Università degli studi di napoli federico ii

Dipartimento di Scienza degli Alimenti

Tesi di Dottorato di Ricerca

Scienze e tecnologie
delle produzioni agro-alimentari
XXI ciclo

Application of High Resolution NMR
in the study of oils and fats

TUTOR:
PROF. RAFFAELE SACCHI

DOTTORANDO:
ANTONELLO PADUANO

COORDINATORE:
PROF. GIANCARLO BARBIERI

Non si riceve la saggezza, bisogna scoprirla da sé,
dopo un tragitto che nessuno può fare per noi, né può
risparmiarci, perché essa è una visuale sulle cose.

(M. Proust, *Alla ricerca del tempo perduto*)

To my family

INDEX

1 THE NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

The Nuclear Magnetic Resonance Spectroscopy (NMR) is a powerful analytical method that allows obtaining detailed information on molecular structure of organic compounds. It's one of the most common analytical methods in chemistry and the related sciences due to the wealth of information available from the spectra. Accordingly, both ^{1}H- and ^{13}CNMR spectroscopy are routinely used in lipid chemistry (Gunstone *et al.*, 2007). Also being the more recent spectroscopic technique, it has exceeded all the other techniques for potentiality of application in several fields of chemistry, finding employment also in other fields which medical sciences (Lanzetta, 2002).

NMR is an analytical method that makes use of the fact that nearly all molecules contain magnetic nuclei and can therefore be detected in a strong magnetic field when irradiated with a specific radiofrequency (Falch, 2006).

In 1945 was observed for first time the physical phenomenon of the Nuclear Magnetic Resonance, and that it made to win the Nobel Prize in physics to Bloch (Stanford University) and Purcell (Harvard University) in 1952. In 1953 the first commercial spectrometer was constructed operating to Continuous Wave (CW-NMR) and used to observation of proton only. In years '70 the second generation of spectrometers to Fourier Transform (FT-NMR) has allowed the observation of less abundant nuclei: ^{1}H, ^{13}C, ^{15}N, ^{19}F, ^{29}Si, and ^{31}P. The contemporary evolution of computers has widened the NMR applications with the possibility to obtain two and three dimensions spectra (2D and 3D-NMR). In organic chemistry NMR spectroscopy have wide application in the definition of structure of natural substances and in the study of reaction mechanisms (Lanzetta, 2002).

NMR spectroscopy involves a transition of nuclear *spin* induced from an opportune radio-frequency after to have subordinate the nucleus in examination to the influence of an external magnetic field. NMR studies a magnetic nucleus, like that of a hydrogen atom (^{1}H is the most receptive isotope at natural abundance), by aligning it with a very powerful external magnetic field and perturbing this alignment

using an electromagnetic field. The response to the field (the perturbing), is what is exploited in NMR spectroscopy. Therefore with the NMR the atomic nuclei are examinated directly and not the electrons. Every information on around chemical comes deducted observing the behaviour of the atomic nuclei (Falch, 2006).

The simplest atom, hydrogen, is found in almost all organic compounds and is composed of a single proton and a single electron. The hydrogen atom is denoted ^{1}H, in which the superscript signifies the sum of the atom's protons and neutrons (i.e., the atomic mass of the element). For the purpose of NMR, the key aspect of the hydrogen nucleus is its angular momentum properties, which resemble those of a classical spinning particle. Because the spinning hydrogen nucleus is positively charged, it generates a magnetic field and possesses a *magnetic moment μ*, just as a charge moving in a circle creates a magnetic field (**Figure 1.1**). The *magnetic moment μ* is a vector, because it has both magnitude and direction, as defined by its axis or spin in the figure. The NMR experiment exploits the magnetic properties of nuclei to provide information on molecular structure (Lambert and Mazzola, 2003).

Charge moving
in a circle

Spinning spherical
nucleus
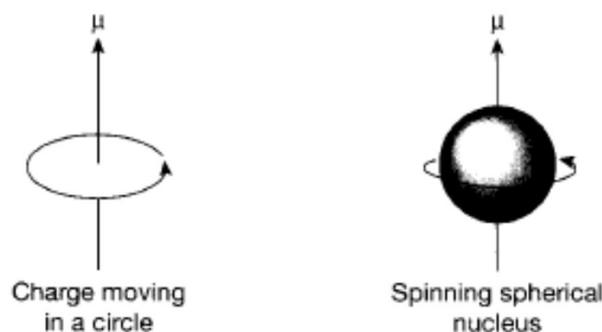
Figure 1.1. Analogy between a charge moving in a circle and a spinning nucleus

The spin properties of protons and neutrons in the nuclei of heavier elements combine to define the overall spin of the nucleus. When both the atomic number (the number of protons) and the atomic mass (the sum of the protons and neutrons) are even, the nucleus has no magnetic properties, as signified by a zero value of its *spin quantum number I* (**Figure 1.2**).
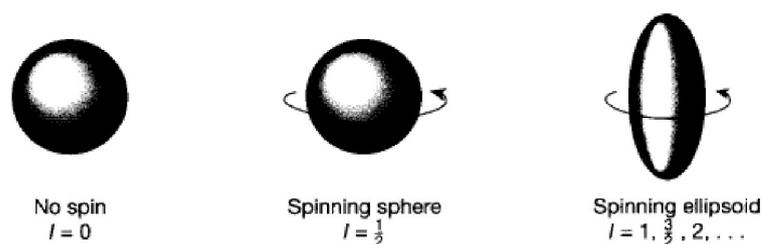
Figura 1.2. Three classes of nuclei.

Such nuclei are considered not to be spinning. Common nonmagnetic (nonspinning) nuclei are carbon (^{12}C) and oxygen (^{16}O), which therefore are invisible to the NMR experiment. When either the atomic number or the atomic mass is odd, or both are odd, the nucleus has magnetic properties and is said to be spinning. For spinning nuclei, the *spin quantum number* can take on only certain values. Those nuclei with a spherical shape have a spin of ½, and those with a nonspherical, or quadrupolar, shape have a spin of 1 or more (in increments of ½). Common nuclei with a spin of ½ include ^{1}H, ^{13}C, ^{15}N, ^{19}F, ^{29}Si, and ^{31}P. Thus, many of the most common elements found in organic molecules (H, C, N, P) have at least one isotope with $I = ½$ (although oxygen does not). The class of nuclei with $I = ½$ is the most easily examined by the NMR experiment. Quadrupolar nuclei ($I > ½$) include ^{2}H, ^{11}B, ^{14}N, ^{17}O, ^{33}S, and ^{35}Cl.

The magnitude of the magnetic moment produced by a spinning nucleus varies from atom to atom in accordance with the equation $\mu = \gamma hI$. The quantity h is Planck's constant divided by 2π, and γ is a characteristic of the nucleus called the *gyromagnetic* or *magnetogyric ratio*. The larger the gyromagnetic ratio, the larger is the magnetic moment of the nucleus. Nuclei that have the same number of protons, but different numbers of neutrons, are called *isotopes* ($^{1}H/^{2}H$, $^{14}N/^{15}N$). The term *nuclide* is generally applied to any atomic nucleus.

All *nuclei* that have a *spin quantum number* value $I \neq 0$ possesses a *magnetic moment* μ and, when are subordinated to the action of an external magnetic field and irradiated by a radio frequency, they turn out active to the NMR, that is give useful

and interpretable marks for the definition of the molecular structure.

The most common magnetic nuclei are ^1H, ^{13}C, ^{31}P, ^{19}F, ^{14}N with natural abundances close to 100%, except ^{13}C which has 1.1% natural abundance. In lipid research ^1H, ^{13}C and ^{31}P are the most studied nuclei. Nuclei in different chemical environments have slightly different resonance frequencies. The resonances obtained from NMR are expressed as *chemical shift* values (δ) in ppm units relative to a reference compound (tetramethylsilane ($\delta = 0.0$ ppm) for ^1H).

While resonance frequency is given in Hz, which is dependent on the acquisition conditions, the *chemical shift* scale is a dimensionless quantity and a more convenient way of presenting the spectra. The *chemical shift* values are dependent on molecular structure (Hunter *et al*, 2005), but do also vary based on solvent, concentration (Mannina *et al*., 2000; Gunstone, 2004) and pH (Fan, 1996).

1.1 ULTRA-HIGH RESOLUTION NMR (HR-NMR)

The application of Nuclear Magnetic Resonance (NMR) spectroscopy to the analysis and quality control in foods also shows great developments in the last few years. The increase of new applications and the attention to this technique by scientists, official control institutions and food industries can be attributed both to the high specificity and versatility of the NMR technique and to the improvement of NMR instrument performances and availability (Sacchi and Paolillo, 2007).

Ultra High-Resolution NMR (HR-NMR) utilises frequencies above 1 GHz and is available together with cryo-probes in recent years. HR-NMR has been recently applied in many more food authenticity studies. The advantage of HR-NMR is that permits the observation of very detailed spectral parameters of a food sample (high resolution of signals in natural food mixtures and used without any previous treatment) as well as a very high sensitivity. These two factors are indeed quite important in assigning components and measuring their intensity ratios. In general qualitative and quantitative criteria are considered in HR-NMR. Qualitative criteria are discussed in terms of linearity and selectivity. NMR is definitely the best analytical method from the linearity point of view since the intensity of resonances is

4

strictly proportional to the number of nuclei resonating at a certain frequency. Selectivity is also extremely good because NMR differentiates all the isotopes of the elements and even for a given isotope is able to yield measurable differences in chemical shift for different chemical environments.

From a quantitative point of view sensitivity, precision and accuracy are crucial criteria. Sensitivity depends on the signal to noise ratio (S/N) which can be considered acceptable when it is higher than 10. Modern NMR spectrometers easily meet these requirements. Precision and accuracy can be determined from mean standard deviations on replicates. It has been shown that by using ultra-high field spectrometers precision and accuracy are comparable to HR-GC (high resolution gas chromatography) techniques (Sacchi and Paolillo, 2007). The major disadvantage of HR-NMR is that it is one of the most expensive analytical techniques to employ, both in terms of the initial capital outlay and running costs (Reid *et al.*, 2006).

1.2 APPLICATIONS OF NMR TO OILS AND FATS ANALYSIS

Food quality, authenticity and traceability are becoming very important aspects in the food chain and market. Safety, nutritional and sensory quality are very important for both fresh and processed food products and recent issues are related to the assurance of the food geographic origin, raw material used (botanical variety or cultivar, animal species) and processes applied to produce it (Aparicio *et al.*, 1999; Sacchi and Paolillo, 2007).

Authentication of food products is of primary importance for both consumers and industries, at all levels of the production process, from raw materials to finished products. From the legislative point of view, quality standards have been established through the requirement of quality labels that specify the chemical composition of each product. From the economic point of view, product authentication is essential to avoid unfair competition that can create a destabilized market and disrupt the regional economy and even the national economy. All food products targeted for adulteration are high-commercial-value products and/or produced in high tonnage around the world (Cordella *et al.*, 2002).

One of the main fields in which High Resolution NMR has been largely applied is food lipids. The use of NMR in lipid science has begun some forty years ago with the pioneering applications of proton NMR to oils and fats made with the aim of assess the global unsaturation (Johnson and Shoolery, 1962). These Researchers measured the degree of unsaturation of fatty acids and their triglycerides. Area measurements of signals assignable to olefinic protons in the fatty acid chains and protons in the glyceryl radical allowed a determination of average molecular weight. This measure were used to calculate an iodine number which are compared to that obtained on the same sample by the Wijs method.

Since 1962 to 2008 many NMR applications in the study of oils, fats and lipids appear in the scientific literature and also several reviews on ^{13}C and ^{1}H NMR applications have been recently published. The results and applications relevant for our study will be briefly discussed in the following paragraphs.

1.2.1 ^{1}H-NMR spectroscopy.

The term "proton" is routinely used to indicate ^{1}H-NMR spectroscopy even though the atoms, the hydrogens, in the molecules are the species studied. ^{1}H NMR spectroscopy has become an extremely valuable research tool in elucidating the structure of molecules as the chemical shifts (position of signals in the spectrum) of the protons are often very sensitive to minor changes in molecular structure.

For the purpose of obtaining NMR spectra, lipid samples are usually dissolved in a deuterated solvent or one that does not contain hydrogen. Deuterated chloroform ($CDCl_3$) is currently the most common solvent used for this purpose. With about 1 mg of oil, a ^{1}H NMR spectrum can be recorded in few minutes. All values reported in **Table 1.2.1.1** use the δ scale (ppm), in which to the signal of tetramethylsilane (TMS) has been assigned the value $\delta = 0$. In practice, many spectra are now obtained using the known shift values of the solvent signal(s) as reference.

Table 1.2.1.1 contains information on the most common functional groups in lipid chemistry and their ^{1}H-NMR chemical shifts. To illustrate these values, the ^{1}H-NMR spectrum of methyl linoleate (**Figure 1.2.1.1**) is given as an example since it contains most of the common functional groups in a fatty acid chain. The assignments are shown in **Figure 1.2.1.2**. The abbreviations in parentheses refer to the splitting of the signals, s = singlet, d = doublet, t = triplet, q4 = quartet, q5 = quintet, m = multiplet (usually broad; br) (Gunstone *et al*., 2007).

[1]H nuclear magnetic resonance spectroscopy is a technique that can be very useful to determine the proportion of the different acyl groups in oils and fats, in a very short period of time. The area of the signals of the ^{1}H NMR spectra is proportional to the number of hydrogen atoms that produce the signal (Sacchi *et al*., 1989, 1996; Guillèn *et al.,* 2003a). High-resolution ^{1}H NMR spectroscopy has limited use in fatty acid analysis owing to the small range of chemical shifts covered by protons, which resulted in the small number of signals in the proton spectrum. The splitting patterns of proton signals, however, can provide unique structural properties of lipid molecules under investigation (Lie Ken Jie and Mustafa, 1997).

Table 1.2.1.1. Assignments of proton signals in the ^{1}H-NMR spectra of fatty compounds; all values relative to tetramethylsilane (TMS) = 0 ppm

Structure	Shift values [a]
— CH_2 — (cyclopropane)	(-0.3) – 0.6
— CH_2 — (cyclopropene)	0.6 (singlet)
— CH_3 (terminal methyl in alkyl chain)	0.85–0.90 (triplet)
— CH_3 (branched, saturated isoprenoid)	0.85–0.90 (singlet or doublet)
— $C(CH_3)_2$ isopropyl methyl	1.2–1.3
(ω1)CH_2, saturated alkyl chain	1.21.3
— CH_2 — , acyl C-3, saturated chains	1.58
— CH_2 — , acyl C-4 to C-(ω3). saturated chains; (ω2)CH_2, saturated chain	1.2–1.3
RSH (sulfhydryl)	1.1–1.5[b]
RNH_2 (amino)	1.1–1.5 (1.8)[b]
R_2NH (imino)	0.4–1.6 (2.2)[b]
R_3C-H (saturated)	1.4–1.7
— C=C — CH_3 (allylic methyl)	1.6–1.9 (doublet)
— C=C—CH_2 — (allylic methylene)	2.04 (doublet)
— C=C — CH_2 — C=C — (diallylic methylene)	2.8 (triplet)
— CH_2 — COOR, acyl C2	2.1–2.3 (triplet)
— CH_2 — CO — (α-metbylene in ketone)	2.2–2.5
COOR-CH_3 (methyl in acetoxy)	1.9–2.6 (singlet)
Ar — CH_3	2.1–2.5
— C–C — H (terminal acetylene, nonconjugated)	2.5–2.7
— O — CH_3 (methoxy ether, aliphatic)	3.3–3.8 (singlet)
— O — CH_3 (methyl ester, aliphatic)	3.6–3.8 (singlet)
— CH — OH, sn-2 in glycerol	3.75 (multiplet)
— CH_2 — OH, sn-1 or sn-3 in glycerol	3.6 (doublet)
— O — CH_2 — (aliphatic saturated alcohol or ether)	3.4–3.7
— CH_2 — O — CO — R (sn-1 or sn-3 esterified glycerol)	4.2–4.4
— CH — O — CO — R (sn-2 esterified glycerol)	5.1–5.2 (quintet)
— CH_2 — O — R (sn-1- or sn-3-O-alkylglycerol)	3.5–3.6
— CH — O — R (sn-2-O-alkylated glycerol)	3.6–3.7
— CH — O — P (O-acylglycerol; sn-1 or sn-3)	3.9
— CH_2 — O — P (O-alkylglycerol; sn-1 or sn-3)	3.9
— CH_2 — O — P (choline or sulfocholine)	4.3–4.4
R — OH (hydroxyl proton)	3.0–5.3
R — CH = CH — O (vinyl ether)	5.8 (cis), 6.0 (trans)
C=CH_2 (terminal vinyl, nonconjugated)	4.6–5.0
H — C = C — H (olefinic or cyclic; nonconjugated)	5.1–5.9 (multiplet)
— CH = CH — R, cis-Δ^2 in fatty acid chain	7.0 (-), 5.8 (-)
cis-Δ^3 in fatty acid chain	5.6
cis-Δ^4 in fatty acid chain	5.5
cis-Δ^5 in fatty acid chain	5.4
cis-Δ^6 in fatty acid chain	5.4
cis-Δ^9 in fatty acid chain	5.3
cis-Δ^{12} in fatty acid chain	5.3
— (CH$_3$)C=C — H (olefinic isoprenoid)	5.0–5.1
— C = CH_2 (terminal vinyl, conjugated)	5.3–5.7 (6.2)
H — C = C — H (olefinic, conjugated; diene or triene)	5.8–6.5 (7.1)
— CO — N — H (amide NH and CO)	5.5–8.5
Ar - H (benzenoid)	7.3 - 8.5
RCHO (aldehyde proton aliphatic saturated)	(9.5) 9.7–9.8
aliphatic, α,β-unsaturated	9.5–9.7
R-COOH (carboxyl)	10.5–12.0

[a] Values in parentheses apply to compounds that may absorb outside this range.
[b] Concentration-dependent; higher δ when diluted.
Source: From Gunstone F.D., Harwood J.L. and Dijkstra A.J. (2007). In The Lipid Handbook - Third Edition CRC Press Taylor & Francis Group.
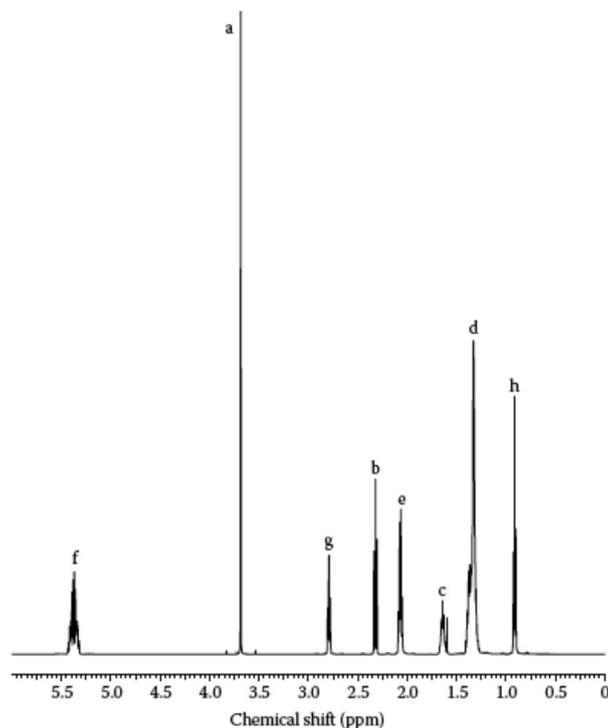
Figure 1.2.1.1. ^{1}H-NMR spectrum of methyl linoleate.
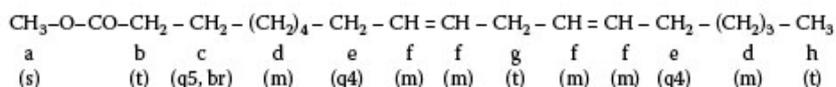
Figure 1.2.1.2. Example of the common functional groups in a fatty acid chain.

