the outer coil is tunable over a frequency range that permits decoupling of nuclei  $^{31}\text{P}$  and  $^{15}\text{N}$ , depending on the probe configuration. The advantage of inverse experiments over direct  $^{13}\text{C}$ ,  $^{15}\text{N}$ , or  $^{31}\text{P}$  detection is that the  $^1\text{H}$  nucleus with the highest gyromagnetic ratio is detected, yielding the highest possible sensitivity. Further sensitivity gain (three to four times) is achieved by cryogenically cooling (approximately 20 K) the radio-frequency coils and first-stage receiver electronics of the NMR probe reducing the thermal noise.

The new-generation NMR spectrometers include a very effective digital lock for excellent magnet stability and second-generation analog-to-digital converter (ADC) (converts the analog NMR signal, a voltage, to a binary number proportional to the magnitude of the signal that is stored in the computer memory for further processing) to obtain the highest possible digital spectral resolution and dynamic range. The dynamic range describes the ability of an ADC to sample a small signal in the presence of a large one and depends on the ADC resolution. The latter is expressed in bits; a 16 bit corresponds to a resolution of  $2^{16} - 1$  and is able to represent values in a range of  $-32,767$  to  $+32,767$  ( $2^{16} = 66,536$ ) with one bit reserved to represent the sign of the signal. This means that the smallest signal that can be detected has a maximum amplitude of  $1/32,767$ . Signals smaller than this amplitude will be lost, whereas too strong signals, like those of solvents, can cause ADC overflow. Moreover, the word length of the computer of the spectrometer must exceed the ADC resolution; otherwise memory overflow will result, with a consequent loss of information. Modern spectrometers (600 MHz) are equipped with ADCs with 32- or even 64-bit resolution.

Depending on the experiment, different resolutions or sensitivities are required. Resolution is important for the integration of small signals, such as those in the carbonyl region of  $^{13}\text{C}$  NMR spectra of olive oil. Since the digital resolution is proportional to the reciprocal of the acquisition time, its enhancement can be achieved either by increasing the memory size or by decreasing the spectral width. For older NMR spectrometers, the user should be cautious in adopting the latter remedy to avoid signal aliasing (where the signal becomes indistinguishable).

A large number of 1D NMR experiments, such as the measurement of relaxation times and NOEs, and all 2D NMR experiments involve several pulses separated by timed variable delays and are controlled by pulse programs written in a high-level language. A dynamic software package implemented in the computer of the spectrometer is indispensable in order to interpret these complicated pulse sequences. Moreover, the software should offer a well-designed and user-friendly interface for acquisition, processing, and analysis of the NMR data.

Screening of a large number of samples is of great interest for industrial applications. Various automation options, including autosamplers, robotic sample preparation systems, and probes with automated probe tuning, are available on the market. The automation procedure should be monitored and controlled with appropriate software.

The instrumentation for LC-NMR experiments should be conducted on a dedicated NMR spectrometer equipped with an ultra-shielded magnet to allow hyphenation with the nearby HPLC system. The detection system of HPLC is selected according to the programmed analysis. For instance, UV or diode array detection systems are suggested for the analysis of phenolic compounds. The outlet of the detector is connected to the flow NMR probe, whereas a computer-controlled valve-switching interface regulates the transfer of each fraction separated by HPLC to the NMR probe. The stop-flow or the on-flow process is used depending on the availability of a loop storage device that allows the collection of fractions. The LC-SPE-NMR system requires a SPE unit, whose utilization was described previously. Since the

volume of the liquid from the SPE unit to the flow probe is small (approximately 200  $\mu\text{L}$ ), the use of deuterated solvent is both practical and economical, thus making it possible to avoid stringent solvent suppression requirements. Furthermore, the fact that deuterated solvents are only needed for the final step means that chromatographic separation can be performed using the less expensive protonated solvents.

## 11.5 Sample Preparation

For  $^1\text{H}$  NMR spectra recorded on a 600 MHz spectrometer, 1–2 mg of the oil are dissolved in 0.5 mL of chloroform-*d* and placed in 5 mm NMR tubes. A tiny amount (approximately 10  $\mu\text{L}$ ) of DMSO- $\text{d}_6$  may be added to facilitate dissolution of olive oil polar compounds (phenols, volatiles). Since proton exchange in DMSO is slow relative to the NMR scale, signals caused by hydroxyl protons emerge in the spectrum, complicating the analysis. In this respect, the hydroxyl hydrogens should be replaced by deuterium atoms (hydrogen-deuterium exchange) by shaking the sample with  $\text{D}_2\text{O}$  before dissolution in DMSO- $\text{d}_6$ . For the less sensitive nuclei (e.g.,  $^{13}\text{C}$ ) the amount of olive oil is increased to 5–10 mg. Regarding olive oil minor constituents – e.g., phenolic compounds and their hydrolysis products – the first step is the extraction of the desired metabolites. In general, the choice of solvent depends on the nature of metabolites and the extraction efficiency. For the extraction of phenolic compounds, a mixture of methanol:water 80:20 v/v was found to be most effective (Montedoro et al. 1992). Olive oil lipophilic components (e.g., triacylglycerols, diacylglycerols, sterols) are removed with nonpolar solvents such as hexane. The final extract is lyophilized to concentrate the phenolics and remove water.

Sample preparation for  $^{31}\text{P}$  NMR analysis is somewhat lengthy since it requires prior derivatization by the phosphorus reagent 1 according to the reaction scheme in Fig. 11.1. Compounds are dissolved in a mixture of pyridine-chloroform-*d* that contains a small amount of the paramagnetic reagent  $\text{Cr}(\text{acac})_3$  [chromium (III) acetylacetonate] to diminish the phosphorus spin–lattice relaxation time. Pyridine is added to capture the released HCl during the phosphorylation reaction (Fig. 11.1).