In **Figure 1.2.1.3** the spectrum of an olive oil is shown as an example. The labelled signals can be observed in all triglyceride seed oils that contain usual fatty acids, including linolenic acid. Signal groups at δ (ppm) 0.85 ($CH=CHCH_2CH_3$ all acids except linolenyl); 0.95 ($CH_2CH_2CH_2CH_3$ Linolenyl); 1.28 (CH_2 All acyl chains), 1.6 (CH_2-CH_2-COOH, All acyl chains); 2.05 ($CH_2CH=CH$, All unsaturated fatty acids), 2.2 (-CH_2-COOH, All acyl chains); 2.8 ($CH=CHCH_2CH=CH$, Linoleyl and linolenyl); 4.19 (CH_2OCOR, Glycerol triacylglycerols); 5.15 ($CHOCOR$, Glycerol triacylglycerols); 5.29 ($CH=CH$, All unsaturated fatty acids); 7.26 ($CHCl_3$,

9

Chloroform solvent) can be observed in the spectrum. As only a relatively small number of signals at $\delta > 9$ ppm can be observed, the information impact of ^{1}H NMR spectra is limited in comparison with that of ^{13}C NMR spectra (Sacchi *et al.*, 1997; Spitzer, 1999).
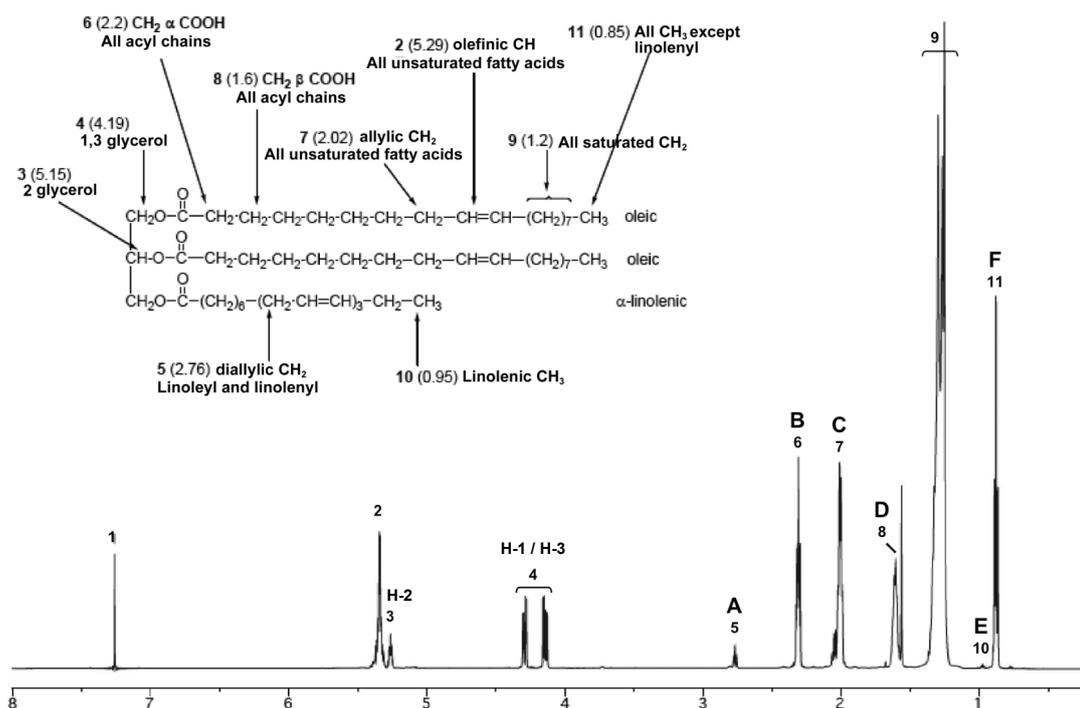
Figure 1.2.1.3. 400 MHz ^{1}H NMR spectrum of a virgin olive oil. (*from* Sacchi *et al.*, 1997)

The proton resonances were explained in terms of the long-range deshielding effects which are produced by the functional groups of *cis*-unsaturated fatty acids and esters, i.e. the double bonds and carboxylic groups, upon methylenes up to five or six carbons distant from the functional groups. The chemical shifts were predicted by adding to the basic unperturbed value for a middle chain methylene ($\delta = 1{,}25$) and for a methyl group ($\delta = 0{,}88$); the α- to ζ- substituent effects of the functional groups (Vlahov, 1999). The configuration of the double bond protons could be determined by the coupling constant of methyne protons which is always larger for *trans* than for *cis* bonds, especially for monounsaturated compounds, even if this is not possible in

practice because the chemical shift differences of methyne protons of isolated double bonds are small and produce a signal envelope that is very difficult to analyse (Vlahov, 1999). In conjugated linoleic acid (CLA) the peaks of the olefinic protons are split. The number of signals depends on the double bond configuration. Two peaks are observed when the two double bonds have identical configuration and four signals when the configuration is not identical (Lie Ken Jie *et al.*, 1997; Gunstone *et al.*, 2007).

Glycerides. The glycerides, 1- and 2-monoglycerides, 1,2-diglycerides, 1,3-diglycerides and triglycerides can be detected on the basis of the resonances of the protons attached to glycerol carbons. Three well resolved signals appear for the H-1, H-2 and H-3 protons in 1-mono and 1,2-diglycerides, whereas the H-1 and H-3 protons resonate as a single peak shifted at lower frequency from H-2 in 2-mono and triglycerides (**Figure 1.2.1.3**). Only one signal appears for the glycerol protons in 1,3-diglycerides (Sacchi *et al.*, 1991, 1996; Gunstone *et al.,* 2007). The resolution of proton resonances of 1,3-diglycerides and triglycerides, is improved by acylating the diglyceride hydroxyl groups with trichloroacetyl isocyanate which makes the α,α'-CH_2 of 1,3-diglycerides shift to higher frequency. As a consequence, they appear better resolved from the triacylglycerol resonances (Sacchi *et al.,* 1991). The method was used for the quantitative determination of 1,2- and 1,3-diglycerides in virgin olive oils and, in particular, to detect the adulteration of virgin olive oils by refined olive oils whose diglyceride content is substantially larger.

Fatty acids. The amounts of individual fatty acids can be obtained by means of ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectroscopy in an oil or fat sample (Knothe and Kenar, 2004). Probably the first report on the ^{1}H-NMR spectra of fatty compounds was published in 1959 (Hopkins and Bernstein). Three years later, the presumably first report using ^{1}H-NMR for quantitatively determining unsaturation and average molecular weight followed (Johnsthon and Shoolery, 1962).

Further, the molar percentages of unsaturated fatty acids in triglycerides can be measured by comparing the peak areas of the allylic methylenes with those of the methyl signals. Considering that each mole of monoenoic acid or polyunsaturated

fatty acid contains four allylic protons, the ratio of peak areas of allylic methylenes (δ = 2.05 ppm) and terminal methyl of all chains (δ = 0.8 - 1.0 ppm) lower than 4:3 indicates the presence of saturated fatty acids, whereas the ratio is zero for triacylglycerols containing only saturated chains (Sacchi *et al.*, 1989, 1997; Vlahov, 1999). The use of ^{1}H-NMR has since expanded to include the identification of vegetable oils as well as identifying individual vegetable oils in mixtures thereof (Fauhl *et al.,* 2000; Sacco *et al.*, 2000; Guillén and Ruiz, 2003b; Brescia and Sacco; 2008). Since vegetable oils contain various proportions of saturated, oleic, and polyunsaturated acyl groups, the resulting signals have different chemical shifts and shapes depending on these proportions. A careful observation of the shape and number of peaks of each signal present in the spectra and the use of multivariate statistical method allows to find significant differences between vegetable oils of different composition (Guillén and Ruiz, 2003b). The adulteration of olive oil with seed oils such as soybean and rapeseed oils, whose linolenic acid content is higher than that of olive oil, can be detected by using the methyl signal of n-3 fatty acids resonating at 0.94 ppm, in olive oil the only n-3 fatty acid detected is linolenic acid. Owing to its low intensity, the resonance at 0.94 ppm has to be compared with the 13C satellites of the methyl resonance at 0.84 ppm whose amount is 0.57% of the methyl signal (Sacchi *et al.*, 1996). Nevertheless, the full fatty acid pattern of olive oils can be derived from the proton spectrum by measuring the intensities of the methyl resonance at 0.84 ppm as a total of saturated, monounsaturated and polyunsaturated (linoleic) chains, whereas the allylic (1.97 ppm) and the bis-allylic (2.73 ppm) proton resonances represent the sum of oleic plus linoleic acid and of linoleic plus linolenic acid, respectively (Sacchi *et al.,* 1996).

The analysis of the ^{1}H NMR spectrum of fish oils permits the quantitative determination of of specific fatty acids (DHA and EPA) in materials such as fish oils (Sacchi *et al.*, 1993a; Igarashi *et al.*, 2000, 2002; Tyl *et al.,* 2008).

Aldehydes and Volatile Compounds. The first ^{1}H NMR studies regarding oxidation processes of edible oils have been conducted on pure triacylglycerols, like trilinolein, trilinolenin, and triolein (Frankel, 1987). The oxidation of unsaturated

fatty acids by oxygen, generally known as autoxidation, is important in the development of rancidity and other "off-flavours" in edible fats. The secondary reactions occurring during the autoxidation produce by chain scission, shorter-chain carbonyl compounds (mainly saturated and unsaturated aldehydes). The volatile compounds of virgin olive oils have been studied by high field (600 MHz) NMR spectroscopy (Sacchi *et al.,* 1996). The aldehydic protons of saturated aldehydes, hexanal and heptanal resonate as a singlet at 9.74 ppm, whereas *trans*-2-hexenal, which represents more than 50% of the headspace above fresh extra virgin olive oils, resonates at 9.46 ppm as a doublet ($J = 7.96$ Hz) (Vlahov, 1999). In model sistems, the heating of oils in presence of pure oxygen formed principally *mono*hydroperoxides, together with *bis*-hydroperoxides and *tris*-hydroperoxides (Neff *et al*., 1988), that produce a characteristic signal at 8-8.9 ppm, due to the hydroperoxide group. Other authors (Sacchi *et al*., 2006a; Guillén and Ruiz, 2005a,b, 2006, 2008) have directly monitored by ^{1}HNMR spectroscopy the deterioration process of edible oils submitted to thermal stressing, in order to study the nature and levels of potentially toxic products, which were produced during heating at frying temperature. They detected the presence of signals due to hydroperoxides and saturated, *mono*-unsaturated, *di*-unsaturated, and hydroxy *mono*-unsaturated aldehydes. These aldehydes can be easily detected in the low-field region of the ^{1}H NMR spectrum (Sacchi *et al*., 2006a; Guillén and Goicoechea, 2007) and can be used to follow the oxidation process of an oil and to evaluate its oxidative stability (Brescia and Sacco, 2008).

The composition of volatiles plays an important role in defining the sensory quality of virgin olive oils. Positive (green-fruity odor, etc.) and negative attributes (bitter tasting, etc.) have been correlated with the presence of aldehydes, alcohols, polyphenols, terpenes, and acetic acid. The very low concentration of these compounds in virgin olive oil makes possible their direct determination only by high field (500-600 MHz) ^{1}HNMR (Mannina and Segre, 2002) further studies are needed to establish more strict correlations between minor compounds determined directly by high field NMR and sensory profile of virgin olive oils.

Sterols. The signal due to methyl CH_3 in position 18 of the sterols resonates between 0.6 and 0.7 ppm. The signal due to methyl 18 signal of β-sitosterol, resonating at 0.62 ppm, has been clearly identified in virgin and refined olive oils (Sacchi *et al.,* 1996).

Phenolic Compounds. Phenolic compounds contribute to olive oil sensory quality, since they are responsible for its bitter and pungent flavor. The prevalent phenolic constituents of olive oil are secoiridoid derivatives, such as the dialdehydic form of the elenolic acid linked to hydroxytyrosol or to tyrosol (ligstroside) and the elenolic acid linked to hydroxytyrosol (oleuropein aglycon) (Sacchi *et al*., 1997; Brescia and Sacco, 2008). Their structure has been identified using NMR (Montedoro *et al*., 1993), after extraction in absolute methanol and separation by HPLC. Other compounds detected in olive oil phenolic fraction, whose structure was elucidated with NMR, are demethyl-oleuropein, glucosides of hydroxytyrosol (Bianco and Uccella, 2000) and also two soluble lignans-pinoresinol and its derivative, 1-acetoxypinoresinol (Brenes *et al*., 2000).

A detailed assignment of ^{1}H NMR spectra of phenolic compounds extracted by SPE from virgin olive oils and analyzed in methanol-*d* solution by NMR was reported by Dais and Christophoridou (2008).

1.2.2 ^{13}C-NMR spectroscopy

The chemical shift values of ^{13}C normally ranges from 0 to 200 ppm and spectra are normally broadband, proton decoupled and therefore show the resonances as single lines. The lower abundance of ^{13}C compared to ^{1}H, makes it is 400 times less sensitive to the NMR phenomena. The number of scans is usually 1000 or more. The sample size for a routine ^{13}C-NMR spectrum is 50-100 mg but by investment in a large number of scans high quality spectra can be obtained with as little as 1 mg. It therefore takes a longer time to acquire ^{13}C spectra, though they tend to look simpler. Overlap of peaks is much less common than for ^{1}H NMR which makes it easier to distinguish among different carbon atoms (Sacchi *et al.*, 1989; Falch, 2006).

Similar to ^{1}H-NMR a ^{13}C-NMR data for linoleic acid have been shown (**Figure 1.2.2.1.**). The ^{13}C-NMR spectrum of the methyl ester of linoleic acid is shown in **Figure 1.2.2.2**.
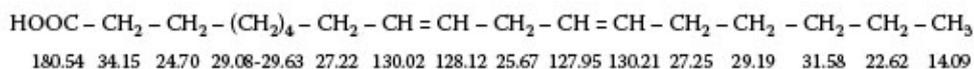
Figure 1.2.2.1. Example of the common functional groups in a linoleic acid chain and their ^{13}C-NMR chemical shift in CDCl$_3$.
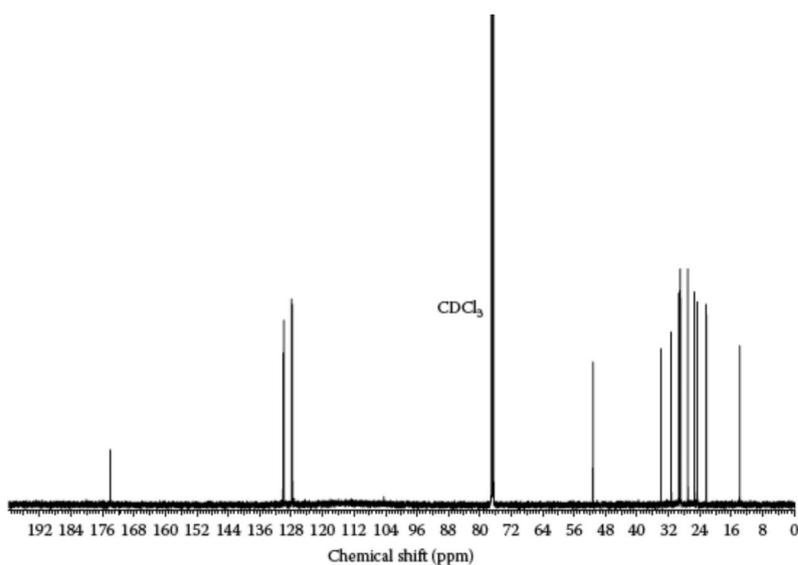
Figure 1.2.2.2. ^{13}C-NMR spectrum of methyl linoleate.

Generally, many effects observed in ^{1}H-NMR are also found in ^{13}C-NMR. For example, the methyl and methylene signals are upfield in the spectrum, while signals of olefinic carbons are farther downfield. The number and nature of double bonds affects the chemical shifts as does the proximity of multiple double bonds to each other and the presence of functional groups. **Table 1.2.2.1** is a compilation of the chemical shifts of functional groups in ^{13}C-NMR (Gunstone *et al.*, 2007).

Table 1.2.2.1. Assignments of carbon signals in the ^{13}C-NMR spectra of fatty compounds (CDCl$_3$)

Acids and Esters	Assignment
HOOC – (CH$_2$)$_x$	179–181
HOOC – CHCH$_3$ – (CH$_2$)$_x$	183–184
CH$_3$O – CO – ; other alkyl esters	172–175
CH$_3$O – CO	51–52
Glycerol esters	
Triacylglycerols	
Glyc-O-CO-(CH$_2$)$_x$ – CH$_3$	173–173.2 (-chain); 172.7-172.9 (-chain)
Glyc-O-CO-(CH$_2$)$_x$ – CH = CH - R	172.5–172.9 (-chain); 172.1–172.5 (-chain)
CH$_2$ – O – CO – R	62–62.5
CH – O – CO – R	68.5–69
CH$_2$ – O – CO – R	62–62.5
Diacylglycerols	
CH$_2$ – O – CO – R	ca. 61.5–62
CH – O – CO – R	72–72.5
CH$_2$OH	ca. 62
CH$_2$ – O – CO – R	ca. 65
CH$_2$	68–68.5
CH$_2$ – O – CO – R	ca. 65
Monoacylglycerols	
CH$_2$ – O – CO – R	63–63.5
CHOH	70-70.5
CH$_2$OH	65.65.5
CH$_2$ OH	61.5–62
CH – O – CO – R	74.5–75
CH$_2$OH	61.5–62
Methyl esters	
– (CH$_2$)$_x$ – CH$_3$; x > 1	13.5–14.5
Methylene in saturated chains	
(CH$_2$)$_x$	29–30
HOOC – CH$_2$ – (CH$_2$)$_x$ – or CH$_3$O – CO – CH$_2$ – (CH$_2$)$_x$ –	34-35
HOOC – CH$_2$ – CH$_2$ – (CH$_2$)$_x$ – or CH$_3$ O – CO – CH$_2$ – CH$_2$ – (CH$_2$)$_x$ –	23–26
– (CH$_2$)$_x$ – CH$_2$ – CH$_3$	22–23
– (CH$_2$)$_x$ – CH$_2$ – CH$_2$ – CH$_3$	31.5–32.5
Double bonds	
– CH = CH –	125–135 (exceptions given below)
(CH$_2$)$_x$ – CH = CH$_2$	114–115
(CH$_2$)$_x$ – CH = CH$_2$	139–140
(CH$_2$)$_x$ – CH$_2$ – CH = CH$_2$	33–35
(CH$_2$)$_x$ – CH$_2$ – C = C – CH$_2$ – (CH$_2$)$_y$	27–28 (*cis*); 32–33 (*trans*)
HOOC – CH$_2$ – C = C	33–34 (*cis*)
HOOC – CH$_2$ – CH$_2$ – C = C	22–23 (*cis*)
– CH=CH – CH$_2$ – CH$_3$	20–21(*cis*); 25–26 (*trans*)

(Continued)

Table 1.2.2.1. Continued

	Assignment
– CH=CH – CH_2 – CH_2 – CH_3	29-30 (*cis*); 34–35 (*trans*)
– C = C – CH_2 – CH = CH –	25–26 (all *cis*); 35–36 (all *trans*); 30–31 (*cis, trans*)
– CH = C = CH –	200–205
– CH = C = CH –	90–92
Triple bonds	
$(CH_2)_x$ – $C \equiv C$ – $(CH_2)_y$	79–81
$(CH_2)_x$ – CH_2 – C $\equiv$ C – CH_2 – $(CH_2)_y$	18–19
– C $\equiv$ C – CH_2 – $C \equiv$ C –	74–75
– C $\equiv$ C – CH_2 – C $\equiv$ C –	9–10
– C $\equiv$ C – CH_2 – CH_2 – C $\equiv$ C –	19–20
– $C \equiv$ C – C $\equiv$ C –	77–78
– C $\equiv$ C – $C \equiv$ C –	65–66
–CH_2-C $\equiv$ C –	18–20
Hydroxy groups	
$(CH_2)_x$ – $CHOH$ – $(CH_2)_y$	71–73
$(CH_2)_x$ – CH_2 – CHOH – CH_2 – $(CH_2)_y$	37–38
$(CH_2)_x$ – CH = CH – $CHOH$ – $(CH_2)_y$	67–68 (*cis*); 73–74 (*trans*)
$(CH_2)_x$ – CH = CH – CHOH – $(CH_2)_y$	131–134; (*trans*; CH adjacent to CHOH downfield)
$(CH_2)_x$ – CH = CH – CH_2 – CHOH – $(CH_2)_y$	133–134 (*cis*)
$(CH_2)_x$ – CH = CH – CH_2 – CHOH – $(CH_2)_y$	125–126 (*cis*)
$(CH_2)_x$ – CH = CH – CH_2 – CHOH – $(CH_2)_y$	35–36 (*cis*)
$(CH_2)_x$ – CH = CH – CH_2 – CH_2 – CHOH – $(CH_2)_y$	130–131 (*cis*)
$(CH_2)_x$ – CH = CH – CH_2 – CH_2 – CHOH – $(CH_2)_y$	129–130 (*cis*)
$(CH_2)_x$ – CH = CH – CH_2 – CH_2 – CHOH – $(CH_2)_y$	23–24 (*cis*)
$(CH_2)_x$ – CH = CH – CH_2 – CH_2 – CHOH – $(CH_2)_y$	37–38 (*cis*)
$(CH_2)_x$ – $CHOH$ – $CHOH$ – $(CH_2)_y$	74–75 (erythro slightly > threo)
$(CH_2)_x$ – CH_2 – CHOH – CHOH – CH_2 – $(CH_2)_y$	31–32 (erythro), 33–34 (threo)
$(CH_2)_x$ – CHOH – CH_2 – CH_2 – CHOH – $(CH_2)_y$	32–33 (erythro), 33–34 (threo)
$(CH_2)_x$ – CH_2 – CHOH – CH_2 – CH_2 – CHOH – CH_2 – $(CH_2)_y$	37–38 (threo slightly > erythro)
$(CH_2)_x$ – CHOH – CH = CH – CHOH – $(CH_2)_y$	133–134; (*trans*; threo slightly > erythro)
Hydroperoxy compounds	
$(CH_2)_x$ – CH_2 – CH(OOH) – CH = CH – CH= CH – $(CH_2)_y$	86–88
$(CH_2)_x$ – CH_2 – CH(OOH) – CH = CH – CH = CH – $(CH_2)_y$	25–26
Oxo compounds	
$(CH_2)_x$ – CO – $(CH_2)_y$	209–213
$(CH_2)_x$ – CH_2 – CO – $(CH_2)_y$	42–43
$(CH_2)_x$ CH = CH – CH_2 – CO – $(CH_2)_y$	41–42
$(CH_2)_x$ CH = CH – CH_2 – CO – $(CH_2)_y$	133–134
$(CH_2)_x$ CH = CH – CH_2 – CO – $(CH_2)_y$	121–122
$(CH_2)_x$ – CO – CH_2 – CH_2 – CO – $(CH_2)_y$	36–37
$(CH_2)_x$ – CO – CH = CH – CO – $(CH_2)_y$	203-204 *cis*; 200–201 *trans*
$(CH_2)_x$ – CO – CH = CH – CO – $(CH_2)_y$	135–136 *cis*; 136–137 *trans*
Epoxy and furanoid compounds	
$(CH_2)_x$– CH–CH–$(CH_2)_y$ (with O bridge)	56–57 (*cis*); 58–59 (*trans*)
$(CH_2)_x$–CH_2–CH–CH–CH_2–$(CH_2)_y$ (with O bridge)	27–28 (*cis*); 31–32 (*trans*)
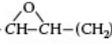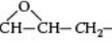
(Continued)

17

Table 1.2.2.1. Continued

	Assignment
$(CH_2)_x - CH=CH - CH_2 - CH \overset{O}{\triangle} CH - CH_2 - (CH_2)_y$	25–26 (cis; cis-epoxy)
$(CH_2)_x - CH=CH - CH_2 - CH \overset{O}{\triangle} CH - CH_2 - (CH_2)_y$	132–133 (cis; cis-epoxy)
$(CH_2)_x - CH=CH - CH_2 - CH \overset{O}{\triangle} CH - CH_2 - (CH_2)_y$	123–124 (cis; cis-epoxy)
$-(CH_2)_2 - C \overset{O}{\underset{C-C}{\diamond}} C - (CH_2)-$	104–105
	154–155
Acetoxy	
$(CH_2)_x - CO(COCH_3) - (CH_2)_y$	73–75
$(CH_2)_x - CO(COCH_3) - (CH_2)_y$	170–171
$(CH_2)_x - CO(COCH_3) - (CH_2)_y$	20–22
$CH_3 - O - CO - CO(COCH_3) - (CH_2)_y$	72–73
$CH_3 - O - CO - CO(COCH_3) - (CH_2)_y$	70–71
$(CH_2)_x - CH_2 - COCH_3$	64–65
Cyclic compounds (cyclopropene fatty acids)	
$(CH_2)_x - \overset{\overset{CH_2}{\diagup \diagdown}}{CH = CH} - (CH_2)_y$	7–8
	109–110
$(CH_2)_x - CH_2 - \overset{\overset{CH_2}{\diagup \diagdown}}{CH = CH} - CH_2 - (CH_2)_y$	25.5–26.5
$HOOC - (CH_2)_x - CH_2 - \overset{\overset{CH_2}{\diagup \diagdown}}{CH = CH} - CH_2 - (CH_2)_y; x = 2–3$	107–109
$HOOC - (CH_2)_x - CH_2 - \overset{\overset{CH_2}{\diagup \diagdown}}{CH = CH} - CH_2 - (CH_2)_y; x = 2–3$	110–112
Branched compounds	
$(CH_2)_x - CH(CH_3) - (CH_2)_y$	19–20
$(CH_2)_x - CH(CH_3) - (CH_2)_y$	32–33
$(CH_2)_x - CH_2 - CH(CH_3) - CH_2 - (CH_2)_y$	3637
$HOOC - CH(CH_3)_3 - (CH_2)_x - \text{ or } CH_3 - O - CO - CH(CH_3)_3 - (CH_2)_x$	39–40
$HOOC - CH(CH_3)_3 - (CH_2)_x - \text{ or } CH_3 - O - CO - CH(CH_3)_3 - (CH_2)_x$	16.5–17.5
$CH_3 - O - CO - CH_2 - CH(CH_3)_3 - (CH_2)_x$	30–31
$CH_3 - O - CO - CH_2 - CH(CH_3)_3 - (CH_2)_x$	19–20
$CH_3 - O - CO - CH_2 - CH(CH_3)_3 - (CH_2)_x$	41–42
$CH_3 - O - CO - CH_2 - CH_2 - CH(CH_3)_3 - (CH_2)_x$	31–32
$CH_3 - O - CO - CH_2 - CH_2 - CH(CH_3)_3 - (CH_2)_x$	19–20

Source: From Gunstone F.D., Harwood J.L. and Dijkstra A.J. (2007). In The Lipid Handbook - Third Edition CRC Press Taylor & Francis Group.

The fact that ^{13}C-NMR spectra cover a wider range of chemical shifts (0 to 200 ppm and even beyond) facilitates some aspects of evaluation compared to ^{1}H-NMR. A major aspect is that some functional groups impart shift values in parts of the spectrum where they do not overlap with the peaks of other moieties.

^{13}CNMR spectroscopy is routinely used in lipid chemistry. Edible lipids are composed of more than 95% of triacylglycerols. Therefore, the analysis of triacylglycerol components (fatty acids) and, more recently, triacylglycerols themselves, was primarily used to determine oil authenticity. Thus, one of the first

applications of NMR to oil analysis was the simultaneous determination of the fatty acid composition and positional distribution of triacylglycerols by high-resolution ^{13}C NMR (Pfeffer *et al.,* 1977b; Ng, 1983, 1985; Ng and Ng, 1983; Wollenberg, 1990; Sacchi *et al.*, 1992; Knothe *et al.*, 1995). When an oil is submitted to NMR analysis, the spectrum obtained is that of triacylglycerols. In **Figure 1.2.2.3**, the ^{13}C NMR spectrum of a virgin olive oil is shown. ^{13}C NMR resonances can be grouped into four well-defined spectral regions: carbonyl carbons ranging from 173.3 to 172.8 ppm; unsaturated carbons ranging from 132.0 to 127.1 ppm; glycerol carbons ranging from 69.1 to 61.6 ppm; aliphatic carbons ranging from 34.3 to 14.0 ppm. The assignment of the different signals has been the objective of many studies and is nowadays clearly resolved (Ng, 1983; Bonnet *et al.*, 1990; Sacchi *et al.*, 1992; Gunstone, 1993a,b; Van Calsteren *et al.*, 1996; Mannina *et al.*, 1999; Vlahov *et al.*, 1999; Alemany, 2002; Simova *et al.*, 2003; Vlahov, 2006). The main resonances observed in the ^{13}C NMR spectra of edible oils are collected in **Table 1.2.2.2** (Gunstone *et al.*, 2007).
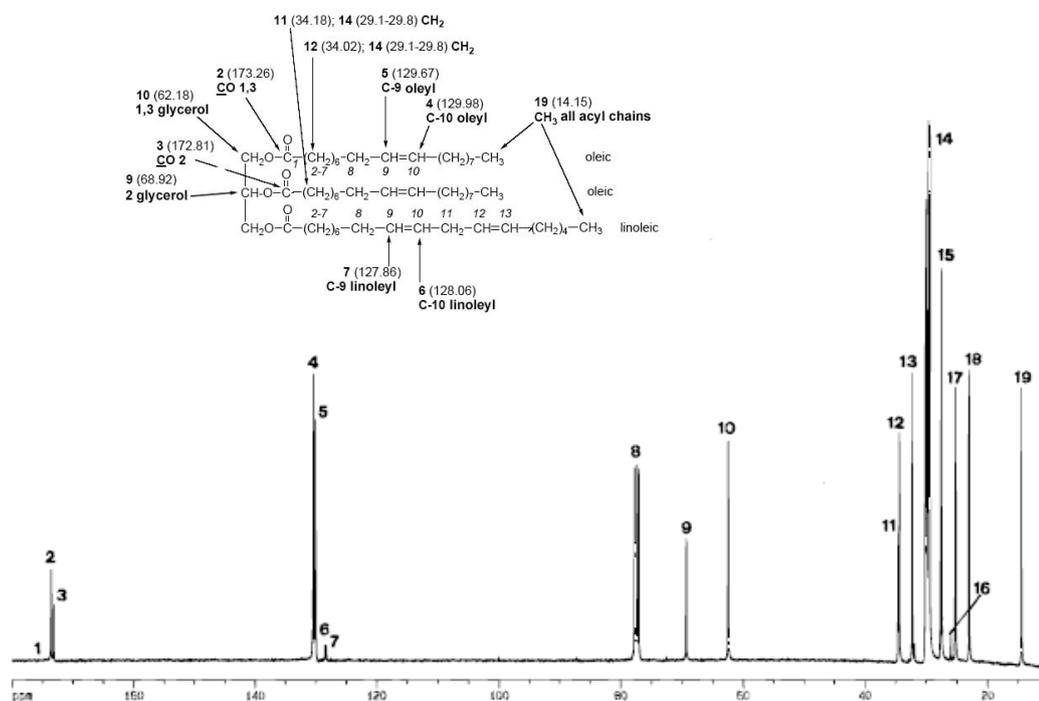
Figure 1.2.2.3. Typical ^{13}C NMR spectrum of a virgin olive oil. (*from* Sacchi *et al.*, 1997)

Table 1.2.2.2. Chemical shift assignments of the ^{13}C NMR signals of the main components of edible oils[a]

Chemical shift (ppm)	Assignment	Chemical shift (ppm)	Assignment	Chemical shift (ppm)	Assignment
176–174	Fatty acids	34.20	L2β/Ln2β	29.19	L5α/Ln5α
173.29	P1α	34.09	P2α/St2α	29.17	O6β/St4α
173.26	O1α	34.06	O2α	29.16	P4α
173.25	St1α	34.04	L2α/Ln2α	29.15	O6α/L6β
173.22	L1α/Ln1α	31.98	St16αβ	29.14	L6α/Ln6β
172.88	P1β	31.96	P14αβ	29.13	O4α/St4β/Ln6α
172.85	O1β/St1β	31.94	O16αβ	29.12	P4β
172.82	Ln1β	31.55	L16αβ	29.11	L4α
172.81	L1β	29.80	O12αβ	29.10	Ln4α
131.96	Ln16αβ	29.76	O7β/St11αβ/St12αβ/St13αβ/St14αβ		
130.23	Ln9α	29.74	O7α/P11αβ/P12αβ/St10αβ	29.09	O4β
130.22	L13β	29.73	P10αβ	29.07	L4β
130.21	L13β/Ln9β	29.72	St9αβ/St8αβ	29.06	Ln4β
130.06	O10β	29.70	P8αβ/P9αβ/St7β	27.26	O11αβ
130.04	O10α	29.68	P7β/St7α	27.23	L14αβ/Ln8αβ
130.01	L9α	29.66	P7α	27.21	O8αβ/L8αβ
129.98	L9β	29.65	L7β	25.66	L11αβ
129.74	O9α	29.63	L7α	25.65	Ln11αβ
129.71	O9β	29.62	Ln7β	25.56	Ln14αβ
128.32	Ln12β	29.61	Ln7α	24.95	P3β/St3β
128.31	Ln12α	29.56	O14αβ/St5β	24.92	O3β
128.26	Ln13α	29.54	P5β	24.91	St3α
128.25	Ln13β	29.53	St5α	24.90	L3β/P3α/Ln3β
128.12	L10β	29.52	P5α	24.88	O3α
128.10	L10α	29.42	St15αβ	24.86	L3α/Ln3α
127.93	L12α	29.40	P13αβ	22.73	St17αβ
127.92	L12β	29.37	L15αβ	22.72	P15αβ
127.80	Ln10β	29.36	O13αβ/O15αβ	22.71	O17αβ
127.79	Ln10α	29.35	St6β	22.59	L17αβ
127.15	Ln15α	29.33	P6β	22.57	Ln17αβ
127.14	Ln15β	29.32	St6α	14.29	Ln18αβ
68.93	β-Glycerol	29.31	P6α	14.13	P16αβ/St18αβ
62.13	α-Glycerol	29.24	O5β	14.12	O18αβ
34.25	P2α/St2β	29.22	L5β	14.08	L18αβ
34.23	O2β	29.21	O5α/Ln5β		

[a] Assignments are abbreviated by fatty acid, carbon number, and position in the glycerol. 1(3)- and 2-positions of glycerol are designated by the Greek symbols α and β, respectively. Labeling of acyl chains: P, palmityl; St, stearyl; O, oleyl; L, linoleyl; Ln, linolenyl chain. Depending on the oil composition and experimental conditions, a lower number of signals is usually observed.
Source: From Gunstone F.D., Harwood J.L. and Dijkstra A.J. (2007). In The Lipid Handbook - Third Edition CRC Press Taylor & Francis Group.

The different oils usually exhibit some differences in quantitative chain composition rather than in qualitative acid profile, therefore exhibiting analogous signals in the ^{13}C NMR spectrum, although with varying intensities which are characteristic for each oil. Positions and intensities of the signals are a consequence of the fatty acid profile of the oils and the position of these acyl chains in the triacylglycerols (Hidalgo and Zamora, 2003). Thus, when studying the carbonyl region (**Figure 1.2.2.4**), the substituents at the positions *sn*-1,3 and *sn*-2 of the triacylglycerol can be clearly distinguished.
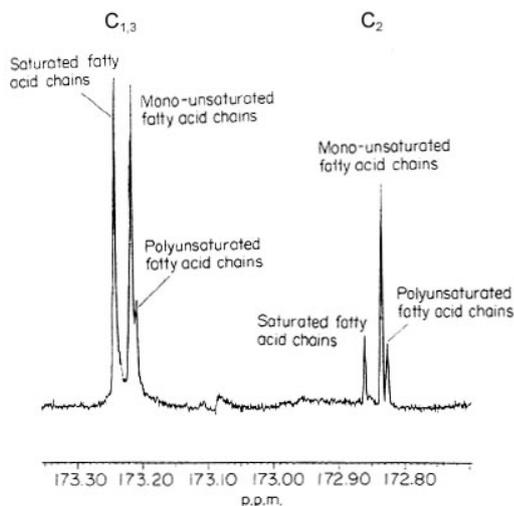
Figure 1.2.2.4. ^{13}C NMR carbonyl spectra of ester carbons of triglycerides. The carbonyl peaks are defined as attached to the *sn*-1,3 or *sn*-2 position. The acyl groups are saturated, monounsaturated and polyunsaturated (*from* Bonnet *et al.,* 1990).

Analogous results can also be obtained when analyzing the olefinic region. Thus, the different olefinic carbons of the different fatty acids and in the different positions of the triacylglycerol molecule appear at different chemical shifts (**Figure 1.2.2.5**).
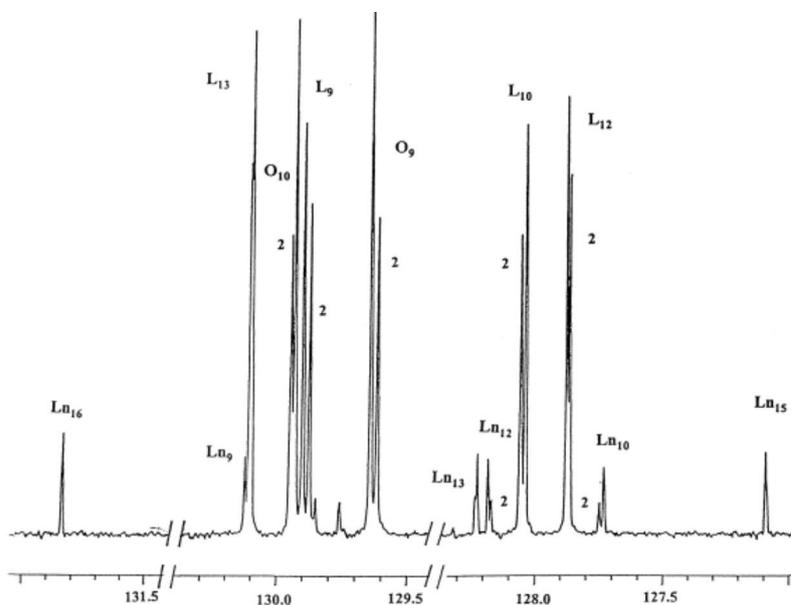
Figure 1.2.2.5. The olefinic carbon region 124–134 ppm of the 300 MHz ^{13}C spectrum of an olive oil sample. The resonances of the unsaturated carbons of oleyl (O), linoleyl (L) and linolenyl (Ln) chains esterified at 2-glycerol positions are indicated (*from* Vlahov, 1999).

21

All this information can be used for fatty acid determination. For this purpose HR-^{13}C-NMR was applied to the study of fish lipid composition and structure (Aursand and Grasdalen, 1992; Aursand *et al.,* 1993, 1995, 2000; Vatèle *et al.*, 1998; Aursand and Alexon 2001; Sacchi *et al.*, 1993a,b, 1994, 2006b; Scano *et al.*, 2008) and for the analysis of conjugated linoleic acid isomers (Davis *et al.*, 1999).

^{13}C NMR is also useful for distinguishing different glyceride classes and for verifying the presence of non triacilglycerol compounds such as mono-, diglycerides and phospholipids (Sacchi *et al.*, 1990, 1993b, 1995; Medina and Sacchi, 1994; Ng, 2000). The glycerol carbons of mono-, di- and triacylglycerols, resonate in the spectral region from 60 to 72 ppm. The chemical shift assignments were based on the assumption that acylglycerol symmetry or asymmetry determines the number of resonances and their relative intensities. Therefore, 2-monoacylglycerols, 1,3-diacylglycerols and triacylglycerols give two signals for the glycerol moiety with intensity ratios 1:2, whereas the asymmetrical 1-monoacylglycerols and 1,2-diacylglycerols give three separate signals (Vlahov, 1999). The spectral region of glycerol carbons of a standard mixture of glycerides, is reported in **Figure 1.2.2.6** and the chemical shift assignments in **Table 1.2.2.3**. The shift data demonstrate that the length and degree of unsaturation of the acyl chains does not influence significantly the chemical shifts of glycerol carbons.

Table 1.2.2.3. ^{13}C NMR chemical shifts (ppm) of glycerol carbon atoms in glycerides at different esterification degree (*from* Vlahov, 1999).

Glycerol ester	1 CH$_2$	2 CH	3 CH$_2$
1-Monopalmityl	63.34	70.26	65.11
1-Monooleyl	63.35	70.26	65.13
1,2-Dipalmityl	61.54	72.11	62.02
1,2-Dioleyl	61.56	72.12	62.00
1,3-Dipalmityl	65.04	68.38	65.04
1,3-Dioleyl	65.03	68.34	65.03
1,2-1,2,3-Tripalmitin	62.10	68.87	62.10
1,2,3-Trilinolein	62.10	68.88	62.10
1,2,3-Triolein	62.08	68.87	62.08
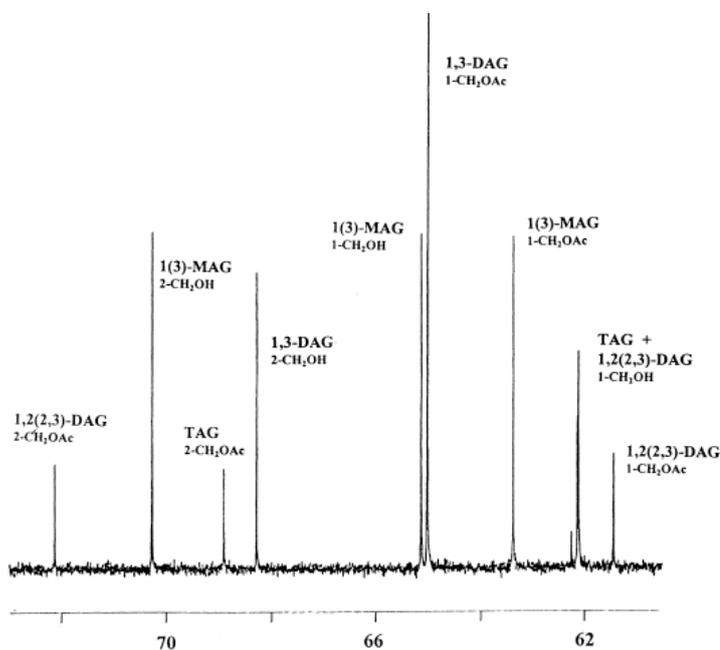
Figure 1.2.2.6. The glycerol carbon region 60-72 ppm of the 300 MHz ^{13}C spectrum of a standard mixture of glycerides at different esterification degree. The resonances of glycerol carbons are labelled as -CH$_2$OAc and -CH$_2$OH in correspondence of an acylated and a non-acylated alcoholic group of the glycerol molecule (*from* Vlahov, 1999).

The fatty acids composition can be evaluated in the ^{13}C NMR spectrum by evaluating the intensities of the signals of methylene and olefinic carbons. The resonances of methylene and methyl carbons of saturated and unsaturated acyl chains are reported in **Figure 1.2.2.7.**

From the observation of the methylene region (**Figure 1.2.2.8**), saturated, *cis*-monoene, *cis*-diene and *cis*-triene levels can be determined. In addition, the total *trans/cis* ratio and the amounts of *trans*-monoene (*t*), *cis-trans* (*ct*), *trans-cis* (*tc*) and *trans-trans* (*tt*) diene can be calculated. This possibility arises from thew very large chemical shift difference (Δδ=5 ppm) occurring in the *cis*-allylic carbons (27.12 ppm) with respect to the *trans*-allylic ones (32.53 ppm) in monoenes as well as from the presence of resolved signals for dienes (**Table 1.2.2.4**) (Pfeffer *et al.*, 1977a; Sacchi *et al.*, 1995).
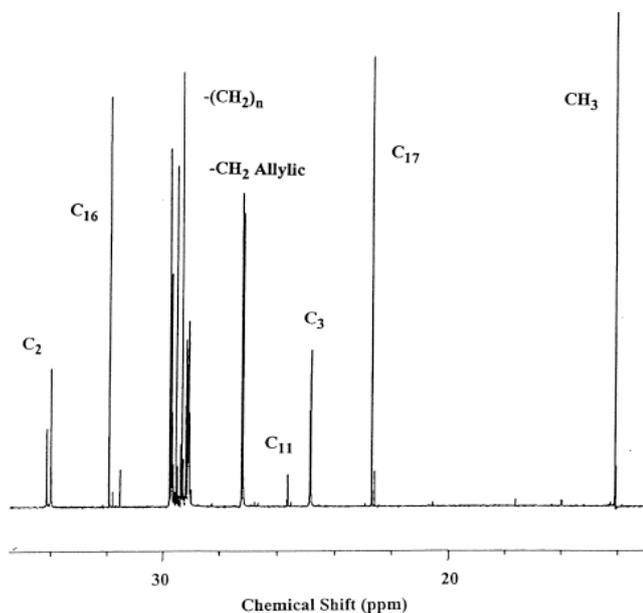
Figure 1.2.2.7. The methylenic and methyl carbon region 10-35 ppm of the 300 MHz 13C spectrum of an olive oil sample. The resonances of carboxy chain end methylenes C-2 and C-3, of methyl chain end carbons C-16/C-18, of allylic and bis-allylic methylenes, are reported except for the methylene envelope –$(CH_2)n$ which is indicated as a whole (*from* Vlahov, 1999).
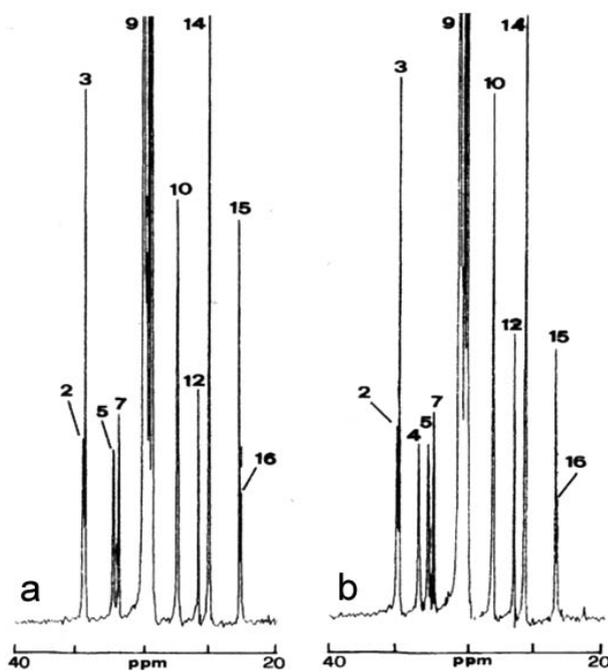
Figure 1.2.2.8. The methylene region of the 100.64 MHz 13C-NMR spectra of fractionated-interesterified (**a**) and hydrogenated (**b**) margarine samples (*from* Sacchi *et al.*, 1995).