## 11.6 Olive Oil Analysis

Analysis of olive oil using NMR spectroscopy has been described in several good review articles mentioned in the introductory section; the reader is advised to consult them for details. Table 11.3 summarizes olive oil constituents that have been determined by NMR methods. The second to last column of this table describes the pros and cons of the NMR methods relative to official or well-recognized analytical techniques. A comparison of the NMR methods with conventional analytical techniques in determining certain olive oil constituents was done recently (Dais et al. 2007).

**Table 11.3** Olive oil constituents determined by NMR spectroscopy; advantages and disadvantages with official or other recognized methods

Constituent	Magnetic nucleus	Comparison with official or well-recognized methods	References
Fatty acid profile ( <i>cis</i> and <i>trans</i> ) ( <i>positional distribution</i> )	$^1\text{H}$ and $^{13}\text{C}$	<i>Pro</i> : faster and simpler than official method (GC) <i>Con</i> : no determination of individual saturated fatty acids	1, 2
Triacylglycerols profile	$^{13}\text{C}$	<i>Pro</i> : much faster and simpler than enzymatic procedure of official method <i>Con</i> : requires high spectral resolution	2
Mono- and diacylglycerols	$^1\text{H}$ and $^{31}\text{P}$	<i>Pro</i> : faster than official method (GC) <i>Con</i> : no determination of acyl-chain profile	1, 3
Phenolic compounds profile	$^1\text{H}$ and $^{31}\text{P}$	<i>Pro</i> : faster and simpler than HPLC; identification at molecular level; new phenolic compounds have been discovered <i>Con</i> : requires derivatization for $^{31}\text{P}$ NMR	4, 5, 6
Total, free, and esterified sterols	$^1\text{H}$ and $^{31}\text{P}$	<i>Pro</i> : much faster than official method (GC) <i>Con</i> : no determination of individual sterols; requires strong magnetic fields ( $\geq 500$ MHz)	7
Free glycerol	$^{31}\text{P}$	<i>Pro</i> : much faster than periodate oxidation method <i>Con</i> : requires derivatization	8
Phospholipids	$^{31}\text{P}$	<i>Pro</i> : faster and more accurate than TLC, GC-MS <i>Con</i> : requires derivatization	9
Free acidity	$^{31}\text{P}$	<i>Pro</i> : faster and more accurate than official methods of titration <i>Con</i> : requires derivatization	10
Water content	$^{31}\text{P}$	<i>Pro</i> : much faster and more accurate than Karl Fischer, dry-oven method and azeotropic distillation <i>Con</i> : requires derivatization	11
Iodine value	$^1\text{H}$ and $^{13}\text{C}$	<i>Pro</i> : much faster than official methods of titration and GC <i>Con</i> : less accurate than official method	10, 12
Squalene	$^1\text{H}$	<i>Pro</i> : much faster than TLC, GC, HPLC <i>Con</i> : complex spectra (observation of single signal in spectra), less accurate; requires strong magnetic fields ( $\geq 600$ MHz)	13
Cycloartenol	$^1\text{H}$	<i>Pro</i> : much faster than TLC, GC, GC-MS <i>Con</i> : Complex spectrum (observation of only two signals), less accurate, requires strong magnetic fields ( $\leq 600$ MHz)	13
Chlorophyll	$^1\text{H}$	<i>Pro</i> : much faster detection of adulteration with defective chlorophyll (lacking Mg) <i>Con</i> : observation of a single signal (NH of porphyrin), requires strong magnetic fields ( $\leq 600$ MHz)	13

(continued)

**Table 11.3** (continued)

Constituent	Magnetic nucleus	Comparison with official or well-recognized methods	References
Volatiles	$^1\text{H}$	<i>Pro</i> : much faster than GC, GC-MS, LC-GC <i>Con</i> : detection of a small number of volatiles, requires strong magnetic fields ( $\geq 600$ MHz)	1
Oxidation process	$^1\text{H}$ and $^{13}\text{C}$	<i>Pro</i> : monitoring at molecular level <i>Con</i> : less sensitive than optical and conventional methods (rancimat, oxidation analytical indices)	14, 15
Oxidation stability	$^{13}\text{C}$	<i>Pro</i> : determination at molecular level, better prediction than conventional methods <i>Con</i> : requires lengthy sample pretreatment (chromatographic fractionation)	16
Color determination	$^{13}\text{C}$	<i>Pro</i> : determination at molecular level <i>Con</i> : Lengthy sample pretreatment, lack of validation	17
Sensory quality	$^1\text{H}$	<i>Pro</i> : sensory attributes (odors and tastes) have been associated with characteristics signals in the spectrum <i>Cons</i> : lack of validation	13

References: 1, Sacchi et al. (1997); 2, Vlahov (1999); 3, Spyros and Dais (2000); 4, Christophoridou et al. (2005); 5, Christophoridou and Dais (2006); 6, Christophoridou and Dais (2009); 7, Hatzakis et al. (2010a); 8, Hatzakis et al. (2010b); 9, Hatzakis et al. (2008); 10, Fronimaki et al. (2002); 11, Hatzakis and Dais (2008); 12, Miyake et al. (1998); 13, Mannina and Segre (2002); 14, Guillen and Ruiz (2006); 15, Medina et al. (1998); 16, Hidalgo et al. (2002); 17, Zamora et al. (2003)