24

Table 1.2.2.4. Chemical shift (ppm/TMS) assignement of methylene carbons (*from* Sacchi *et al*., 1995).

Peak number	ppm/TMS	Carbon	Fatty acid[a]
1	35.56	C-11	18:2,tt
2	34.10	C-2 (β)	All
3	33.95	C-2 (α)	All
4	32.53	C-8,C-11	18:1t
		C-8,C-14	18:2tt
5	31.84	ω3	SFA and n-9 acids
6	31.43	ω3	n-6 *cis* acids
7	31.35	ω3	n-6 *trans* acids
8	30.37	C-11	18:2,ct and 18:2,tc
9	29.6-29.0	Cn	All
10	27.12	C-8,C-14	18:2,cc
		C-8,C-11	18:1,c
11	27.01	C-8,C-14	18:2,cc
		C8,C-11	18:1,c
12	25.55	C-11	18:2,cc
13	25.48	C-14	18:2,cc
14	24.77	C-3	All
15	22.61	ω2	n-6 acids
16	22.48	ω2	SFA and n-9 acids
17	20.0	ω2	18:3,ccc

[a] SFA, saturated fatty acids; c, cis-monoene; t, trans-monoene; tt, trans,trans-diene; cc, cis,cis-diene; ct, cis,trans-diene; tc, trans,cis-diene; ccc, cis,cis,cis,-triene.

Unsaturated fatty acid composition can be in detail assessed also by studying the olefinic region (120-135 ppm) (**Figure 1.2.2.5**).

1.2.3 Other NMR experiments

Besides acquiring the "normal" ^{1}H- and ^{13}C-NMR spectra, other NMR experiments can prove to be very helpful in assigning peaks and removing remaining ambiguities. For example **DEPT** (distortionless enhancement of polarization transfer) is an experiment that is useful for determining the number of protons attached to a carbon atom. In the usual DEPT experiment (DEPT-135; the number referring to the pulse angle of the experiment), the ^{13}C signals appear either as positive or negative peaks or disappear. Positive peaks correlate with carbons carrying an odd number of protons (methine or methyl carbons). Negative peaks correlate with carbons carrying an even number of protons (methylene carbons). Carbons without an attached hydrogen do not give a visible peak. This experiment, therefore, is useful for distinguishing carbons in areas of the spectrum where there can be significant overlap of the type of signalcausing carbons (Gunstone *et al.*, 2007).

Two-dimensional experiments provide a means of identifying nuclei that are mutually coupled. In the **COSY** (COrrelation SpectroscopY) experiment, homonuclear coupled protons are identified. The ^{1}H spectrum serves as *x*- and *y*-axis for a contour plot. The diagonal of the contour plot results from the cross peaks of an individual signal with itself. Besides the peaks in the diagonal, various cross peaks "scattered" across the contour plot, but symmetrical to the diagonal as mirror plane, signal coupling of specific protons. This is often very useful in identifying which kind of carbon a specific functional group is bound.

Because of the low natural abundance of ^{13}C (ca. 1.1%), correlation spectroscopy between carbons is of no significance. Instead, heteronuclear correlation (**HETCOR**) between ^{1}H and ^{13}C is now a common experiment yielding useful information. The common experiment is termed **HMQC** (heteronuclear multiple-quantum correlation). The experiment yields a contour map similar to the one described for COSY above, except that one axis is now the ^{13}C spectrum instead of the ^{1}H spectrum, and, of course, there is no diagonal of cross peaks. The contour plot

indicates which ^{1}H-NMR peaks correlate with which ^{13}C peaks (Gunstone *et al.*, 2007).

Thus, the combination of "regular" spectra, DEPT and 2D experiments is a powerful tool for determining the structure of lipids and organic compounds in general.

2   MILK FAT CONTENT IN MIXED FATS

2.1   INTRODUCTION

In food production the quality assessment of raw materials and final products is a fundamental parameter for having and maintaining high quality standards. There are different aspects determining the overall quality of foods: the "quality" (in terms of sensory characteristics, stability and nutritional value), the "safety" (with respect to microbiology, contaminants and toxins) and the "authenticity".

An authentic product, whether raw material or a product on the supermarket shelf, is one which strictly complies with the declaration given by the producer in terms of ingredients, natural components, absence of extraneous substances, production technology, origin, production year and genetic identity. Authenticity is an important issue for the food industry due to legal compliance, economic reasons ("right goods for the right price"), guarantee of a constant well-defined quality, use of safe ingredients (Kamm, 2002). The problem how to guarantee/ensure food authenticity can be tackled by applying accurate specifications for raw materials and selecting suppliers that have a quality assurance system in place. The compliance with the specification is assured by regular audits of the supplier(s), certificates of analysis and periodical analytical checks. Hence it is necessary to identify and anticipate emerging authenticity issues, to confirm authenticity of potentially adulterated products, to develop methods adapted to solve new problems and to organize and/or perform analytical surveys of sensitive products (Kamm, 2002).

According to EC regulations, in the blends of milk fat with other fats (mixed fats or yellow fats) the milk fat content has to be indicated on the label (Council Regulation (EC) No 2991/94; Council Regulation (EC) No 445/07). The proportion of milk fat in the product has to be labelled to protect the consumer from fraudulent malpractice, since the price of milk fat is higher than that of other relevant raw materials. Due to the considerable price differences between milk fat and other fats (animal or vegetable), there is a strong interest in verifying the declared milk fat content in products obtained with a mixed fat. Adulteration has always been

practised and it is carried out for economical purposes (e.g. to increase the bulk volume, to over-evaluate a product of inferior quality or to subtract/save expensive ingredients). On the other hand, contaminations may occur accidentally, e.g. in factories, where several fats are produced or used at the same time (Kamm, 2002).

To assess the authenticity of fats and oils it is fundamental to know the technologies applied, the fat modification techniques used and the chemical composition of the authentic oil(s) and of the potential adulterants (Kamm *et al.*, 2001). The fundamental problem for the authenticity assessment of fats and oils is to define one or more parameters within the lipid fraction which allow to check for the identity and purity of the specified fat or oil. Ideally, such markers are chemical compounds which are present in the adulterant fat and absent in the original one. Therefore, profiles of authentic fats must be compared with the fat to be tested. For the definition of authenticity, the natural variations of the markers, *e.g.* due to climate, soil and breeding must be taken into account.

Chemically, the main constituents of fats and oils are triacylglycerols (commonly referred to as triglycerides), lower levels of diacylglycerols (diglycerides), monoacylglycerols (monoglycerides) and free fatty acids, accompanied by other minor components like the sterols (Kamm, 2002).

Methods for the determination of the milk fat content of fat mixtures are of considerable practical importance, as can be seen from three relevant Regulations published by the European Commission and the Council (Commission Regulation (EEC) No 4056/87, No 4154/87, Council Regulation (EC) No 2991/94). In order to check the correct labelling of mixed spreads, food inspection authorities need reliable analytical methodologies to determine the percentage of milk fat in the spread. The International Dairy Federation (IDF) developed an accurate and reliable method for this purpose, based on the fatty acid profile of the raw materials and the finished product (Muuse and Martens, 1993). However, this approach is not used widely, since in modern commerce the raw materials are mostly not available for testing. As a consequence, the most distinctive feature of milk fat, i.e. the unique occurrence of

butyric acid, exclusively present in milk fat, is most often determined by chromatography, and used as an indicator for calculating the milk fat content in foodstuffs (Ulberth, 1998).

Milk fat contains predominantly short-chain fatty acids (chains of less than eight carbon atoms) built from acetic acid and β-hydroxibutyrate units derived from fermentation in the rumen (Alais, 2000). So butyric acid (BA) occurs exclusively in milk fat from cows and other ruminant mammals, thus is not found in fats of vegetable origin or animal adipose tissues. This fact can be used for distinguishing between these kinds of fat, and also for the determination of proportions of milk fat in mixed fats. Thus, the quantitative determination of BA levels is particularly useful for controlling the declared composition of mixed-fat products, as well as of processed foods like chocolate, bread, cakes, pastries and ice-cream. (Molkentin and Precht, 1998).

The butyric acid content of milk fat and butterfat, although subject to a significant natural variation, can be assumed for practical purposes to be constant within certain limits. This fact allows the butyric acid content of a fat to be used as a criterion for the measurement of the amount of butterfat in the fat (Pocklington and Hautfenne, 1986; Molkentin and Precht, 2000; Glaeser, 2002). But while it is generally accepted that measurement uncertainty has to be taken into consideration when evaluating analytical results, the variation of the butyric acid content of milk fat is usually not taken into consideration. A conversion factor is normally used to calculated the milk fat content of fat mixtures from the results of butyric acid determination (Molkentin and Precht, 1997).

This procedure can lead to considerable errors. An approach based on the actual butyric acid content of the milk fat used for the production of fat mixtures therefore appears to be preferable. Ideally, when performing the analysis, information on the butyric acid content of the milk fat used for the production of mixed fats should be available. This information could be part of the milk fat specification and passed by the processor to the control authority (Glaeser, 2002).

A procedure based on the calculation of several fatty acid methyl esters

apparently did not find widespread application. Several authors have proposed methods for the determination of milk fat based on the content of fatty acids typical for ruminant milk fat such as butyric acid or the ratio between myristic and palmitic acids (Muuse and Martens, 1993). Other strategies to detect adulterated milk fat are based on the concentration ranges of even more than two fatty acids using multivariate statistical evaluation tools (Ulberth, 1995). Currently, the analysis of butyric acid in a mixed fat is a widely applied method and has, for instance, already been applied to quantitate small amounts of milk fat in cocoa butter or chocolate fats (Buchgraber *et al*, 2007). Nevertheless, depending on feeding conditions and lactation, the amount of butyric acid varies considerably. If a sample of the pure milk fat is not available, an average of butyric acid content may be used instead, which could assist in accurately determining the milk fat content fats (Buchgraber *et al*, 2007).

There are numerous methods for the measurement of butyrate in fats that are mainly based on gas chromatography (GC) of the methyl ester or the free acid. Because of different dimensions of BA data, e. g. the butyric acid content may be expressed as free acid or as methyl ester and may be related to total fat or to fatty acids, and also because of inaccuracies associated with certain methods, varying and partly contradictory results may be found with different methods for the measurement of butyric acid. However, the expression of the butyrate content in g/100 g fat is most advantageous for purposes of food control, as it is frequently used in food regulations (Molkentin and Precht, 1998).

In the last decade the use of Nuclear Magnetic Resonance (NMR) in food science has consistently grown (Alberti *et al*., 2002). This non-invasive technique preserves food structure and extracts useful information from such a chemically complex and highly heterogeneous system. The increase of new applications and the attention to this technique by scientists, official control institutions and food industries can be attributed both to the high specificity and versatility of the NMR technique and to the improvement of NMR instrument performances and availability. In the last 10 years, a strong diffusion of new NMR equipments occurred in research centres and

industries, thus stimulating new applications, and a similar trend can be expected in the near future (Sacchi and Paolillo, 2007).

Taking advantage of the development of digital computers, pulse Fourier transform techniques and the availability of superconducting magnets, ^{13}C NMR technique complemented ^1H NMR technique in many ways. The natural abundance of ^{13}C (1.1%) in nature was compensated by the low possibility of ^{13}C-^{13}C spin-spin coupling and by broadband heteronuclear decoupling (i.e., elimination of all ^1H-^{13}C couplings) which resulted in sharp singlets for all ^{13}C absorptions, permitting small chemical shift differences to be measured. With advanced electronic techniques, it was also possible to run a ^{13}C NMR spectrum under conditions that allow for a quantitative integral, as in the case of ^1H NMR. Inverse Gated decoupling was used to remove the nuclear overhauser enhancement, and longer pulse delays were used to allow ^{13}C nuclei with long spin-lattice relaxation time to relax completely.

The first quantitative applications of ^{13}C NMR spectroscopy on lipid molecules were reviewed by Shoolery (1977) with which the iodine values of fats and oils were determined accurately. As a complement to ^1H NMR, ^{13}C NMR offered a means of determining the composition of mixtures of fatty acids and lipid molecules in much greater detail. Gunstone published several valuable reviews, which dealt specifically with the use of high-resolution ^{13}C NMR technique in the analysis of lipid mixtures (Gunstone, 1991b, 1993a; 1994). ^{13}C NMR spectra of fatty acids provided a large number of signals spread over a wide range of chemical shifts, which made the spectrum appear complicated but much more informative. Techniques of correlating signals between ^1H and ^{13}C NMR spectra provided two-dimensional correlation spectra (2D COSY), which permitted confirmation of signals. Other techniques, such as INADEQUATE (incredible natural abundance double quantum transfer experiment), HSQC (heteronuclear single quantum correlation), HMQC (heteronuclear multiple quantum correlation) and HMBC (heteronuclear multiple bonds correlation), are some of the latest techniques in NMR spectroscopy from which structural details can be derived in great detail (Lie Ken Jie and Mustafa, 1997).

NMR supports the food industry in its increasing need to understand and be innovative in products and process and provides a new method to enforce legislation and quality control. A well-known example is the authentication of olive oil by using ^{13}C NMR spectroscopy (Sacchi *et al.*, 1992, 1997). From a single ^{13}C NMR spectrum, the fatty acid composition, the saturated, monounsaturated and polyunsaturated fatty acid ratios can be determined. In addition, the presence of unsaturated *trans* isomers and the distribution of fatty acids on the glycerol chain can also be detected (Wollenberg, 1990; Sacchi *et al*., 1995; Lie Ken Jie and Mustafa, 1997).

Although widely studied, because of its complexity, milk and dairy products have not yet been subjected to thorough examination by NMR (Alberti *et al*., 2002). The use of ^{1}H and ^{13}C NMR has been limited to the description of the non random distribution of butyric acyl group in triacylglycerols from butter oil fraction (Pfeffer *et al.*, 1977b; Gunstone, 1993a; Kalo *et al*., 1996; Van Calsteren *et al*., 1996). Recently, NMR spectroscopy has been successfully applied to milk directly without any pretreatment. Various organic compounds in whole milk were quantified simultaneously by one- (^{1}H, ^{13}C) and two-dimensional (^{1}H-^{13}C HSQC 2D) NMR spectra (Hu *et al.*, 2004, 2007)

No NMR methods have as yet been proposed as analytical tools in analysis of butyric acid (BA) in a mixed fat although ^{13}C-NMR spectroscopic analysis of milk fat triacylglycerols show well-resolved resonances of butyrate carbons (Pfeffer *et al*, 1977; Lie Ken Jie and Mustafa, 1997; Andreotti *et al*., 2000, 2002) (**Figure 2.1** and **2.2**).
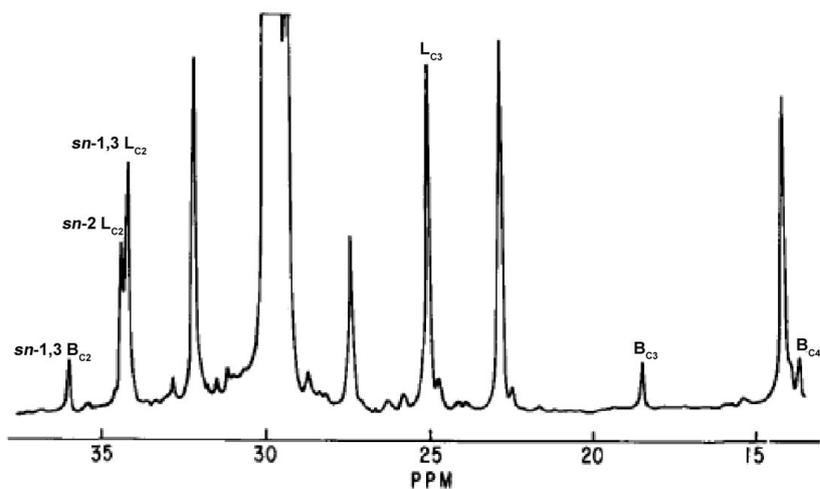
Figure 2.1. 22.63 MHz ^{13}C spectrum (methyl and methylene region) of butter oil (0.5 g in 1.3 ml of CDCI3). Sweep width of displayed spectrum is 500 Hz. The letter (B) identifies shifts due to butyrate and (L) those due to long chain species (Pfeffer *et al*, 1977b).
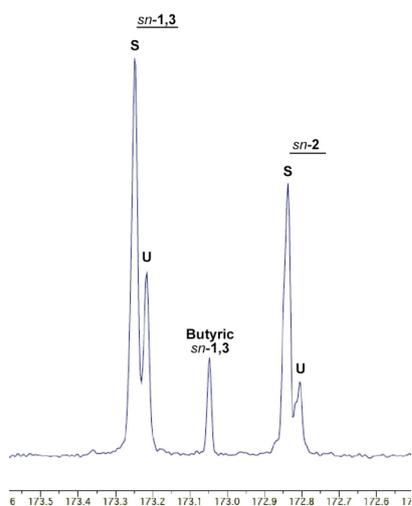
Figure 2.2. 100 MHz 13C nuclear magnetic resonance (NMR) spectrum of the C1 region of triacylglycerols from buffaloes' milk fat in CDCl3 at 25°C. Saturated, S, and unsaturated, U, fatty acids signals are labeled referring to the glycerol backbone, i.e., position *sn*-1,3 and position *sn*-2. The C1 signal for butyric acyl group in *sn*-1,3 position is labeled.

Considering that non-milk animal and vegetable fats contain very low amounts of short chain fatty acids and no butyric acid, the direct quantitative evaluation of BA by NMR could be an useful method to quantify the milk fat content.

In this study the butyrate content in milk fat and mixed fats (obtained by mixing milk fat with known amounts of pork fat and vegetable margarines) was determined using ^{13}C NMR. The NMR data have been compared to those obtained by capillary gas chromatography of fatty acid methyl esters.

2.2 MATERIALS AND METHODS

2.2.1 Fat samples

Fat mixtures containing different amounts of milk fat were prepared by mixing a butter milk fat sample with some amount of genuine pork fat sample, obtained by melting a pork adipose tissue, and a commercial vegetable margarine (fractionated and interesterified fats). The fat samples were dried on sodium sulphate, weighed and mixed in order to obtain two series of mixed fat samples containing known amounts of milk fat (5, 10, 25, 50 % w/w). Mixtures were melted at 40°C for 1 h and homogenized (**Figure 2.1.1**).
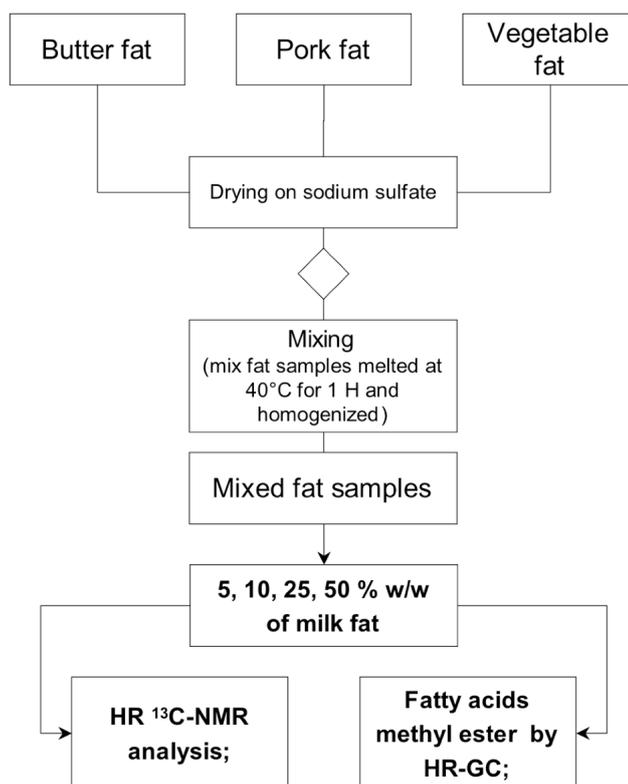
Figure 2.1.1. Experimental design relative to the preparation and analytical determination of the mixed fat samples.

2.2.2 Standards and reagents

Chloroform-*d* (with 0.03 % v/v internal tetramethylsilane, TMS) and thribromobenzol (TBB) were obtained from Aldrich Chemical Co. (Milwaukee, WIS, USA). All other chemicals were HPLC or reagent grade and were purchased from Fluka (Buchs, Switzerland).

2.2.3 Preparation of FAMEs and Gas chromatography

Analysis of fatty acid methyl esters (FAMEs) were carried out by cold transmethylation in KOH/methanol (Christie, 2003; Ichihara *et al*., 1996).

A quantity of 100 mg fat was weighed (to the nearest 0.1 mg) and dissolved in 10 ml *n*-hexane. 1 ml of this solution was mixed with 500 μl sodium methylate solution (2 N in methanol) in a sample vial, shaken vigorously for 1 min using a vortex mixer, and centrifuged for 1 min. The clear supernatant was used for GC analysis.

Analyses of FAME were performed using a Shimadzu GC17A gas chromatography (Shimadzu Italia, Milan, Italy) equipped with split/splitless injection port, flame ionization detector and a 60 m fused-silica capillary column (Quadrex Corporation, New Heaven, U.S.A.) coated with cyanopropyl methyl silicone (0.25 μm film thickness). Helium was used as the carrier gas and the flow rate was 1.8 ml min±1 at a split ratio of 1:60. The temperature of both the injector and detector was 250 °C. The initial oven temperature was set at 70 °C for 4 min; it was then programmed to increase at a rate of 10 °C min±1 until it reached 170 °C, maintained for 10 min, and thereafter to increase at a rate of 10 °C min±1 until it reached 220 °C, maintained for 10 min. The samples (1 μl) were injected using an automatic injector Shimadzu AOC-20i (Shimadzu Italia, Milan, Italy).

2.2.4 High Resolution ^{13}C-NMR spectroscopy

The ^{13}C-NMR analysis was carried out on dissolving in 5 mm NMR-tubes 50 mg of sample in 500 μl chloroform-*d* with internal 0.03% v/v tetramethylsilane. The ^{13}C-NMR spectra were recorded on a Bruker AV 400 spectrometer (Bruker, Germany)

operating at a ^{13}C-frequency of 100.62 MHz. Spectra were recorded with 32K data points, spectral width of 200 ppm, pulse width 45°, acquisition time 0.81 sec, relaxation delay 4 sec (with a digital resolution of 2 Hz/pt). Quantitative spectra were recorded with 128-3000 scans and the acquisition was stopped when a good S/N ratio was observed for the butyric acid minor C4 peak (experimental time of 20-120 min in relation to the milk fat amount in the samples). High resolution carbonyl spectra were recorded at 30 ± 1°C with 32K data points, a spectral width of 10 ppm, pulse width 90°, an acquisition time of 23.2 sec and a relaxation delay of 2 sec. The resulting digital resolution was 0.04 Hz/pt.

Nuclear Overhauser Effects (NOEs) were measured only for those signals relevant in the quantitative analysis by comparing spectra recorded in Broad Band (BB) decoupling mode (with NOE enhancement) with those recorded by using the inverse gate decoupling sequence (without NOE). Inverse gated spectra were recorded with the same acquisition parameters and with the same number of scans used in the BB experiments, with an additional delay of 20 sec. Spectra were transformed in absolute intensity mode. Carbon-13 spin-lattice relaxation times (T_1) were measured using the inversion-recovery (180-τ-90) pulse sequence[1]. Spectra recorded at

---

[1] The inversion-recovery method is a convenient way to measure T_1 values of both ^{1}H and ^{13}C nuclei. In a moderately complex molecule (15–30 carbons), the T_1 values of all positions in the molecule can be determined simultaneously, with spectral overlap the only limitation. The method is a multiple-pulse experiment in which net magnetization of the sample nuclei is first inverted with a 180◦ pulse ("inversion") and then allowed to relax along the z axis with the characteristic time constant T_1 ("recovery"). The effect of the 180◦ pulse is to interchange all of the spins between the upper and lower energy levels, so that now the higher energy spin state has a slight excess of population and the lower energy spin state has a slightly depleted population. This causes the net magnetization vector to be turned upside-down so that Mz now equals $-Mo$. Recovery begins immediately according to the exponential law, with characteristic rate $R1 = 1/T_1$. Because z magnetization is not a directly observable quantity, the recovery period is followed by a 90◦ pulse that "samples" or "reads" the z magnetization by converting it into observable x–y magnetization. The magnitude of the FID signal that results from this x–y magnetization (and the peak height in the spectrum) should be directly proportional to the sample's z magnetization just before the 90◦ pulse. By repeating the experiment with different time delays after the 180◦ pulse, we can monitor this return of z magnetization to equilibrium and determine the value of T_1 by curve fitting of the data to an exponential function. The phase correction parameters are first set using a simple 90◦ pulse acquisition (starting with equilibrium magnetization, along+z) and then applied to a series of inversion-recovery spectra acquired with increasing values of the delay $τ$. For $τ = 0$ we should see an upside-down spectrum, with each peak at its maximum height but inverted. As the delay is increased

different delays between pulses (τ values) were transformed in absolute intensity mode without any phase correction. In order to obtain the T_1 values, peak intensities were fitted to an exponential curve via a three-parameter minimization (Jacobsen, 2007). Chemical shift values were referenced to internal TMS and assignments made by comparison with standard compounds and data in the literature (Pfeffer *et al*, 1977b; Van Calsteren, 1996; Lie Ken Jie and Mustafa, 1997; Andreotti *et al*., 2000, 2002).

---

each peak will become less intense, pass through zero, and finally become positive. At very long τ delays the spectrum should look just like a normal spectrum (Jacobsen, 2007).

2.3 RESULTS AND DISCUSSION

Figures 2.3.1 and **2.3.2** show the ^{13}C-NMR spectra (expansion of carbonyl and methylene regions) of the milk fat sample, used in this study. Labelled signals were assigned as shown in **Table 2.3.1**. Four butyril chain signals were completely resolved in the milk fat spectrum. No butyrate was detected in pork fat and margarine. This suggests that all butyrate signals can be used to quantify the milk fat contents. For this reason the work has been focused on the C1 and C2 resonances which being well resolved provide, in addition to the total amount of butyrate, also useful information on the α–β distribution on triacylglycerols. The position of butyrate was first determined by NMR methods by Pfeffer *et al.* (1977) who indicated that butyrate is predominantly present in the α-position. Our high resolution spectral data confirm these previous findings, and the C1 or C2 α-butyril peaks can, infact, be used for the determination of butyrate amount which can be expressed as molar fraction % of the total fatty acid chains or referred only to the α-acyl chains.
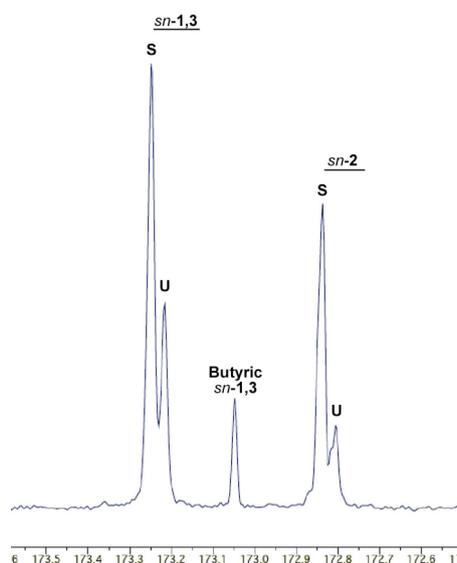
Figure 2.3.1. 100 MHz ^{13}C nuclear magnetic resonance (NMR) spectrum of the carbonyl region of triacylglycerols in butter fat in CDCl3 at 25°C. Saturated, S, and unsaturated, U, fatty acids signals are labelled referring to the glycerol backbone, i.e., position *sn*-1,3 and position *sn*-2. The C1 signal for butyric acyl group in *sn*-1,3 position is labelled.
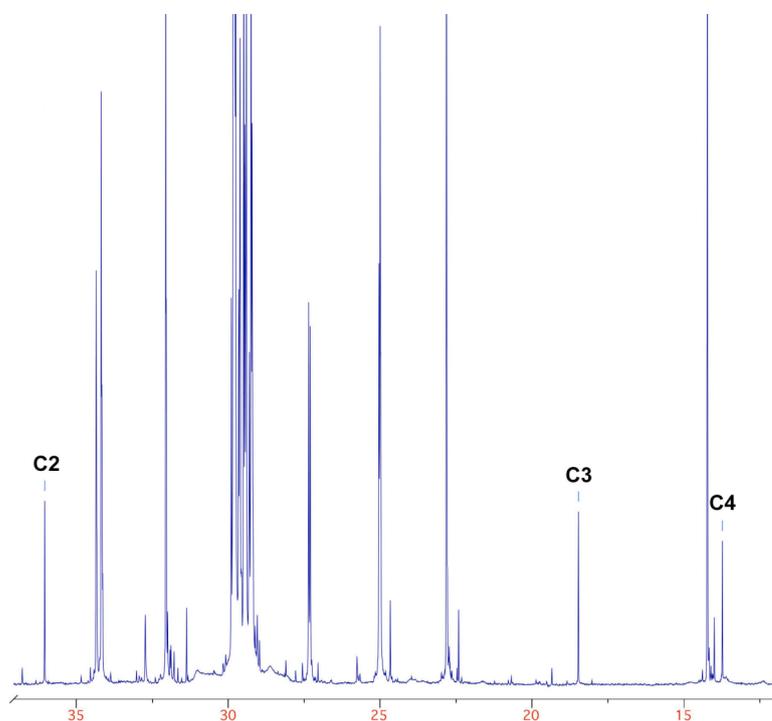
Figure 2.3.2. 100 MHz ^{13}C nuclear magnetic resonance (NMR) spectrum of the methylene region of triacylglycerols in butter fat in CDCl3 at 25°C. The C4, C3 and C2 signals for butyric acyl group are labelled.

Table 2.3.1. ^{13}C NMR chemical shift values of Butyrate

Carbon nuclei	Butyrate	
	(α)	(β)
C-1	173.136	-
C-2	35.938	-
C-3	18.366	-
C-4	13.630	-
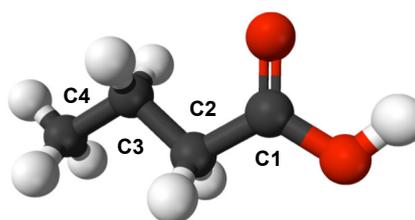
The quantitative analysis of butyrate can be performed by comparing the intensity of the C1 or C2 peaks at 173,13 and 35,94 ppm, with the corresponding signals of other long, medium and short chain fatty acids (C$_6$-C$_{22}$), using the following expressions:

$$\text{Butyrate\% (mole fraction)} = B_1*100/ (B_1 + A_1 + C_1) \qquad (1)$$

where: B_1= intensity of C1 butyrate signal (173,13 ppm); A_1= intensity of C1 α-long chain fatty acids (C_6-C_{20}) (173,36 ppm); C_1 = intensity of C1 β-long chain fatty acids (C_6-C_{20}) (172,95 ppm) (**Figure 2.3.3**).
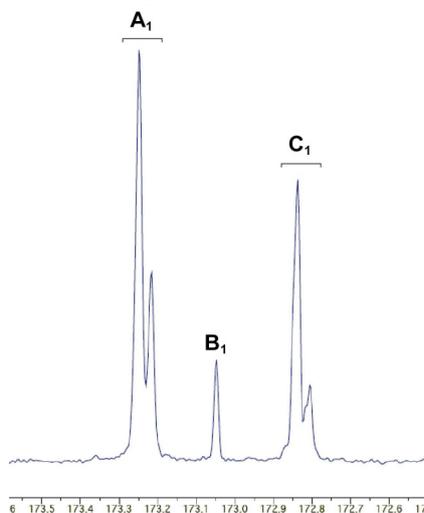
Figure 2.3.3. Carbonyl carbon signals of acyl group in butter fat. The C1 signal in *sn*-1,3 and *sn*-2 position are labelled.

$$\text{Butyrate\% (mole fraction)} = B_2*100/ (B_2 + A_2 + C_2) \qquad (2)$$

where: B_2 = intensity of C2 butyrate signal (35,94 ppm); A_2 = intensity of C2 α-long chain fatty acids (C_6-C_{20}) (34,18 ppm); C_2 = intensity of C2 β-long chain fatty acids (C_6-C_{20}) (34,35 ppm) (**Figure 2.3.4**).
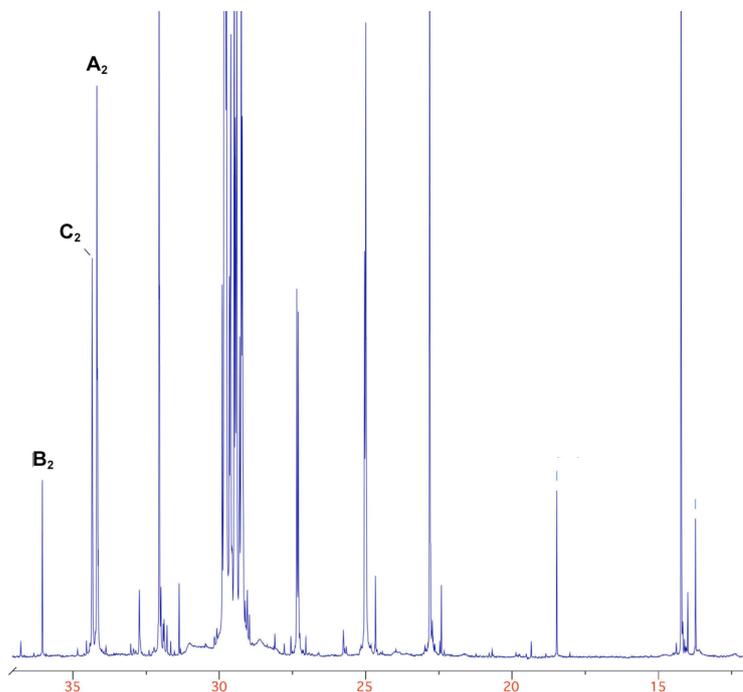
Figure 2.3.4. C2 carbon signals of acyl group in butter fat. The C2 signal in *sn*-1,3 and *sn*-2 position are labelled (A$_2$, B$_2$, C$_2$).

It's important to mention at this point that, in order to obtain an NMR quantitative response, spectra have to be acquired under experimental conditions ensuring the same recovery of each NMR resonance used in the quantitative measurements. In fact, the linearity between NMR signal intensity and the concentration of the component can be distorted by the different relaxation rate on Nuclear Overhauser Enhancement (NOE) (Wollenberg, 1990; Lie Ken Jie and Mustafa, 1997; Ng, 2000).

In routine ^{13}C-NMR qualitative analysis, most spectra are recorded using Broad-Band proton decoupling (irradiation of all the protons at the same time with the result of a completely proton decoupled spectrum with a single line for each carbon) and using a short delay time (D$_1$) between two subsequent pulses. When carbons have different relaxation behaviours, the longitudinal relaxation time (T$_1$) has to be known for all carbons to ensure that all carbons are fully-relaxed between two succeeded pulses. As for the NOE factor, NOE enhancement may not be the same for all

resonances, which means that evaluation of the peak areas could not correspond to the true population of the carbon species. For this reason both NOE and T_1 values have been regulated by considering the literature data for the acquisition parameters used for quantitative experiments (Wollenberg, 1990; Ng, 2000). T_1 values ranged between 9 and 11 s for carbonyls and were less than 1 sec for all methylene carbons (Ng, 2000). Based on these T_1 values, and using a 45° pulse, a short relaxation delay of 2 sec was used for recording full quantitative spectra.

The Broad Band mode was used for a faster quantitative analysis using the same acquisition conditions for each sample to ensuring a similar NOE for different experiments. The advantage of using the broad band mode is related both to better sensitivity due to the NOE enhancement, and higher S/N ratio due to the faster recycle delay between pulses (2.37 s) with respect to those used in NOE suppressed spectra (22.37 s). This means a higher number of scans/min (25.3 scans/min) in the broad band accumulation with respect to the inverse gated recording (only 2.7 scans/min). To obtain the same S/N for methylene carbons, 20-30 times smaller accumulation time than the inverse-gated mode is required.

Considering the small NOE effect on carbonyl signal intensities, the α−β acyl distribution on triacylglycerols can be studied from the high resolution spectra of the carbonyl region using the broad band mode. In fact, the complete relaxation of carbonyls is guaranteed by the high acquisition time (12-20 sec) requested for high digital resolution (0.04 HZ/pt) (Wollenberg, 1990).

In this study we have measured the signal intensity of C2 carbon methilen resonances in order to verify the amount of milk fat in the mixed fat samples. To check the quantitative performances of NMR measure, independent GC analysis were performed.

In **Table 2.3.2** is reported the amount of butyric acid obtained by NMR measure of C2 carbon, expressed in mole fraction %, and by Gas Chromatographic analysis of methyl ester fatty acids, expressed in %, of mixed fat samples. NMR spectra were repeated three times and a good reproducibility of relative signal intensities has obtained (st. dev. 0.08).

Table 2.3.2. Butyric acids amount in mixed fat samples (average ± st. dev.).

milk fat %	butyric acid by NMR (fraction molar %)	butyric acid by GC (%)
0	0,00 ± 0,00	0,00 ± 0,00
5	0,55 ± 0,05	0,21 ± 0,02
10	1,02 ± 0,01	0,44 ± 0,01
25	2,13 ± 0,02	0,84 ± 0,02
50	4,36 ± 0,06	1,65 ± 0,04
100	8,38 ± 0,08	3,21 ± 0,07

The NMR quantitative response was verified by comparing the NMR derived data with those obtained from Gas Chromatography (**Figure 2.3.5**). A good agreement ($R^2 = 0.9996$) was found between butyric acid of mixed fat samples calculated by means of NMR and GC.

The plot shows butyrate NMR (mf %) versus butyric acid % (GC), with the fitted line $y = 2,6462x + 0,0628$ and $R^2 = 0,9996$.
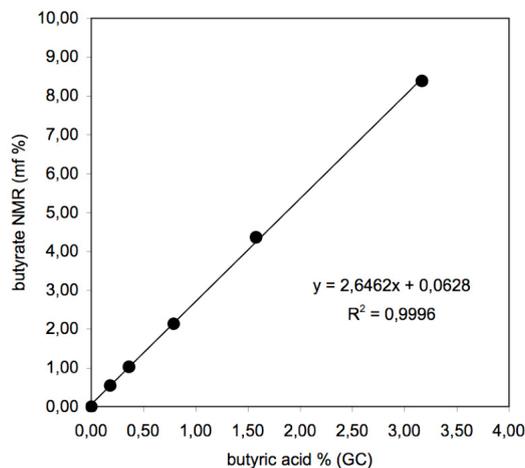
Figure 2.3.5. C2 NMR data vs BA GC data

The NMR values for C2 carbon were also plotted against the amounts of milk fat in the mixtures (w/w%) (**Figure 2.3.6**). The NMR response showed a very good linearity with R^2 of 0.9994
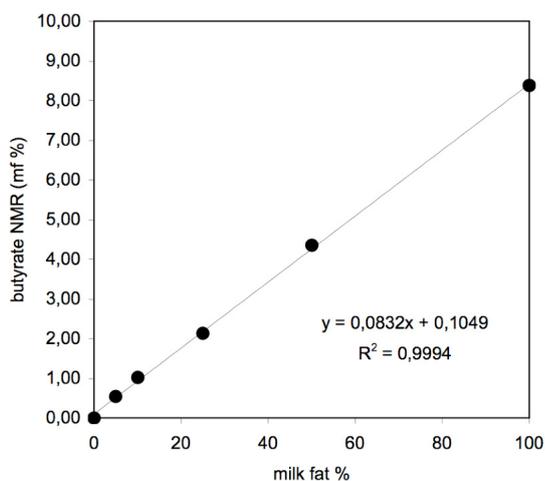
Figure 2.3.6. C2 NMR data vs milk fat amount in mixed fat samples

2.4 CONCLUSIONS

Carbon-13 NMR spectroscopy has the considerable advantage, over the usual methods, of operating directly on the oil sample without any chemical manipulation and it seems to represent the only direct instrumental method by which the butyrate content and position on triacylglycerols can be specifically identified. High resolution NMR analysis, certainly cannot be suggested as a routine method to quantify milk fat in mixed fats, due to the expensive NMR equipment required, but it can certainly be used as a direct, specific and rapid reference method, also to assess the genuineness and purity of butter and the presence of transesterified glycerols, with BA, in which the butyrate is randomly distributed.

3 HYDROPEROXIDE DECOMPOSITION PRODUCTS IN THERMALLY OXIDIZED OILS

3.1 INTRODUCTION

The oxidation of polyunsaturated fatty acids (PUFAs) is an autocatalytic, self-perpetuating chain reaction system and the oxidized PUFAs have been implicated in the pathogenesis of many human diseases, e.g., atherosclerosis and inflammatory joint diseases (Lopez-Varela *et al.*, 1995; Esterbauer, 1993; Dobarganes and Márquez-Ruiz, 2003). Oxidation of lipids is promoted by factors such as elevated temperature, exposure of light, presence of extraneous materials, such as metals or other oxidation initiators. The nature of the radicals also influences the products obtained. Oxidation can be inhibited, but not prevented in the long term, by the presence of antioxidants (Gunstone *et al.*, 2007).

PUFAs are particularly susceptible to oxidative damage by virtue of the easy abstraction of one of their bis-allylic methylene group hydrogen atoms on exposure to light or radical species of sufficient reactivity, a process facilitated by the low bond dissociation energy of the methylene group C–H bonds. Subsequently, one major reaction pathway for the resulting resonance-stabilized carbon-centered pentadienyl lipid radical generated in this manner involves its interaction with molecular oxygen to produce a peroxyl radical which in turn can abstract a hydrogen atom from an adjacent PUFA to form a conjugated hydroperoxydiene (CHPD) and a further pentadienyl lipid radical species. In the absence of sufficient quantities of chain-terminating, lipid-soluble antioxidants such as vitamin E (α-tocopherol), the process is repeated many times. CHPD are subsequently degraded to a wide variety of secondary autoxidation products which include saturated and unsaturated aldehydes, di- and epoxyaldehydes, lactones, furans, ketones, oxo and hydroxy acids, and saturated and unsaturated hydrocarbons (**Figure 3.1.1**).
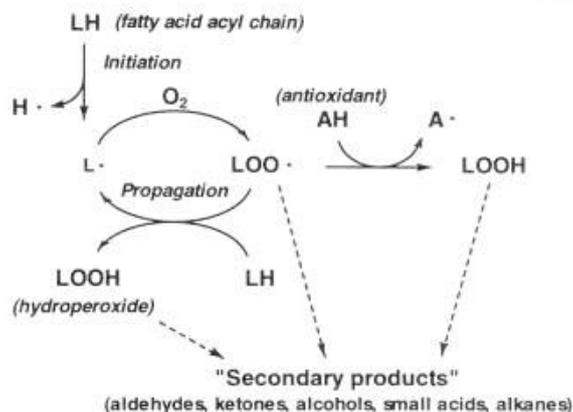
Figure 3.1.1. Oxidation of polyunsaturated lipid

Thermal stressing of culinary oils according to standard frying/cooking practices (domestic or otherwise) gives rise to and/or perpetuates the radical-dependent autoxidation of PUFA therein. Indeed, a wide range of aldehydes arises from the thermally induced decomposition of CHPD *via* several processes, including the β-scission of pre-formed alkoxyl radicals. Such aldehydic fragments (*n*-alkanals, *trans*-2-alkenals, *trans,trans*- and *cis,trans*-alka-2,4-dienals, 4-hydroxy-*trans*-2-alkenals, and malondialdehyde) have the capacity to exert a variety of toxicological effects in view of their extremely high reactivity with critical biomolecules (DNA base adducts, proteins such as the apolipoprotein B moiety of low density lipoprotein, peptides, free amino acids, endogenous thiols such as glutathione, etc). Interaction of these aldehydes with DNA can give rise to genotoxic events and possibly cancer (Silwood and Grootveld, 1999).