### 11.6.1 $^1\text{H}$ NMR

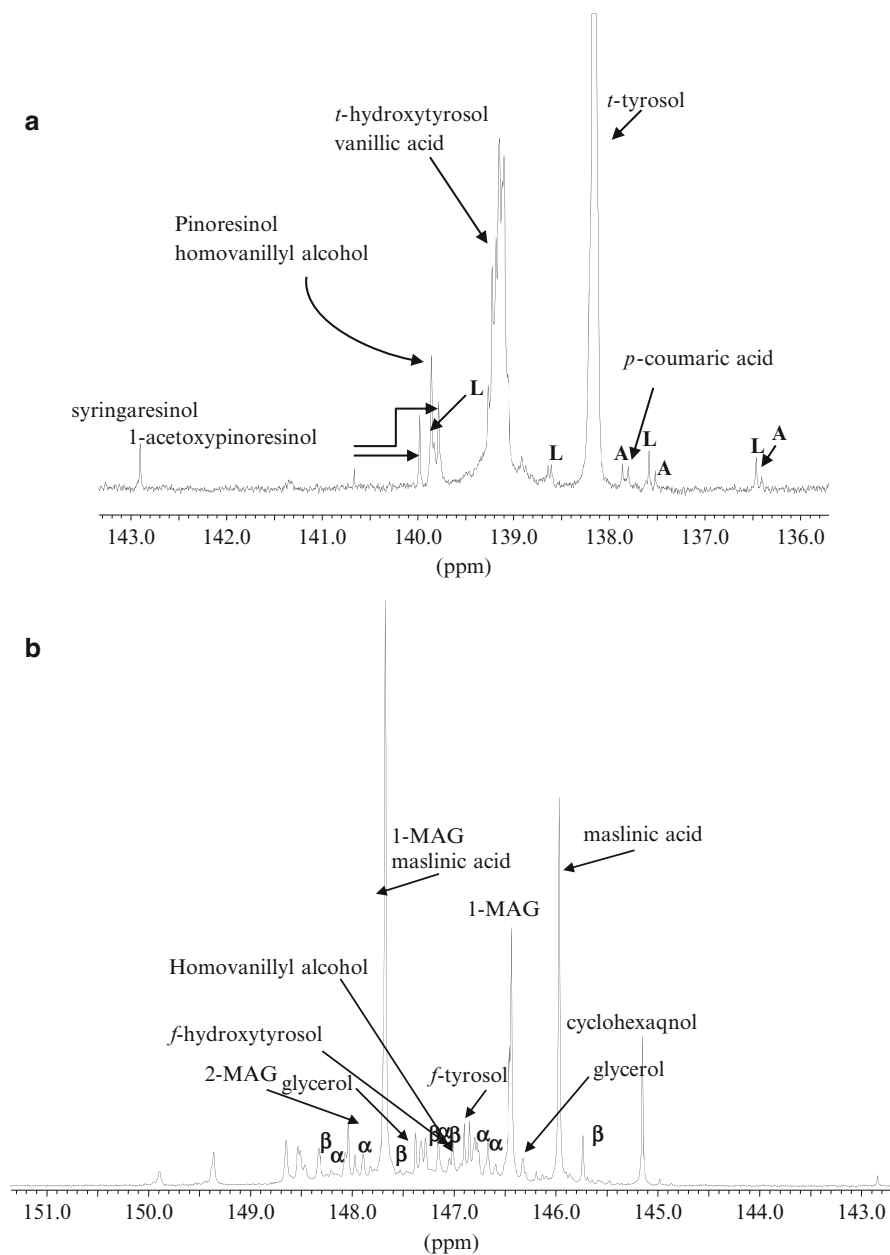
This NMR methodology is the best one for the quantification of fatty acids, despite the fact that it is unable to determine individual fatty acids (unlike gas chromatography). Due to signal overlap, quantification of the sum of saturated fatty acids (SFAs), the monounsaturated oleic acid (MUFA), and the polyunsaturated linoleic and linolenic acids (PUFAs) can be obtained by means of mathematical equations using appropriate signal intensities as variables. This procedure exempts linolenic acid, which can be quantified from the signal of the methyl protons of the linolenyl chain at  $\delta$  0.96. The much higher sensitivity of the proton nucleus makes this technique suitable for detection and direct quantification of minor compounds of olive oil, such as free fatty acids, volatiles (alcohols and aldehydes), the two diacylglycerol isomers, total sterols, the hydrocarbon squalene, the tetracyclic alcohol cycloartenol, and chlorophyll. Recent advances in  $^1\text{H}$  NMR include the detection and quantification of phenolic compounds in the polar part of olive oil mentioned previously, and, in combination with  $^{31}\text{P}$  NMR, it enables the quantification of total, free, and esterified sterols (Hatzakis et al. 2010a).

### 11.6.2 $^{13}\text{C}$ NMR

Almost all analyses performed by  $^1\text{H}$  NMR can also be accomplished by  $^{13}\text{C}$  NMR. Despite the sensitivity disadvantages,  $^{13}\text{C}$  NMR is the preferred technique to obtain information about the positional distribution of the saturated, oleyl, linoleyl, and linolenyl chains on the glycerol moiety (Vlahov 1999; Mavromoustakos et al. 1997; Mannina et al. 1999a). This is achieved from the signal integrals in the carbonyl region of the spectrum as well as from those of the olefinic carbons. Moreover, the 5 ppm distance between the signals of the allylic carbons of *cis* and *trans* double bonds allows an easy and accurate quantification of *trans* fatty acids (Gao et al. 2009). Nevertheless, the insensitivity of the  $^{13}\text{C}$  nucleus, the very small quantity of *trans* lipids contained in foods, and the need for screening a large number of samples render this NMR technology problematic in terms of instrument time and cost. *Cis-trans* isomers have been determined by the  $^1\text{H}$  NMR technique as well using the allylic methylene protons adjacent to *cis* and *trans* double bonds (Sedman et al. 2010). However, the much smaller chemical shift difference (0.15 ppm) between the two signals requires a magnetic field  $\geq 14.1$  T (600 MHz) to obtain a good separation of *cis* and *trans* proton signals. Recently, fractionation of olive oil by means of column chromatography was proposed (Zamora et al. 2002) in order to increase the concentration of minor compounds, thereby boosting the potential of  $^{13}\text{C}$  NMR analysis. As expected, the  $^{13}\text{C}$  NMR spectrum of the oil fraction was more complex than that of intact oil due to the presence of additional signals of minor compounds. Statistical analysis of selected signal intensities in the spectrum of the oil fraction allowed prediction (with great accuracy) of the stability of 66 vegetable oil samples, including virgin and refined olive oil (Hidalgo et al. 2002), and the calculation of oil colors (Zamora et al. 2003).

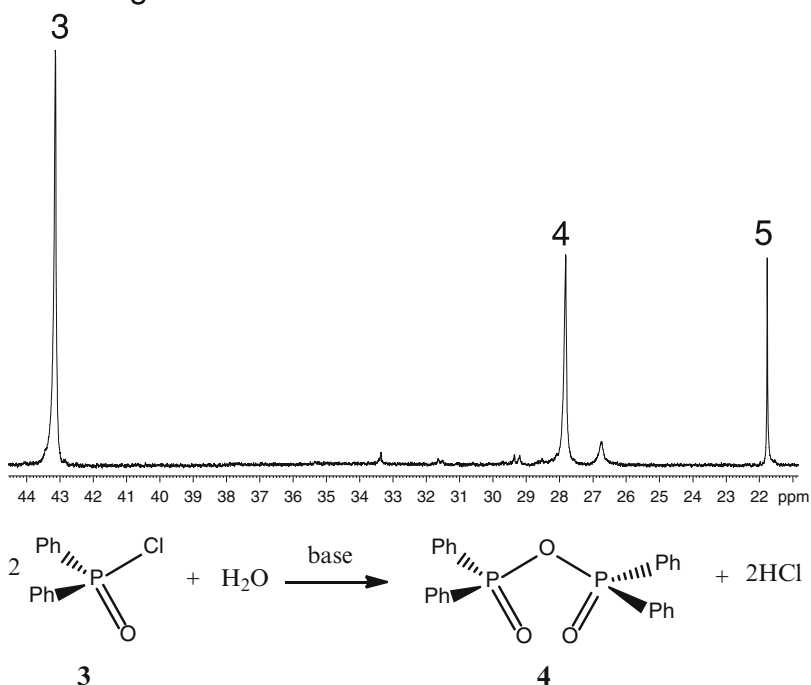
### 11.6.3 $^{31}\text{P}$ NMR

This NMR method was introduced recently as a complementary analytical technique for the analysis of olive oil.  $^{31}\text{P}$  NMR spectra of olive oil were used for the detection and quantification of phospholipids (Hatzakis et al. 2008) extracted from olive oil with a mixture of ethanol:water (2:1 v/v). The main phospholipids found in olive oil were phosphatidic acid, lyso-phosphatidic acid, and phosphatidylinositol. Direct phosphorylation of an olive oil sample was performed for the quantification of diacylglycerols, total free sterols, and free fatty acids (Fig. 11.2), as mentioned previously, whereas phenolic compounds were identified and quantified in the polar part of olive oil (Christophoridou and Dais 2006). The resonances of the phosphorylated aliphatic and aromatic hydroxyl groups of phenolic compounds are illustrated in Fig. 11.13. Fifteen polyphenols were detected and quantified, including simple phenols, lignans, and flavonols. The secoiridoid derivatives were determined in the form of total hydroxytyrosol and total tyrosol. The amounts of phenolic compounds determined by  $^{31}\text{P}$  NMR agree very well with those obtained by applying  $^1\text{H}$  NMR (Christophoridou and Dais 2009).



**Fig. 11.13** 202.2 MHz  $^{31}\text{P}$  NMR spectrum of phosphitylated polar fraction of virgin olive oil sample in chloroform/pyridine solution: (a) aromatic region; (b) aliphatic region. A apigenin, L luteolin, 1-MAG 1-monoacylglycerols, 2-MAG 2-monoacylglycerols,  $\alpha$   $\alpha$ -D-glucopyranose,  $\beta$   $\beta$ -D-glucopyranose, *f*- and *t*- free and total, respectively (Source: Christophoridou and Dais (2006), with permission of American Chemical Society)

Excess of reagent



**Fig. 11.14**  $^{31}\text{P}$  NMR spectrum (at 202.2 MHz) of a phosphitylated sample of olive oil with reagent 3 in pyridine/chloroform solution. The phosphitylation reaction illustrated below the spectrum leads to the formation of the anhydride product 4. Methyltriphenylphosphonium iodide (5) is the internal standard. (Source: Hatzakis et al. (2008), with permission of American Chemical Society)

Another application of  $^{31}\text{P}$  NMR in olive oil analysis is the determination of water content in olive oil (Hatzakis and Dais 2008). Water is transferred to olive oil during olive crushing and malaxation, and its content in olive oil has long been recognized as an important factor determining the quality of olive oil (IOC 2003). Small quantities of water in olive oil are responsible for the creation and persistence of the suspended and dispersed material that constitutes the so-called veiling of olive oil. This oil is not attractive to the consumer, although some investigators have claimed that water and small particles dispersed in the oil have some antioxidant effects (Lercker et al. 1994). At any rate, water inducing degradation of minor compounds during storage contributes to the perception of wine-vinegar and acid flavors, which deteriorates the organoleptic properties of olive oil. Phosphitylation of water molecules with compound 1 or, alternatively, with the reagent diphenylphosphinic chloride (3) was successful in determining the olive oil moisture with high accuracy (Hatzakis and Dais 2008). Reagent 3 reacts cleanly and instantaneously with water molecules under mild conditions with no side reactions, giving a sole product and, hence, a single signal in the  $^{31}\text{P}$  NMR spectrum (Fig. 11.14).