Many humans are continually exposed to oxidized oils and fats in the diet which arise from either shallow or deep fat frying processes, and the possibility that regular consumption of such materials may be deleterious to human health has attracted much interest (Guillén and Ruiz, 2005a).

Deep-fat frying is a common method of food preparation that imparts desired sensory characteristics of fried food flavor, golden brown color and crisp texture. During frying, at approximately 190°C, as oils thermally and oxidatively decompose,

volatile and nonvolatile products are formed that alter functional, sensory, and nutritional qualities of oils (**Figure 3.1.2**) (Warner, 2002).

During this process a great number of changes occur in the sample as consequence of the incorporation of oxygen into the triglyceride structure, and the generation of very reactive species which causes the breakdown of the acyl group chains producing volatile and semi-volatile molecules of different natures, as well as reactions between different acyl groups chains to give oligomeric or polymeric systems (Guillén and Goicoechea, 2007).

With continued heating and frying, the hydroperoxides decompose further until breakdown products accumulate to levels that produce off-flavors and potentially toxic effects, rendering the oil unsuitable for frying. The amounts of these compounds that are formed and their chemical structures depend on many factors, including oil and food types, frying conditions and oxygen availability (Warner, 2002).
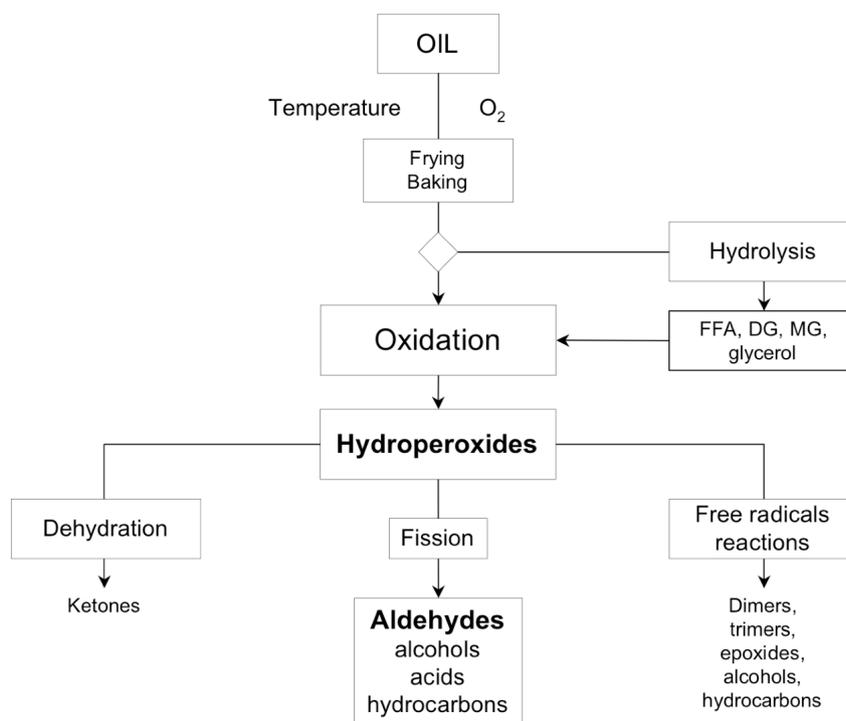
Figure 3.1.2. Physical and chemical reactions that occur during frying.

Besides species with relatively unchanged molecular weight, secondary oxidation products are a variety of polymeric and lower-molecular weight compounds. The complicated decomposition process of hydroperoxides is reflected in this complex variety of products.

Information on the secondary oxidation products that can result from the initially formed hydroperoxides is summarized in **Table 3.1.1**. The secondary products are categorized as monomeric (products with the same chain length as the original material, but with different functional groups), oligomeric (products of higher molecular weight), and volatile (short-chain) compounds. Various types of reactions can occur during the decomposition process including dehydration, cyclization, rearrangement, radical substitution, chain cleavage, dimerization, etc (**Figure 3.1.2**). In many cases, a combination of these reactions occurs to form the final "secondary" oxidation products. The formation of some of the observed volatiles cannot be readily explained by classical cleavage mechanisms, but may be attributed to further oxidation of the unsaturated aldehydes and, thus, may be "tertiary" products. Thermal and metal catalyzed reactions of hydroperoxides give homolytic cleavage while acidic decomposition proceeds by heterolytic cleavage, leading to less complex products consisting mainly of aldehydes (Warner, 2002; Frankel, 1998).

The reactions that occur during the formation of the secondary products are influenced by various parameters (temperature, light, air or extraneous materials) which also affect the initial oxidation step of hydroperoxide formation. It may be noted that the various secondary oxidation products listed in **Table 3.1.1** are formed in different proportions depending from the oxidation conditions.

Table 3.1.1. Secondary oxidation products derived from the decomposition of hydroperoxides (Gunstone *et al.*, 2007)

Secondary products	Fatty compound d			
	Oleate	Linoleate	Linolenate	Triacylglycerols
Monomeric	Allylic keto-oleates (keto at C8-11) Epoxy stearate Epoxy oleates Dihydroxystearates	Keto-linoleate (C9-C13) Epoxyhydroxy oleate Hydroxy dienes Dihydroxyoleates Trihydroxyoleates	Hydroperoxy epidioxides, dihydroperoxides	
Oligomeric		Dimers linked via peroxy or ether groups with OOH, OH or C=O groups C-C linked dimers (under N2) Dimers and oligomers containing conjugated diene-triene, dihydroperoxides, or hydroperoxy epidioxide units		Di- and trihydroperoxides; less tendency to dimerize than with methyl esters; more significant at higher temperatures (frying), giving "thermal" and "oxidative" dimers
Volatiles	Saturated and unsaturated short-chain carbonyl compounds, alcohols, and hydrocarbons; examples given below			
	Heptanal, nonanal, decanal 2-nonenal, 2-undecenal heptane, octane methyl nonanoate	From peroxide-linked dimers pentane, 1-pentanol, pentanal, hexanal, 3-nonenal, 2,4-decadienal, methyl octanoate, methyl 9-oxononanoate Others: acetaldehyde, 2-pentylfuran, methyl heptanoate, 2-octenal, 2,4-nonadienal, 2-4-decadienal, methyl 8-oxooctanoate, methyl 10-oxodecanoate; 2c-octenal, 2t-nonenal, 1-octene-3-one, 3-octene-2-one, 2t-octenal, 4-hydroxy-2-nonenal, 4-oxo-2-nonenal; volatile epoxy acids (t-2,3-epoxyoctanoic acid)	Decatrienal, methyl octanoate, propanal, 2-pentenal, 3-hexenal, 2,4-heptadienal, ethane, acetaldehyde, butanal, methyl 9-oxononanoate, 2-butenal, 2-butylfuran, 2t,6c-nonadienal, 1,5coctad ien-3-one, 3c-hexenal, 3-nonenal, hexanal; t-4, 5-epoxy-2-heptenal	Oxoglycerides or aldehydo-glycerides From trilinolein: pentane, hexanal, 2-heptenal, 2,4-decadienal From trilinolenin: propanal, 2,4-heptadienal, 2,4,7-decetrienal In vegetable oils: ethane, propane, pentane, hexane, dialdehydes, ketones, ethyl esters, nonane, decane, undecane, 2-pentylfuran, lactones, benzene, benzaldehyde, acetophenone
Volatiles from Secondary products	Alkanals, glyoxal, α-keto aldehydes, dialdehydes		Malonaldehyde (typical for lipids with three or more double bonds)	

Source: Adapted from Gunstone, F.D. (Ed.), in *The Chemistry of Fats and Oils*, Blackwell Publishing, Oxford, U.K., 2004, pp. 150–168; and Frankel, E.N., *Lipid Oxidation*, 2nd ed., The Oily Press, Bridgwater, U.K., 2005.

The most important reaction involved in the oxidative deterioration of food lipids is the thermally induced radical-mediated autoxidation of polyunsaturated fatty acids (PUFAs), primarily generating conjugated hydroperoxydiene (CHPD) species. Such PUFA-derived CHPDs are particularly unstable at temperatures associated with standard frying practices (180°C), and are degraded to a wide variety of secondary peroxidation products, including a range of aldehydes (predominantly n-alkanals, *trans*-2-alkenals, alka-2,4-dienals, 4-hydroxy-*trans*-2-alkenals and malondialdehyde) which have the capacity to exert a range of toxicological effects in view of their high reactivity with critical biomolecules *in vivo* (Claxson *et al.*, 1994).

The unsaturated aldehydes formed as volatile secondary products are important because these are responsible for the off-flavour of foods containing oxidized oils. These flavour-affecting compounds are often formed in very small amounts (ppb levels) (Solinas *et al.*, 1988; Gunstone *et al.*, 2007).

Several studies have been caried out in order to indentify mechanisms of volatile compound formation (Frankel, 1998; Katsuta, 2008). Selke *et al.* (1977) identified volatile odor constituents and their precursors from heated soybean oil, using model triglycerides (pure triolein, mixture of triolein 25% -tristearin, and a randomly esterified triglyceride of stearic and 25% oleic acids) heated at 192°C in air for 10 minutes. Each model system produced the same major compounds, identified as heptane, octane, heptanal, octanal, nonanal, 2-decenal, and 2-undecenal. These seven compounds were unique to the oxidation of the oleate fatty acid in each triglyceride sample. Later, Selke and coworkers (1980) analyzed pure trilinolein and mixtures of trilinolein-tristearin, trilinolein-triolein, and trilinolein-triolein-tristearin heated to 192°C in air. Major volatiles detected in heated and frying oils included pentane, acrolein, pentanal, 1-pentanal, hexanal, 2- and/or 3 hexanal, 2-heptenal, 2-octenal, 2,4-decadienal, and 4,5-epoxide-2-enal (Frankel, 1998; Warner, 2002).

Oil thermoxidation is currently assessed using different analytical methods. As early as 1981, a measure of thermoxidation based on the estimation of the total polar compounds (TPC) by silica column chromatography was introduced by the IUPAC. (Walking and Wesserls, 1981; Dobarganes *et al.*, 2000). Determination of polar

compounds in heat-abused oils and fats is a well-accepted method due to its accuracy and reproducibility (Walking and Wesserls, 1981). The level of polar compounds is a quite good indicator of the quality of used frying fats and oils, giving information of the total amount of newly formed compounds having higher polarity than that of triacylglycerols and being the basis of the present recommendations and regulations limiting degradation of used frying fats for human consumption. More recently High Performance Size Exclusion Chromatography (HPSEC) and High Performance Liquid Chromatography (HPLC) were applied to measure the high molecular weight compounds (dimers and polymers of triacylglycerols) formed during oil heating (**Figure 3.1.3**) (White and Wang, 1986; Veazey, 1986; Márquez-Ruiz *et al.*, 1995; Dobarganes *et al.*, 2000). Complementary determination of polymerized triacylglycerols in fats and oils by exclusion chromatography allows quantification of the main groups of compounds formed during the frying process (Dobarganes *et al.*, 2000).
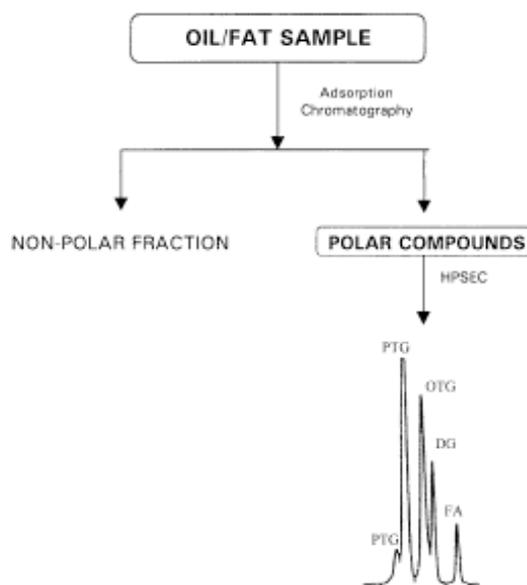
Figure 3.1.3. Analytical procedure for determination of total polar compounds and their distribution. Abbreviations: HPSEC, high-performance size-exclusion chromatography; PTG, polymerized triacylglycerols; OTG, oxidized triacylglycerols; DG, diacylglycerols; FA, fatty acids (Dobarganes *et al.*, 2000.

Márquez-Ruiz and Dobarganes (1996) also proposed a simple High Resolution Gas Chromatography (HRGC) determination of short-chain fatty acids (heptanoate and octanoate) to evaluate hydroperoxide thermal decomposition in heated and fried oils. Decomposition of the alkoxy radical involving carbon-carbon bond scission is a major pathway for the generation of low-molecular-weight compounds. Studies on model systems have shown that methyl octanoate ($C_{8:0}$) is a significant product of the oxidation of fatty acid methyl esters. In fats and oils, oxidation takes place in the unsaturated fatty acyl radicals and, in consequence, $C_{8:0}$ and other short-chain fatty acids remain attached to the glyceridic backbone forming part of a non-volatile molecule (**Figure 3.1.4**).
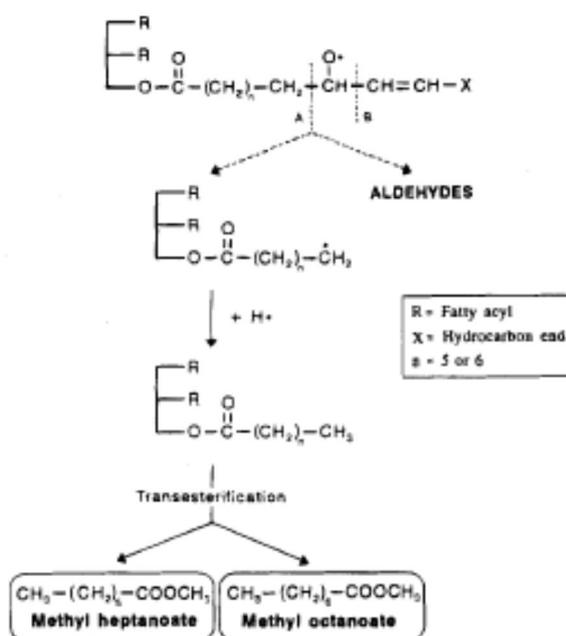
Figure 3.1.4. Schematic representation of bound short fatty acid formation. (Márquez-Ruiz and Dobarganes, 1996)

Quantitation of these resulting short-chain fatty acids is hence an indirect measurement of both oxidation and volatile formation, particularly under frying conditions, wherein the real volatiles are continuously leaving the oil due to the high

54

temperature of the process and the influence of steam while the bound short-chain acids remain as markers of the oxidation intermediates (Márquez-Ruiz *et al*., 1995). Oleic, linoleic and linolenic acids are the major unsaturated fatty acids in edible fats and oils and all of them are capable of forming bound $C_{8:0}$ from their 9-hydroperoxides. The formation of glycerol-bound heptanoate and octanoate correlates with the amount of total polar compounds determined by the official IUPAC method (Márquez-Ruiz *et al*., 1995; Brühl and Matthäus, 2008).

However, the measure of the aldehydes can be also a good way to assess the secondary oxidation products arising from peroxide decomposition (this is very rapid at temperature higher than 140°C), directly responsible of "rancid", "fishy" and "deep-fried" flavours (Solinas *et al*., 1988; Frankel, 1987) (**Figure 3.1.5**).
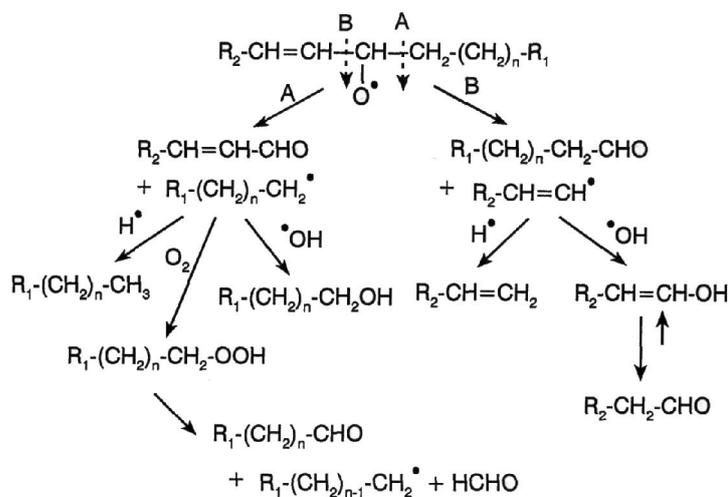
Figure 3.1.5. Thermal decomposition of monohydroperoxides produces alkoxyl radicals undergoing homolytic β-scission to form aldehydes, alkyl and olefinic radicals (Frankel, 1998).

Classical methods of studying these volatile compounds (Head-Space techniques coupled with GC or GC/MS) provide partial information, referring only to the

concentration of one class of compounds present in the complex mixture formed during the oxidation process, but none of them give a picture of the total mixture; this fact, combined with the great number of inherent drawbacks of these methods, such as the poor reproducibility of some, or the difficulty in knowing the real significance of the parameters obtained in others (Frankel, 1998), shows the necessity to search for new methods to study these complex processes in depth. The usefulness of Fourier transform infrared spectroscopy as well as of the ^{1}HNMR has been studied with positive results (Guillén and Goicoechea, 2007; Sacchi et al., 2006a). [1]H nuclear magnetic resonance has proved to be a very valuable tool which allows to follow not only the degradation of acyl groups but also, at the same time, the formation and degradation of primary oxidation compounds as well as the formation and evolution of secondary oxidation products throughout the oxidation process (Guillén and Ruiz, 2005b; Sacchi et al., 2006a).

The first application of ^{1}H-NMR in lipid analysis was performed by Johnston and Shoolery in 1962. These pioneers demonstrated the ability of proton NMR to assess the unsaturation degree in different oils and fats. In recent years, NMR applications in this field concern mainly ^{13}C-NMR, due to the higher resolution of signals.

^{13}C-NMR spectra can provide many important compositional information not easily gained with other analytical tools (Ng, 1985; Gunstone, 1994; Wollenberg, 1990; Sacchi et al., 1997). The direct application of ^{13}C-NMR to the study of lipid oxidation is limited by the low intrinsic sensitivity of this nucleus and by the low amount of oxidation compounds present in oxidized lipids. In particular, secondary oxidation products (i.e. aldehydes) have very low sensory thresholds and their instrumental measure requires very sensitive methods (Frankel, 1998; Guth and Grosch, 1991). For these reasons, the study of lipid oxidation products by ^{1}HNMR has been somewhat ignored and only few applications appeared in the literature, suggesting the possibility of detecting, directly from the proton NMR spectra, the hydroperoxides and aldehydes (Claxson et al., 1994; Aparicio et al., 1999; Guillén and Ruiz, 2003a,b, 2005, 2006, 2007, 2008; Sacchi et al., 2006a).

In this part of the work, high-resolution ^{1}H-NMR spectroscopy (400-600 MHz) was applied to the quantitative analysis of hydroperoxide decomposition products in thermally oxidized oils. A first part of the results obtained as been already pubblished (Sacchi *et al*, 2006a). Further results are in pubblication.

3.2 MATERIALS AND METHODS

3.2.1 Oil samples and thermal stressing

Three oil types (extra virgin olive oil, sunflower oil and soybean oil) were selected for this study and purchased on the Italian market. One litre of each oil was continually heated at 180 °C for 6 hours, using a thermostated household-frying bath (Tefal, Milan, Italy). Oil samples were collected in duplicate at different times during the six-hour heating treatment: 0 (initial time), 60, 120, 240, and 360 min and stored in glass bottle at -20°C until analyses (**Figure 3.2.1.1**).
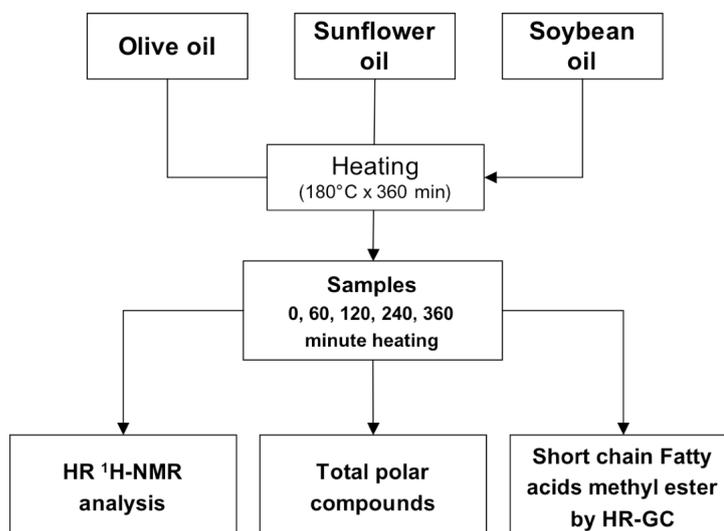
Figure 3.2.1.1. Experimental design relative to the preparation and analytical determination of the oils samples.

3.2.2 Standards and reagents

Standard triacylglycerols, methyl esters and fatty acids were obtained from Fluka (Buchs, Switzerland), Larodan (Malmoe, Sweden) and Nu-Chek-Prep Inc. (Elysian, MIN, USA). Chloroform-*d* (with 0.03 % v/v internal tetramethylsilane, TMS) and thribromobenzol (TBB) were Aldrich Chemical Co. (Milwaukee, WIS, USA)

58

products. All other chemicals were HPLC or reagent grade and were purchased from Fluka (Buchs, Switzerland).

3.2.3 Chemical characterization of oils

The determination of the peroxide value, the specific extinction K_{232} (conjugated dienes) and K_{270} (trienes and carbonyl compounds) were performed on oil samples before the heating tests according to the EC official methods for olive oils (EC Regulation 2568/91).