## 11.7 Nuclear Magnetic Resonance Data Manipulation for Chemometric Analysis

Multivariate statistical analysis of NMR data can be performed on the basis of either selected  $^1\text{H}$  or  $^{13}\text{C}$  signal intensities of the oil samples or suitable chemical parameters determined by NMR. It could be said that the first method resembles the chemometric approach of metabonomics, whereas the second technique is similar to the quantitative method or targeting profiling of metabonomics. In the first method, the selected resonances should show large intensity variability and be independent of one another. In the second method, olive oil constituents are identified and quantified before statistical analysis. For the first method to work, it is critical to have a large number of spectra from many different samples collected and processed identically. The quantitative method does not require identical conditions for spectral collection. Moreover, the latter method allows unambiguous compound identification and precise quantification. Compound identities and concentrations permit explicit recognition of olive oil metabolites and, hence, intermediate interpretation of their influence on olive oil characteristics. However, the quantitative method is not as amenable to automation as the chemometric procedure.

Regardless of the method used, preprocessing and NMR data manipulation are crucial for subsequent multivariate analysis, as well as for the type of statistical method that can be utilized. Data manipulation can be performed in both the time and frequency domain after FT. Briefly, the following steps are necessary for NMR data manipulation and processing:

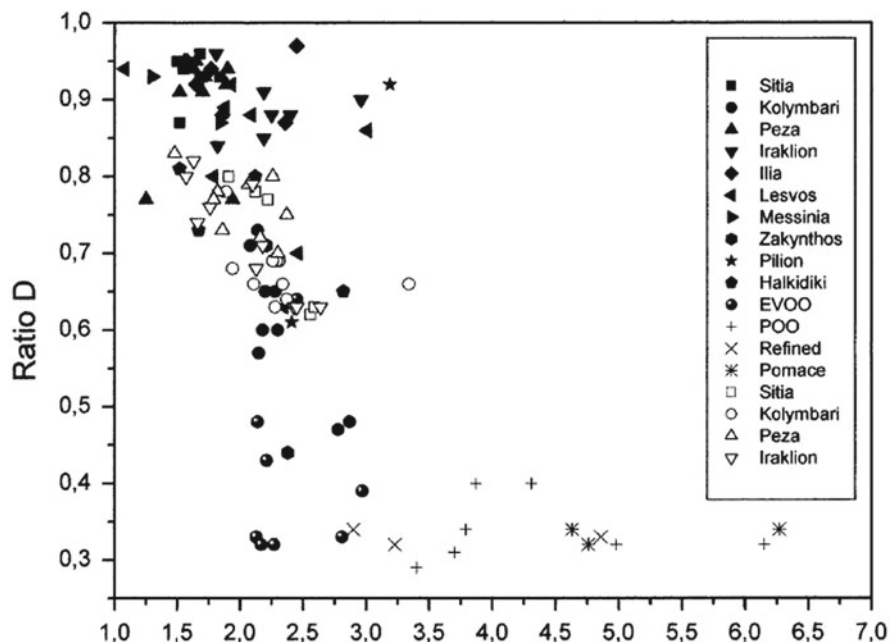
*Time domain:* zero-filling, linear prediction, or both; window function multiplication for resolution or sensitivity enhancement, Fourier transform.

*Frequency domain:* baseline offset correction, phasing, signal alignment, scaling (only for the chemometric method).

The next important step is the choice and application of the multivariate statistical method that can provide the highest possible discrimination of olive oils from different areas or cultivars, as well as olive oils from foreign oils in the context of adulteration studies. Statistical methods used in data analysis and validation of statistical models for prediction are discussed in details in Chap. 12.

## 11.8 Olive Oil Quality and Authentication

A proposed index to monitor the freshness of olive oil is the ratio of diacylglycerol isomers (Sacchi et al. 1997). The diminution of the 1,2-DAG concentration during olive oil storage owing to its isomerization to more stable 1,3-DAG is indicative of VOO aging. The ratio  $D$  (1,2-DAG over total DAG) has been used as



**Fig. 11.15** Plot of ratio  $D$  versus total diacylglycerols for virgin olive oils of various regions of Greece, commercial extra virgin (EVOO), commercial pure olive oils (POO), refined olive oils, and pomace oils. For the four regions of Crete (Sitia, Kolymbari, Peza, and Iraklion) the *solid symbols* correspond to crops of the period 2000–2001, whereas the *empty symbols* correspond to crops of the period 1999–2000 (Source: Fronimaki et al. 2002, with permission of American Chemical Society)

a secondary quality index to map the quality of a large number of EVOO samples (Fronimaki et al. 2002). The plot of  $D$  values versus total DAG in Fig. 11.15 can be considered as a quality map for Greek EVOOs collected in two harvest periods. EVOO samples pointing toward the upper left corner of the diagram are considered fresh oils, whereas those characterized as old oils have low  $D$  values and, depending on the total DAG, are oriented toward the lower left or right corner of the diagram. Refined oils, olive-pomace oils, and mixtures of olive oil and refined olive oil known as pure olive oil or blended olive oil, which are not EVOOs, tend clearly to the lower right corner of the diagram. Further studies (Spyros et al. 2004) have succeeded in giving quantitative information about olive oil aging through a mathematical equation that connects the storage time (age) of EVOO with the ratio  $D$  and free acidity.