The phenolic compounds were extracted from oils and analyzed by reversed phase HPLC following the method previously described (Sacchi *et al.*, 2002) The chromatographic separation was achieved on a Shimadzu (Milan, Italy) liquid chromatograph (model LC-10AD) equipped with a Spherisorb (Bedfordshire, UK) ODS-3 5μ column (250 x 4.6 mm i.d.). The HPLC peaks were detected using a diode array detector (model SPD M10A VP) identified by comparing their relative retention times and UV spectra with those of pure compounds (simple phenol acids and alcohols). Major peaks due to complex phenols (hydrolysable phenols) were assigned to the molecular structures identified by Montedoro *et al.* (1993) and Brenes *et al.* (2000). Quantitative analysis of the main phenol peaks was made with the aid of tyrosol as an external standard according to the procedure indicated by Tsimidou *et al.* (1992).

Tocopherols were determined by reversed- phase HPLC (Tonolo and Marzo, 1989) using the same column and equipment employed for the phenol analysis and d-α-tocopheryl acetate (Fluka, Switzerland) as internal standard. Quantitative data were expressed as α-tocopherol (response factor calculated using a standard solution of α-tocopherol and d-α-tocopheryl acetate).

3.2.4 Total polar compounds and short chain fatty acids analysis

Total polar compounds were determined by column silica gel chromatography according to the official IUPAC method (Waltking and Wessels, 1981; AOAC, 2000; Dobarganes *et al.*, 2000).

The analysis of fatty acid methyl esters (FAMEs) in starting oils and that of short chain fatty acids in heated oils were carried out by gas chromatography according to the method described by Màrquez-Ruiz and Dobarganes (1996) (**Figure 3.2.4.1**) on a Shimadzu GC17A chromatograph (Shimadzu Italia, Milan, Italy) equipped with a 60 m fused-silica capillary column (Quadrex Corporation, New Heaven, U.S.A.) coated with cyanopropyl methyl silicone (0.25 μm film thickness). Temperature was set at 80°C for 5 min, then to 170°C at a rate of 10°C min-1, kept at 170°C for 12 min, then to 220°C at a rate of 10°C min-1, and finally 6 min at 220°C. The injection temperature was 250°C and the detector temperature was 250°C. The individual FAMEs were identified by comparison with the retention times of pure standards (mixture Olive – cod. 90-5518, Larodan, Malmoe, Sweden). Quantification of methyl-octanoate and methyl-heptanoate was made by referring to an internal standard (methyl-nonanoate) (Màrquez-Ruiz and Dobarganes, 1996).
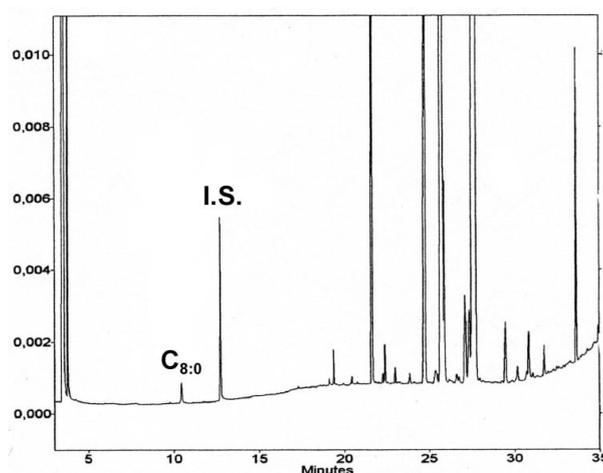
Figure 3.2.4.1. Example of Gas-liquid chromatogram showing fatty acid composition of a sample added $C_{9:0}$ as internal standard. The corresponding peaks of $C_{8:0}$ and I.S. have been indicated.

3.2.5 ^{1}H-NMR spectroscopy

Oil samples were prepared by dissolving in 5 mm NMR tubes 100 μL of oil in 700 μL of Chloroform-d (Euriso-Top, 99.8% isotopic purity) containing 1.1 mmol/L

of 1,3,5-tribromobenzene (TBB, Fluka) as an internal reference for the quantitative analysis of aldehydes. All spectra were recorded at a temperature of 25°C. Chemical shifts were referred to internal tetramethylsilane (TMS). 1D proton NMR spectra were acquired on a Varian Unity 400, operating at a proton frequency of 400 MHz, located at the "IBB-CNR, Istituto di Biostrutture e Bioimmagini del C.N.R." at the University Federico II of Naples, equipped with a 5 mm Indirect Detection probe (Varian, Palo Alto, CA, USA). 400 MHz proton NMR spectra were recorded with the following acquisition parameters: 32K data points (zero-filled to 64K data points prior to Fourier transformation), spectral width 13 ppm, acquisition time 3.0 s, pulse width 10°, 3000 scans were used for a total acquisition time of 2.5 hours. Total accumulation time was reduced to 2 min on a Varian UNITY-Inova 600 NMR spectrometer operating at a proton frequency of 600 MHz, also located at the IBBCNR, equipped with a 5mm PFG Penta Probe (Varian, Palo Alto, CA, USA).

3.3 RESULTS AND DISCUSSION

The chemical composition of vegetable oils used in this study is reported in **Table 3.3.1**. The samples presented oxidation parameters, fatty acids composition, tocopherols and phenolic antioxidants contents quite different. No phenols were detected in two seed oils, whilst virgin olive oil used in this study was characterized by a total phenolic content of 224 mg tyrosol /Kg oil as determined by HPLC (sum of the identified compounds). The phenol composition is reported in **Table 3.3.1**.

Table 3.3.1. Chemical composition of unheated oils

	Virgin Olive Oil	Soybean Oil	Sunflower Oil
PV (meqO$_2$/kg)	6.2	0.6	0.5
K$_{232}$	1.98	5.76	4.00
K$_{270}$	0.15	3.56	4.95
Fatty acid composition (%)			
Palmitic acid	9.86	10.37	7.26
Palmitoleic acid	0.19	0.86	nd
Stearic acid	2.15	3.79	3.75
Oleic acid	76.84	19.28	26.88
Vaccenic acid	1.84	1.55	1.00
Linoleic acid	6.42	55.27	59.26
Linolenic acid	0.64	7.21	0.11
Tocopherols (mg/kg oil)			
α-tocopherol	87	55	503
β + γ -tocopherols	7	378	27
δ -tocopherol	7	187	nd
Phenol compounds (mg tyrosol/kg oil)			
Hydroxytyrosol	38	nd	nd
Tyrosol	16	nd	nd
Dialdehydic form of the acetoxy-methyl oleuropein aglycone	29	nd	nd
Dialdehydic form of the ligstroside aglycone	76	nd	nd
Lignans	32	nd	nd
Oleuropein aglycone	33	nd	nd
Total phenol compounds	224		

Oil samples collected at regular intervals during heating at 180°C, were submitted to the determination of the total polar compounds, to the HRGC and ^{1}H-NMR analyses of short chain fatty acids and aldehydes, respectively. The comparison of the data obtained from each independent method was made following the formation of the different classes of compounds. In fact, the homolytic β-scission of hydroperoxides formed during the thermoxidation of vegetable oils generates different products, depending on the structure of the starting fatty acids, on the position of the peroxyl group and on the cleavage site (Màrquez-Ruiz and Dobarganes, 1996; Frankel, 1991). It is known that temperature-induced cleavage on the side of the acyl carbonyl head of the oleyl-8-hydroperoxide gives rise to the formation of heptanoate and n-alkenals; the decomposition of oleyl-9, linoleyl-9 or linolenyl-9- hydroperoxides gives rise to the formation of octanoate and unsaturated aldehydes like *trans*-2-alkenals, alka-2,4- dienals and 4-hydroxy-2-alkenals (Frankel, 1987).

In **Figure 3.3.1** is shown the formation of octanoate **1** and aldehydes **2**- **5** from oleyl-9 **6**, linoleyl-9 hydroperoxides **7**.

The formation of the octanoyl chain **1** bound to the glyceryl moiety of triacylglycerols is due to oleyl-9 **6** and linoleyl-9 hydroperoxide **7** cleavage, that also produces unsaturated aldehydes 2 and 3 **6**. The formation of saturated aldehydes **4** and **5** originates from the decomposition of oleyl-10 **8** and linoleyl-13 hydroperoxides **9**. In these cases no octanoyl chains are formed (Frankel, 1998).

Therefore, the amount of saturated aldehydes cannot be correlated with the HRGC determination of methyl octanoate **1**, while a possible relationship can only be checked between the total amount of aldehydes determined by NMR and the total amount of polar compounds.
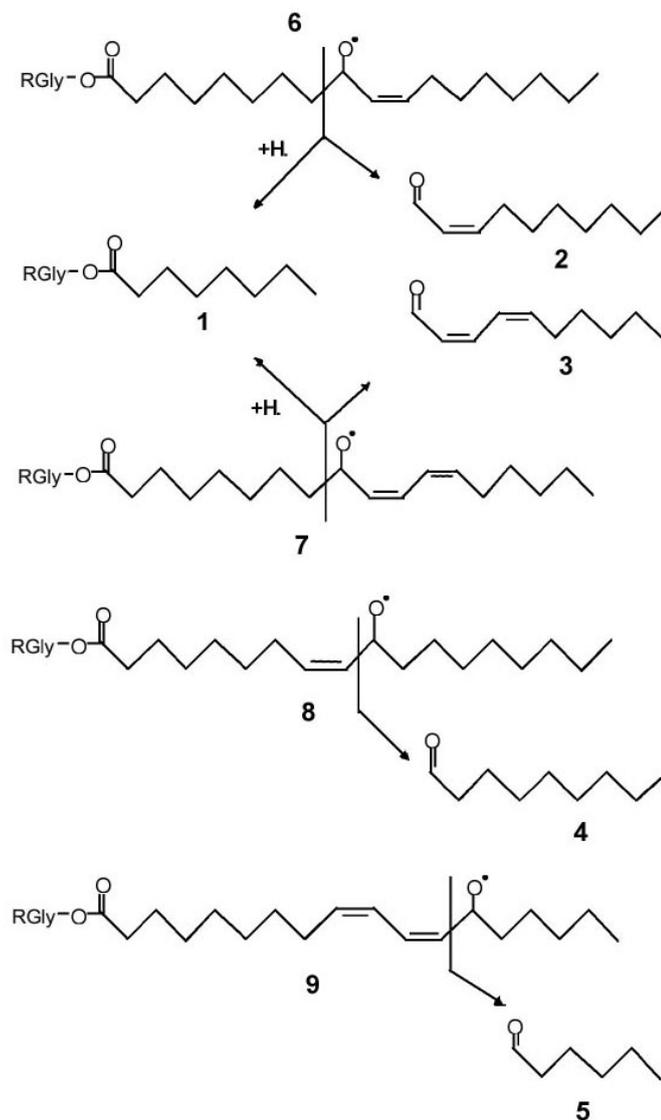
Figure 3.3.1. Formation of octanoate (**1**), 2-undecenal (**2**), 2,4-decadienal (**3**) and n-alkanals (**4** and **5**) from the homolytic b-scission of oleyl-9 hydroperoxy (**6**), linoleyl-9 hydoperoxy (**7**), oleyl-10 hydroperoxy (**8**), and linoleyl-13 hydroperoxy (**9**) radicals.

3.3.1 ^{1}H-NMR quantitative analysis of aldehydes

The formation of aldehydic compounds during the oil thermoxidation was followed in the ^{1}H-NMR spectra by measuring the absolute intensity of carbonyl proton (-C**H**O) resonances in the low field spectral region (**Figure 3.3.1.1**).

According to literature data (Claxson *et al.*, 1994; Guillén and Ruiz, 2005a,b, 2006, 2008), -**CHO** proton resonances have been assigned to *n*-alkanals (triplet at 9.76 ppm), 4- hydroxy-*trans*-2-alkenals (doublet at 9.61 ppm), alka-2,4- dienals (doublet at 9.54 ppm) and *trans*-2-alkenals (doublet at 9.51 ppm).

The quantitative data were expressed as mmol/L oil, using the tribromobenzene (TBB) as an internal standard (7.7 mmol per Litre of oil) added to the oil sample in chloroform-*d* solution. This compound was chosen for its solubility in chloroform and for its proton chemical shift around 7.6 ppm not overlapping other proton NMR signals usually occurring in vegetable oils (Sacchi *et al.*, 1997).
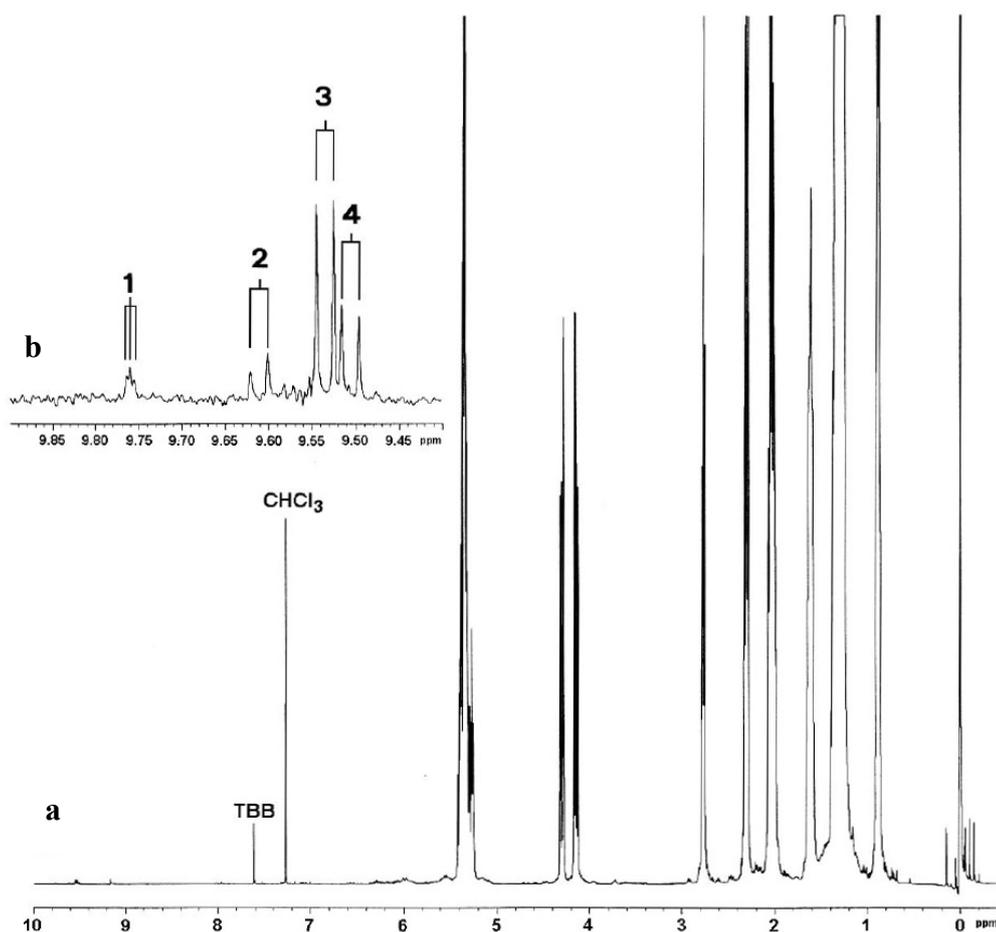
Figure 3.3.1.1. ^{1}H NMR spectrum of a sunflower oil thermoxidized for 6 hours, acquired at 400 MHz in CDCl3 (**a**). In the expansion of the low field region (**b**) the aldehyde proton signals are assigned as follows: 1) *n*-alkanals, 2) 4-hydroxy-*trans*-2-alkenals, 3) alka-2,4-dienals, 4) *trans*-2-alkenals.

The formation of the hydroperoxidienes was also investigated by the proton patterns centred around 8.5 ppm, corresponding to the hydroperoxide protons (-OOH), and at 5.5-6.6 ppm, due to the hydroperoxidiene conjugated olefinic protons (R-C**H**=C**H**-C**H**=C**H**-C**H**-OOH-R) (Claxson *et al.*, 1994; Haywood, *et al.*, 1995). No increase in the corresponding resonance was detected during heating, confirming that hydroperoxides at frying temperature are rapidly decomposed and do not accumulate in the oil. Diagnostic resonances of fatty acid dimers (Neff *et al.*, 1988) in this experimental conditions in the NMR spectra of heated oils were also not detected in all examined oils. The time-course of the total aldehydes formation in three oils studied is presented in the **Figure 3.3.1.2**.
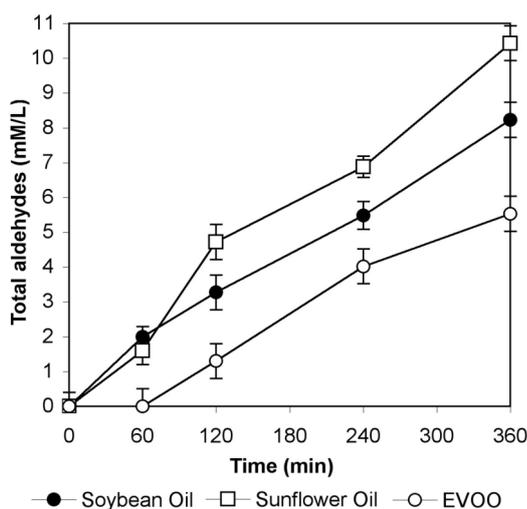
Figure 3.3.1.2. Quantitative evolution of total aldehydes in different vegetable oils subjected to heating at 180°C, as measured by ^{1}H-NMR spectroscopy

In virgin olive oil a detectable amount of aldehydes was observed only after 2 hour heating, whilst sunflower and soybean oils showed 1.99 and 1.60 mM/L respectively of total aldehydes after 1 hour heating. The relative rate of total aldehydes formation was virgin olive oil< soybean oil < sunflower oil. The

behaviour of EVOO can be attribuited both to the fatty acid composition (rich in oleic acid and low level of PUFAs) and to the presence of antioxidants (phenols, tocopherols). The higher degradation of sunflower oil with respect to soybean oil indicate a strong antioxidant role of α-tocopherol at high temperatures (Frankel, 1998) In all vegetable oils, aldehydic components increase quite regularly throughout the thermal treatment. **Figure 3.3.1.3** shows the formation of the different aldehydes during 6 hours heating. Alka-2,4-dienals and *trans*-2-alkenals (**Figure 3.3.1.3a** and **b**) are the major products detected in heated oils. This indicates that the oxidation site in acyl-9 positions is the preferred oxidation site with respect to other positions (acyl- 10 and acyl-11 in oleic acid; acyl-13 in linoleic acid).
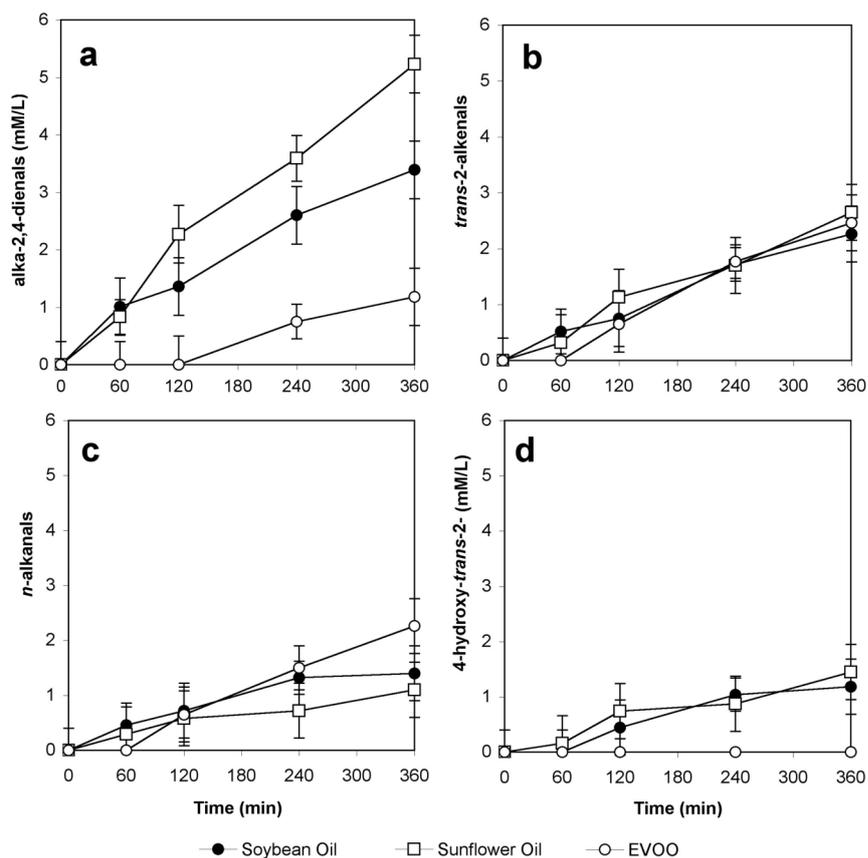
Figure 3.3.1.3. Quantitative evolution of alka-2,4-dienals (**a**), *trans*-2-alkenals (**b**), *n*-alkanals (**c**) and 4-hydroxy-*trans*-2-alkenals (**d**) in different vegetable oils subjected to heating at 180°C, as measured by ^{1}HNMR spectroscopy .

The production of *trans*-2-alkenals was similar in olive oil and seeds oils (**Figure 3.3.1.3b**), while n-alkanals were produced at the end of the heating in a larger amount in olive oil (**Figure 3.3.1.3c**). The 4-hydroxy-2-alkenals were not detected (threshold 0.1 mM/L) in olive oil (**Figure 3.3.1.3d**). The formation of these compounds is, in fact, related to the decomposition of conjugated hydroperoxydienes, arising from the oxidation of polyunsaturated fatty acids (Claxson *et al.*, 1994). In olive oil, the low amount of linoleic and linolenic acids explain this findings. For the same reason, alka-2,4-dienals were also formed in very small amounts (1.1 mM/L oil after 6 hours heating) with respect to polyunsaturated seed oils.