The usual approach to detecting EVOO adulteration is to compare the chemical composition of the suspect olive oil with limits for several of its constituents or physical constants imposed by the European Commission, the International Olive

Council (IOC), and other official food organizations. Any adulterant addition is expected to modify the concentration of these constituents, or at least it will indicate an anomaly in its chemical composition. Along these lines, NMR offers a number of clues for olive oil adulteration, either by simple observation of the NMR spectra or by quantitative analysis. For instance, the appearance of a resonance in the carbonyl region ascribed to saturated fatty acids at the *sn*-2 position of glycerol is considered fraudulent (EC 1991, 1995). Careful observation of the signals in particular regions of the  $^1\text{H}$  NMR spectra of vegetable oils reveals slight differences in the chemical shifts of the saturated and unsaturated acids that allow discrimination of these oils and detection of likely adulteration (Guillen and Ruiz 2003). Another example is the detection of adulteration of EVOO by other oils based on the influence of the added oils on  $^{12}\text{C}$  signal intensities of EVOO samples (Mavromoustakos et al. 2000). Nevertheless, there exist three major problems associated with this procedure; inherent physical variation of olive oil characteristics influenced by extraneous factors is often observed. The cumulative rainfall during the summer period affected the composition of fatty acids and of phenolic compounds, whereas low temperatures during the olive harvest period influenced the contents of chlorophyll, carotenoid pigments, and  $\alpha$ -tocopherol (Morelló et al. 2006). Another example is the concentration of waxes, which should not exceed 250 ppm for EVOO. However, the concentration may exceed this limit during olive oil storage due to an increase in esterified compounds, thus making uncertain the classification of this olive oil sample as genuine (Aparicio and Aparicio-Ruiz 2000). A second problem appears when a foreign oil has a very similar chemical composition to that of EVOO (e.g., hazelnut oil). Another dilemma is caused by the discrepancy observed in the physical concentration of some constituents of local EVOOs with those of official limits. This is attributed to specific climatic conditions prevailing in those regions affecting the ripening of the olive fruits at the time of harvesting. Such examples occur for olive oils originating from certain regions of Argentina and Australia (Ceci and Carelli 2007). Finally, slight differences in the NMR spectral parameters could become less reliable when a real adulteration problem is confronted.

New developments in the detection of fraud adopt chemometric methods. The general scheme of this methodology is as follows: the first step is the buildup of a reliable statistical (classification) model that discriminates EVOO from potential adulterants (seed oils or lower grade olive oils). This is usually done by applying multivariate statistical analysis either to signal intensities or to concentration values of certain olive oil components. The second step involves the preparation of a series of mixtures of EVOO with different adulterant concentrations and the use of the previous classification model to identify the mixtures considered as unknown samples. The adulterated EVOO samples lie between the group of EVOOs and the respective groups of seed oils or olive oils of lower quality. Table 11.4 shows a list of olive oil adulteration studies performed so far using NMR spectroscopy.

**Table 11.4** Olive oil adulteration<sup>a</sup> detected by NMR

Biomarker	Adulterant	Experimental conditions	Nucleus/frequency	Detection limit	Data processing	References
UFA	SOY	DEPT sequence, CDCl <sub>3</sub> solution	<sup>13</sup> C/75.5 MHz	7–20 % depending on FA	Standard	Vlahov (1997)
UFA	VEO	CDCl <sub>3</sub> solutions	<sup>1</sup> H/300 MHz	5 %	DA	Mavromoustakos et al. (2000)
Volatiles and FA	HAZ	NMR for volatiles, GC for fatty acids	<sup>1</sup> H/600 MHz	10 %	LDA	Mannina et al. (1999b)
<sup>1</sup> H NMR <sup>b</sup>	SUN, HAZ	CDCl <sub>3</sub> solutions	<sup>1</sup> H/500 MHz	10 %	ANOVA, LDA	Fauhl et al. (2000)
<sup>13</sup> C NMR <sup>b</sup>	VEO	CDCl <sub>3</sub> solutions	<sup>1</sup> H/300 MHz	5 %	SDA	Zamora et al. (2001)
DAG, FA	VEO	Phosphitylation	<sup>1</sup> H/500 MHz, <sup>31</sup> P/202 MHz	5 %	ANOVA, LDA	Vigli et al. (2003)
<sup>1</sup> H and <sup>13</sup> C NMR <sup>b</sup>	HAZ	CDCl <sub>3</sub> solutions	<sup>1</sup> H/600 MHz	8 %	ANN	García-González et al. (2004)
DAG	ROO, LVOO	Phosphitylation, pyridine/CDCl <sub>3</sub> solutions	<sup>31</sup> P/202 MHz	5 %	ANOVA, LDA	Fragaki et al. (2005)
<sup>1</sup> H NMR <sup>b</sup>	RHAZ	CDCl <sub>3</sub> solutions	<sup>1</sup> H/400, 500 and 600 MHz	≤10 %	ANOVA, PCA, LDA, LMR	Mannina et al. (2009)
<sup>1</sup> H NMR <sup>c</sup>	VEO	Neat samples	<sup>1</sup> H/300 MHz	10 % SUN, SOY; 30 % HAZ	ANOVA, DA	Šmejkalová and Piccolo (2010)
Phenols, FA	RHAZ	Phosphitylation, pyridine/CDCl <sub>3</sub> solutions	<sup>1</sup> H/500 MHz, <sup>31</sup> P/202 MHz	1 %	SCDA, CBT	Agiomyrigianaki et al. (2010)
<sup>13</sup> C NMR <sup>b</sup>	–	CDCl <sub>3</sub> solutions	<sup>13</sup> C/75.5 MHz	–	–	Zamora et al. (1994)
<sup>13</sup> C NMR <sup>b</sup>	–	Fractionation of polar and non-polar part of oils. CDCl <sub>3</sub> solutions	<sup>13</sup> C/75.5 MHz	–	SDA	Zamora et al. (2002)
FA	–	CDCl <sub>3</sub> solutions	<sup>1</sup> H/300 MHz	–	–	Guillén and Ruiz (2003)

LVOO lampante virgin olive oils, ROO refined olive oil, SUN sunflower oil, SOY soybean oil, HAZ hazelnut oil, RHAZ refined hazelnut oil, VEO vegetable oils; UFA unsaturated fatty acids, DAG diacylglycerols, FA fatty acids, ANN artificial neural networks, ANOVA analysis of variance, CBT classification binary trees, DA discriminant analysis, LDA linear DA, SCDA stepwise canonical DA, SDA stepwise DA, LMR linear multiple regression, PCA principle component analysis

<sup>a</sup>Extra virgin olive oil, unless otherwise specified

<sup>b</sup>Fingerprint

<sup>c</sup>Diffusion spectra

## 11.9 Geographical and Varietal Characterization of Olive Oil

The characterization of the geographical or varietal origins of EVOOs is becoming increasingly important. This is because false labeling of the origins of olive oil is considered another facet of fraud. With regard to the geographical origin, EVOO is permitted to be marketed under a Protected Designation of Origin (PDO) or Protected Geographical Indication (PGI) label on the basis of the area, cultivar, and methods of production. This labeling protects the reputation of the regional food and eliminates the unfair competition and misleading of consumers by nongenuine products, which may be of inferior quality. Moreover, olive oils made from a certain cultivar (monocultivar olive oils) are being increasingly introduced in markets, and their quality control requires the development of new and effective analytical methodologies to detect fraud. Monocultivar olive oils have certain specific characteristics ascribed to the olive cultivar and are therefore easier to elaborate. Several groups in different countries, especially from countries located in the Mediterranean basin, have investigated the possibility of discriminating olive oils originating from different areas in the same country or from different countries by employing high-field NMR spectroscopy in combination with multivariate statistical analysis. Their efforts have focused on selecting those biomarkers that are capable of discriminating oils. The biomarkers examined ranged from the major olive oil constituents, fatty acids and triacylglycerols, to minor components, sterols, phenols, volatiles, hydrocarbons, etc., or combinations of both. Minor components provide more useful information than major constituents, and they have been used more often to discriminate olive oils according to their geographical or varietal origin. It appears that minor components are prone to greater concentration changes under the influence of various exogenous and endogenous factors (cultivar, ripening conditions, storage, climatic conditions, agricultural practices, and extraction technology). Table 11.5 lists NMR studies concerning olive oil classification according to geographical origin and cultivar. This table contains the biomarkers used, the chemometric treatment, the cultivars analyzed, and their geographical origins, as well as the most important information obtained from each study.

## 11.10 Future Trends and Perspectives

The NMR techniques described in this chapter and their application to olive oil analysis show the enormous potential of NMR in the quality control and authentication of olive oil. The identification of different olive oil components at the molecular level facilitates current intensive efforts to establish the genuineness and quality of the product in a rapid and reliable way. In addition, application of multivariate statistics to NMR fingerprints increases significantly the efficiency of this technique, especially for the geographical and varietal classification of olive oils. Future trends

**Table 11.5** Geographical and varietal classification of extra virgin olive oil by NMR

Biomarker	Nucleus/frequency	Cultivars	Geographical origin	Data treatment <sup>a</sup>	Observations (discrimination)	References
<sup>13</sup> C NMR <sup>a</sup> (FA)	<sup>13</sup> C/75.5 MHz	Coratina, Dritta, Grossa di Cassano Moraiolo, Picholine	Abruzzo, Calabria, Lazio, Lombardia, Marche, Molise, Puglia, Tuscany	PCA, PLS, PCR, MLR	70–100 % cultivars and 93–100 % origin	Shaw et al. (1997)
<sup>13</sup> C NMR <sup>a</sup> , (DAG, TAG)	<sup>13</sup> C/75.5 MHz	Leccino, Moraiolo, Dritta	Abruzzo, Puglia, Tuscany	PLS, PCR	100 % cultivars, ~100 % origin	Vlahov et al. (1999)
<sup>1</sup> H NMR <sup>a</sup> (FA, phenols)	<sup>1</sup> H/400, 500 MHz	Coratina, Leccino, Oliarola, Olivastro, Simone	Apulia region	PCA, HCA, DA	FA allowed 100 %. Phenols discriminate northern Apulia	Sacco et al. (2000)
<sup>1</sup> H NMR <sup>ab</sup> , <sup>13</sup> C NMR <sup>a</sup> (TAG), GC(FA)	<sup>1</sup> H/600 MHz, <sup>13</sup> C/150.9 MHz	17 monocultivars	Italy, Argentina	TCA, LDA	100 % origin of cultivars by NMR and GC data	Mannina et al. (2001a)
<sup>1</sup> H NMR <sup>ab</sup>	<sup>1</sup> H/600 MHz	Frantoio, Leccino, Moraiolo, Nerino, Quercetana	Tuscany (Seggiano, Arezzo, Florence, Lucca)	HCA, K-means, DA	Origin discrimination	Mannina et al. (2001b)
<sup>13</sup> C NMR <sup>a</sup>	<sup>13</sup> C/75.5 MHz	12 Italian mono- and multicultivar oils	13 Italian areas	PCA	DEPT sequence improved sensitivity; cultivar discrimination	Vlahov et al. (2001)
<sup>1</sup> H NMR <sup>a</sup> , (DAG, TAG) GC (FA)	<sup>13</sup> C/150.9 MHz	Nocellara del Belice, Tonda Iblea, Cerasuola, Biancollila	Castel Vetrano, Trapani, Delia, Paceco, Caltabellota, Ragusa	MANOVA, PCA, TCA, MDS, LDA	100 % cultivars combining <sup>13</sup> C NMR and GC data	Mannina et al. (2003b)
<sup>13</sup> C NMR <sup>a</sup> , GC (FA, TAG)	<sup>13</sup> C/125.7 MHz	Coratina, Leccino, Peranzana, Oliarola	Apulia area	ANOVA, PCA, HCA, DA	92 % with GC and 88 % with NMR data	Brescia et al. (2003)
<sup>13</sup> C NMR <sup>a</sup> , (TAG, sterols, FA)	<sup>13</sup> C/125.7 MHz	Coratina, Oliarola, Peranzana	Terra di Barri, Colline di Brindisi, Foggia	MANOVA, LDA	Origin: 72 % (Dauno) to 90 % (Terra di Bari)	Vlahov et al. (2003)

<sup>1</sup> H NMR <sup>a</sup>	<sup>1</sup> H/500 MHz	Unknown	Greece, Italy, Spain, Tunisia, Turkey	LDA, PLS-DA, ANN, ANOVA, PCA	35–92 % depending on origin and harvest year Fine classification origin	Rezzi et al. (2005) Mannina et al. (2005)
<sup>1</sup> H NMR <sup>ab</sup>	<sup>1</sup> H/600 MHz	Casaliva, multicultivar	Garda, Veneto (Valpolicella, Euganei-Berici, Grappa)	PCA	Satisfactory discrimination by origin	Schievano et al. (2006)
<sup>1</sup> H NMR <sup>ab</sup>	<sup>1</sup> H/600 MHz	Casaliva, multicultivar	Garda Lake, Veneto, Lombardia banks	ANOVA, PCA, LDA	Origin affected by irrigation and altitude	D'Imperio et al. (2007)
<sup>1</sup> H- <sup>13</sup> C-NMR <sup>ac</sup>	<sup>1</sup> H/600 MHz, <sup>13</sup> C/62.9 MHz	22 monocultivars	Lazio	SCDA, CBT	87 % with three and 74 % with six areas, 92 % if harvest year is included	Petrakis et al. (2008)
<sup>1</sup> H NMR (FA,DAG) <sup>31</sup> P NMR (phenols)	<sup>1</sup> H/500 MHz, <sup>13</sup> C/100 MHz	Koroneiki	Crete (Heraklion, Sitia, Chania), Peloponnesus (Messinia, Lakonia), Zakynthos			
<sup>1</sup> H NMR <sup>a</sup> , IRMS	<sup>1</sup> H/500 MHz	Unknown	Italy, Greece, Spain, France, Turkey, Cyprus, Syria	ANOVA, LDA, PLS-DA	Best models for origin and harvest year with PLS-DA; IRMS improves classification results	Alonso-Salces et al. (2010)
<sup>1</sup> H NMR <sup>ab</sup>	<sup>1</sup> H/600 MHz	Unknown	Italy, Liguria, Spain, France, Greece, Turkey	SIMCA, PLS-DA	Selected resonances discriminate Ligurian vs. non-Ligurian oils	Mannina, et al. (2010)

DAG diacylglycerols, TAG triacylglycerols, IRMS isotope ratio mass spectroscopy, ANV artificial neural networks, ANOVA analysis of variance, CART classification and regression trees, CBT classification binary trees, DA discriminant analysis, HCA hierarchical cluster analysis, LDA linear DA, LMR linear multiple regression, MANOVA multivariate analysis of variance, PCA principal component analysis, PCR principal component regression, PLS partial least squares, SCDA stepwise canonical DA, SDA stepwise DA, SIMCA soft independent modeling and class analogy, TCA tree cluster analysis

<sup>a</sup>Fingerprint

<sup>b</sup>Minor compounds

<sup>c</sup>Volatiles, sterols, terpenes, squalene

<sup>d</sup>Bulk oil and unsaponifiable fraction

and perspectives encompass the detailed assessment and standardization of current NMR analytical procedures and accompanying quantitative responses. The NMR analytical approaches discussed in this chapter should be taken properly into consideration by controlling agencies and accredited laboratories in order to support or even substitute some of the old-fashioned analytical protocols now used in olive oil authentication. The MEDEO research project (Development and Assessment of Methods for the Detection of Adulteration of Olive Oils with Hazelnut Oil) funded by the European Union is an important contribution in this direction. Another trend is the development of further innovative analytical NMR procedures that would allow the treatment of more complex problems related to olive oil quality and authentication. For instance, application of NMR metabonomics to more complex systems, such as binary or higher mixtures of olive oils from different monocultivars, would establish subtle variations in the NMR spectral regions (signal intensities or chemical shifts) owing to the presence (or absence) of characteristic metabolites (e.g., phenols) that are unique to each oil component in the mixture. The profiling of the NMR metabonomics data could help to authenticate the coupage (blending) of olive oils, which frequently raises doubts in consumers' minds about the quality of the commercialized olive oil. Another field of exploration would be the investigation of possible existing differences in the deuterium distribution between minor compounds that belong to olive oil and potential adulterants, thus providing a useful NMR index to detect olive oil adulteration. A final trend is the quality examination of olive fruits since the quality of the extracted oils depends ultimately on the quality of olive fruits. A potential, noninvasive method to investigate quality factors or dynamic changes inside the fruit without piercing or slicing is magnetic resonance imaging. This NMR methodology, which has not been exploited in research on olive oil as it should be, could yield information on internal quality defects and disorders due to proharvest treatment and processes leading to quality defects or insect attacks. Also, serial MRI measurements could provide information about developmental processes, such as fruit ripening.

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