3.3.2 Relationship between unsaturated aldehydes and methyl-octanoate

Oil samples collected during the 6 hours heating were analysed by HRGC to follow methyl octanoate **1** and methyl-heptanoate formation (**Figure 3.3.1**). Methyl-octanoate was detected just after 30 min heating for all studied oils, whilst methyl-heptanoate formation was detectable only in virgin olive oil at the end of the 6-hours heating. This finding was in agreement with data reported by Màrquez-Ruiz and Dobarganes (1996), who observed a significant formation of heptanoate only after 10 hours heating in high-oleic oils. In order to correlate the formation of aldehydes measured by NMR to the amount of octanoate **1**, only unsaturated aldehydes like **2** and **3** have been considered on the basis of the hydroperoxide breakdown mechanism described above (**Figure 3.3.1**). The octanoate **1** level determined by HRGC fits well (R^2= 0.941) with that of unsaturated aldehydes measured by NMR as shown in **Figure 3.3.2.1**.

The regression analysis indicates that the aldehyde molar content determined by NMR is almost twice that of the corresponding octanoate content derived from the HRGC data. This is likely a consequence of linoleyl and linolenyl hydroperoxide cleavages in position other than Δ-9, which gives rise to unsaturated aldehydes but to acyl residues different from octanoate (Frankel, 1998).
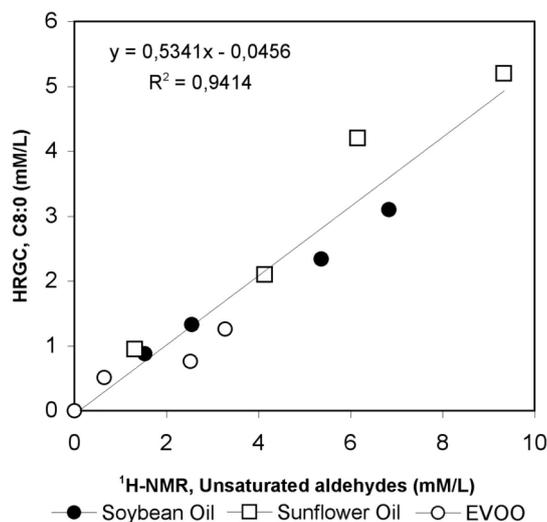
Figure 3.3.2.1. Linear regression plot of methyl octanoate (mM/L of oil) determined by HRGC on total unsaturated aldehyde amount (mM/L of oil) determined by 1H-NMR spectroscopy.

3.3.3 Relationship between total aldehydes and total polar compounds

A good linear relationship (R^2=0.918) was also found between the amount of total aldehydes and total polar compounds (**Figure 3.3.3.1**).
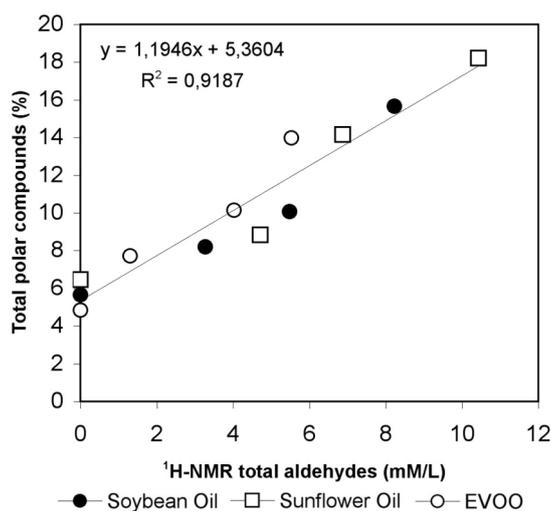
Figure 3.3.3.1. Linear regression plot of total polar compounds (wheight, %) determined by silica column chromatography on total aldehydes amount (mM/L of oil) determined by ^{1}H-NMR spectroscopy.

A consideration arising from these data is that the initial level of total polar compounds can be very different (in our samples was around 5-7%), thus compromising the measure in the early stages of thermal oxidation. On the contrary, the initial level of aldehydes in vegetables oils is very low (**Figure 3.3.1.3**) and its direct detection by ^{1}H-NMR spectroscopy could allow to follow thermal degradation and to evaluate the abuse of vegetable oils during frying without the interference of the initial content of other polar compounds not specifically due to the oxidation occurring during frying.

3.3.4 NMR Sensitivity

A comparison of ^{1}H-NMR spectra samples acquired at 400 MHz and at 600 MHz with different number of scans was made on the same olive oil sample in order to evaluate the time required to reach the same detection limit using two different magnetic fields. The same signal-to-noise ratio was obtained at two different magnetic fields with a very different number of scans. Indeed, while the 400 MHz proton spectrum required a total time of about 150 min using a 5 mm Indirect Detection probe on a Varian UNITY spectrometer, having a proton sensitivity about 130:1, the 600 MHz proton spectrum was obtained within 2 min, using a 5 mm PFG Penta Probe on a modern Varian UNITY Inova system, whose proton sensitivity is about 1100:1. Moreover, both instruments have a 16-bit ADC but the maximum spectral width is 500 kHz at the 600 MHz vs. 100 kHz at the 400 MHz. On the 600 MHz is also possible to use the oversampling during acquisition and this procedure lowers the noise, enhancing the low intensity signals (this is almost the same of a 18-bit ADC). The lower amount of each aldehyde quantified under these experimental conditions was 0.1 mM/L (about 10 mg/Kg oil). Although this quantification limit is 10-fold higher than the odour threshold values reported for the major aldehydes in oils not submitted to thermoxidation (Guth and Grosch, 1991), in thermally stressed or fried oils the amount of these compounds is considerably higher (about 5 folds) than NMR detection limit (Frankel, 1998). In addition, the detection limit can be easily overtaken by increasing the number of scans using a 600 MHz or more

powerful spectrometers, where a remarkably high signal-to-noise ratio can be obtained with a total acquisition time of 15 min or less. In addition, the higher performances of the new multiple resonance probes can greatly improve the ^{1}H sensitivity, allowing a considerable experimental time reduction even on lower magnetic fields (300-400 MHz).

3.4 CONCLUSIONS

The obtained results confirm the possibility of using NMR spectroscopy as an useful method to follow the early stage of lipid thermoxidation and to assess on fried oils the thermal stress. Moreover, the newly available instruments and the use of cold-probes will represent a useful tool to monitor and investigate decomposition products in vegetable oils well below the 1 mM threshold.

Information with the same significance and specificity is actually available only by the chromatographic determination of tryacilglycerol polymers.

4 GEOGRAPHICAL CHARACTERIZATION OF EXTRA VIRGIN OLIVE OILS

4.1 INTRODUCTION

The determination of the geographical origin of extra virgin olive oils is a recent problem: the quality of an olive oil is the result of several factors that can be clustered into four main groups: environmental (soil, climate), agronomic (irrigation, fertilisation), cultivation (harvesting, ripeness), and technological factors (post-harvest storage and extraction system) (Solinas, 1987; Solinas *et al.*, 1987; Fontanazza *et al.,* 1993). Therefore, for the careful determination of the place of production based on chemical composition, many factors need to be taken into account (Montedoro *et al.*, 1992).

The diversity and inter-relationship of these factors makes it tremendously difficult to carry out a complete characterisation of virgin olive oils either by their chemical composition or their sensory descriptors. Consequently, the characterisation cannot be carried out with a few series of chemical compounds or a simple data manipulation. Rather should samples be identified by a large number of variables (chemical compounds and/or sensory descriptors), and data should be analysed by statistical techniques or artificial intelligence algorithms (Aparicio and Luna, 2002).

An important act of legislation, the "Protected Designation of Origin" (PDO) (Reg. EC 2081/92), allows the labeling of some European extra virgin olive oils with the names of the areas where they are produced. This certification improves the commercial value of the product. Since the early nineties, many research institutions devoted to the study of vegetable oils, especially olive oil, have explored and applied various methods potentially capable of characterising PDO oils for their authenticity; almost all these studies were based on the application of chemometrics (Aparicio *et al.*, 1988).

The supposed contribution of the area of olive production to the quality and the peculiarity of the olive oil is particularly important in Italy, where more than 200 different cultivars are grown in different areas. Several attempts have been made to

identify the place of olive oil production by means of multivariate analysis of suitable chemical parameters: using the principal component analysis (PCA) of fatty acid composition and other chemical parameters Forina and Tiscornia (1982), Alessandri *et al*., (1997) and Lanza *et al*., (1998) obtained a first classification of Italian olive oils from different regions; Aparicio *et al.* (1988, 1990, 1996), using an expert system (SEXIA), have studied data from different chemical analyses to classify Spanish oils with respect to their origin and variety; Tsimidou *et al.* (1987) applied the PCA of fatty acids and triacylglycerols for the geographical classification of Greek olive oils. More recent studies have regarded statistical analysis applied to fatty acids, triacylglycerols composition and NIR spectra in order to discriminate French PDO olive oils (Galtier *et al*., 2007), fatty acids composition to ascertain the geographical origin and the cultivar of Sicilian extra virgin olive oil (Di Bella *et al.,* 2007), visible spectra to trace the geographical origin of virgin olive oils coming from several Mediterranean regions (Forina *et al*, 2007), volatiles compounds to characterize virgin olive oils produced in northern Italy (Vichi *et al*., 2003) and to determinate the quality of virgin olive oil applied to the classification of commercial samples (López-Feria *et al*., 2007), electronic nose and electronic tongue analysis to classify and describe extra virgin olive oils belong to Garda PDO (Cosio *et al*., 2006), electronic nose and UV-Vis spectrophotometer data to differentiate the geographical denominations of Liguria (Casale *et al*., 2007) and sensorial and physico-chemical properties to characterize the virgin olive oils produced in the geographic counties of Çanakkale region of Turkey, (Ögütçü *et al*., 2008). Brescia *et al*., 2003,

These authentication methods, usually associated with chemometrics analysis, can be classified as "with identification" and "without identification" (or "fingerprint") techniques. The former are focused on the existence or absence of certain chemical compounds in the sample (e.g. major and minor components of virgin olive oils: triacylglycerols, diacylglycerols, wax esters, free fatty acids, triterpenic alcohols, hydrocarbons, sterols, phenols, flavonoids, pigments, tocopherols, volatile

compounds, etc.), the latter are based on a combination of measurements (e.g. spectral fingerprints, multi-sensor array fingerprints, etc.) (Forina *et al*., 2007).

Chemometrics proved to be a powerful tool for answering complex questions such as the geographical origin and it is generally believed to be applicable to food products (Bianchi, *et al*., 2001). Chemometric techniques are especially suitable for handling the large amounts of data produced by modern analytical methods (Aparicio *et al.*, 1988).

Nowadays, the analysis of vegetable oils is dominated by classic determinations, such as acidity, peroxide value, ultraviolet absorption, etcetera, as well as by the use of chromatographic procedures, including thin layer, gas, and high performance liquid chromatography (Christie, 2003). These techniques are primarily used for quantitative measurement of known compounds, and, with these analytical criteria, different international regulations have been established to define oil genuineness and quality. One drawback to these procedures is that there are too many different assays to be applied to routine analysis. In addition, some of these methods require the isolation and analysis of minor compounds from the unsaponifiable matter by means of procedures that are laborious and time-consuming. Therefore, many studies have been carried out to apply new analytical techniques that, with very little or without any manipulation of the sample, can produce results similar or superior to those obtained by the classical procedures.

In this context, spectroscopic techniques have emerged as potential tools in recent years, although the complexity and intrinsic variability of biological samples such as vegetable oils usually require the application of multivariate calibration or pattern-recognition techniques to aid interpretation of the data obtained using these instrumental methods (Shoolery, 1977; Cordella, 2002; Hidalgo and Zamora 2003; Tapp *et al.*, 2003). One spectroscopic technique with a high potential in this field is High-Resolution Nuclear Magnetic Resonance (NMR) spectroscopy.

Different NMR experiments and equipment, such as High-Resolution NMR, low-resolution NMR and Magnetic Resonance imaging, yield

different information. With High-Resolution NMR one can perform both qualitative and quantitative analyses of samples in solution, which can be used for structural studies and compositional analyses (Mannina *et al.*, 2003b).

So a NMR spectrum contains a great amount of information that can be obtained in a short time period and employed as an alternative to many classic analytical procedures. The different signals present in the NMR spectra provide two kinds of information: the chemical shifts and the relative intensities. The former is of qualitative value and it is related to the different atoms present in the analysed sample. The latter provides quantitative information of the different signals (Hidalgo and Zamora 2003).

In the last year NMR spectroscopy has been widely used in food analysis. With High-Resolution NMR one can perform both qualitative and quantitative analyses of samples in solution, which can be used for structural studies and compositional analyses. To assess the role of NMR in food chemistry we must know which specific problem it has to solve, how conclusive is the evidence that derives from the NMR investigation and how it compares to other methods that can be brought to bear on the same problems. In the published literature, many NMR studies using various NMR techniques have been reported on different types of food and drinks which include wine, olive oil, coffee, fruit juices, tomatoes, milk, meat, egg, starch granules and flour (Alberti *et al.*, 2002; Mannina *et al.*, 2003b; Sobolev *et al.*, 2003; Lachenmeier *et al.*, 2005; Sacchi and Paolillo, 2007).

Numerous application are being described regarding to the determination of authenticity and quality of vegetable oils by using high resolution NMR (Vlahov, 1999; Mannina *et al.,* 2001a,b; Guillén and Ruiz, 2003; Hidalgo and Zamora 2003: Mannina *et al.,* 2005; Rezzi *et al.*, 2005; Petrakis *et al.*, 2008). High-resolution NMR plays an important role in oil analysis. This is firstly due to the absence of sample pretreatment, since oils are simply added to a deuterated solvent before analysis. ^{1}H NMR studies of oils have increased because of the great amount of information that

high field NMR instruments can provide with a single experiment and in a very short time (Sacchi *et al*., 1996, 1997; Brescia and Sacco 2008). High-field ^{1}HNMR spectroscopy has been successfully employed to characterize olive oil, to the extent of identifying many of its minor components including many volatile compounds and discriminate oils from different varieties and from different regions of Italy (Sacchi *et al*., 1996, 1997, 1998; Mannina, 2001a, 2005; Brescia and Sacco, 2008). ^{13}C NMR has been used to monitor adulterations in olive oil and classify the samples according to variety and geographical origin (Vlahov *et al*., 1999; Vlahov *et al*., 2001). ^{13}C-NMR has been applied to the study of the triacylglycerol structure of palm oils (Ng, 1983, 1985, 2000), olive oils (Sacchi *et al*, 1992; Vlahov, 2006), seed oils (Wollemberg, 1990) and some hydrogenated fats (Cengarle *et al*. 1992; Pfeffer *et al*., 1977a; Gunstone, 1991a,b; 1993a,b).

All existing data suggest that with only one analysis, NMR allows the determination of a large number of components with very little or without any manipulation of the oil samples that nowadays need many different analyses. In addition, the application of multivariate statistics to NMR spectral data increases considerably the potential of the technique. However, because minor components of the oils are playing an essential role in defining oil authenticity and quality, concentration of these compounds (either by using a chromatographic procedure or by the use of unsaponifiables) or their observation during routine analysis by using more sensitive probes, seem to be a necessary requisite to achieve a routine application of NMR to most aspects of oil analysis (Hidalgo and Zamora 2003).

Chemometrics is habitually used for exploring patterns of association in data, and preparing and using multivariate classification models. The arrival of Chemometrics techniques has allowed the quantitative as well as qualitative analysis of multivariate data and, in consequence, it has allowed that we can now analyse and model a lot of different types of experiments (Aparicio, 2002). Chemometrics methods have been commonly applied for matching and discrimination, classification and prediction in assessing authenticity of vegetable oils (Zhanga, 2006). The general concept of

authentication, on the other hand, is not only circumscribed to adulteration but also include aspects as characterisation, geographical origin of foodstuffs, extraction and processing systems among others. GC and LC methods in combination with multivariate statistical techniques such as principal component analysis (PCA), discriminant analysis (DA), cluster analysis (CA), K-nearest neighbor, partial least squares (PLS) and artificial neural networks (ANN) have been applied successfully to classify and discriminate the oils (Aparicio, 2002). Moreover, there have been several other researches, using different chemical indices as descriptors, which focused on the use of supervised chemometric techniques for discriminating olive oil samples almost always in terms of their geographical origin (Marini *et al*., 2004).

In this part of the work NMR analysis has been applied to discriminate oils of various geographic and varietal origin and to estimate the contribution of NMR spectroscopy to the traceability of virgin olive oils. The study has been based on ^{1}H-NMR analysis of some minor compounds and on gas chromatographic analysis (GC) of fatty acids composition of olive oils. This last information, in fact, already has allowed to the discrimination of some Italian POD oils (Scarpa *et al*., 2005) and has been applied in combination to NMR spectroscopy for the varietal and geographic classification of olive oils (Mannina *et al*., 2001a,b, 2003a). ^{1}H-NMR and GC data obtained in this study were processed by means of statistics analysis (Principal Component Analysis, Discriminant Analysis and Cluster analysis) to attempt of classify oils obtained from different varieties and geographic areas (Calabria, Campania, Basilicata, Puglia).

4.2 MATERIALS AND METHODS

4.2.1 Oil samples

EVOO samples produced in Southern Italy, 46 monovarietal olive oils obtained by a benchtop oil mill and by proper technological extraction methodologies, obtained within the project RIOM ("Ricerca ed Innovazione per l'Olivicoltura Meridionale") and 9 PDO protected olive oil samples have been analyzed (**Figure 4.2.1.1, Table 4.2.1.1**). NMR and GC data were processed using the XlSTAT software for Mac (version 2007.1, Addinsoft, France).
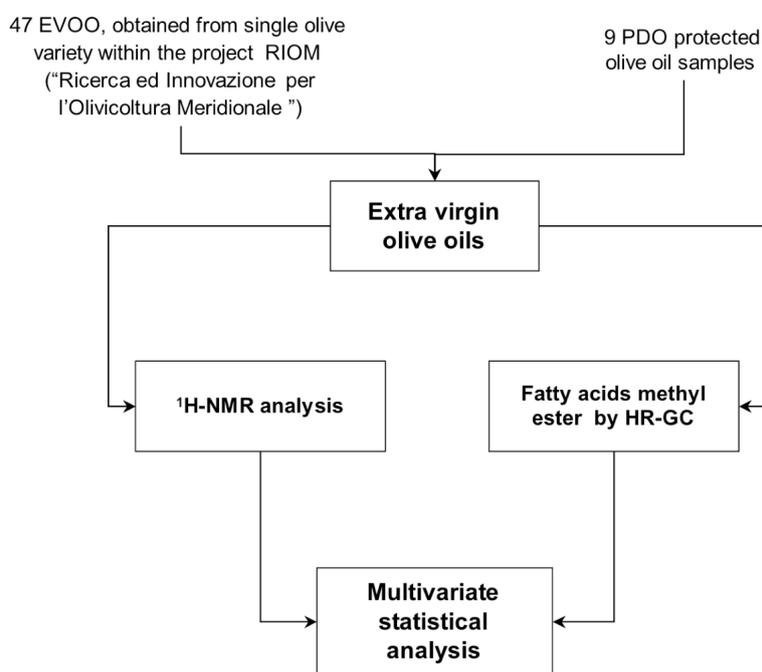
Figure 4.2.1.1. Experimental design relative to the analytical determination of the oil samples.

Table 4.2.1.1. Origins and Cultivars of Extra Virgin Olive Oils

sample	cultivar	origin	sample	cultivar	origin
Rav	Ravece		Car	Carolea	
Rav	Ravece	Avellino	Car	Carolea	Catanzaro
Rav	Ravece		Car	Carolea	
Ogl	Ogliarola		CdM	Cima di Melfi	
Car	Carolea		CdM	Cima di Melfi	
Car	Carolea		CdM	Cima di Melfi	
Cor	Coratina	Bari	CdM	Cima di Melfi	Potenza
Cor	Coratina		MdF	Maiatica di Ferrandina	
Cor	Coratina		MdF	Maiatica di Ferrandina	
Cor	Coratina		MdF	Maiatica di Ferrandina	
Cai	Caiazzana		Car	Carolea	
Cai	Caiazzana		Ott	Ottobratica	Reggio Calabria
Cai	Caiazzana	Caserta	Ott	Ottobratica	
Cai	Caiazzana		Ott	Ottobratica	
Ton	Tonda		Rot	Rotondella	
Ton	Tonda		Rot	Rotondella	
Car	Carolea		Rot	Rotondella	Salerno
Car	Carolea		Rot	Rotondella	
Cas	Cassanese				
Cas	Cassanese			**POD**	
DdR	Dolce di Rossano		AC1	Alto Crotonese	Calabria
DdR	Dolce di Rossano	Cosenza	BZ1	Bruzio	
DdR	Dolce di Rossano		Ci 03	Cilento	Campania
Tond	Tondina		CS 83	Collina Salernitane	
Tond	Tondina		CB1	Colline di Brindisi	Puglia
Tond	Tondina		ME 1	Monte Etna	
Rog	Roggianella		MI 14	Monti Iblei	Sicilia
Car	Carolea	Krotone	VM 6	Val di Mazara	
Car	Carolea	Catanzaro	VT 4	Valli Trapanesi	

4.2.2 Standards and reagents

Chloroform-*d* was obtained from Aldrich Chemical Co. (Milwaukee, WIS, USA). All other chemicals were GC or reagent grade and were purchased from Fluka (Buchs, Switzerland).

4.2.3 Preparation of FAMEs and Gas chromatography

Analysis of fatty acid methyl esters (FAMEs) were carried out by cold transmethylation in KOH/methanol (EC Regulation No. 2568/91; Christie, 2003).

A quantity of 100 mg oil was weighed and dissolved in 10 ml *n*-hexane. 2 ml of this solution was mixed with 1 ml sodium methylate solution (2 N in methanol) in a sample vial and shaken vigorously for 1 min using a vortex mixer. The clear supernatant was used for GC analysis.

Analyses of FAME were performed using a Shimadzu GC17A gas chromatography (Shimadzu Italia, Milan, Italy) equipped with split/splitless injection port, flame ionization detector and a 60 m fused-silica capillary column (Quadrex Corporation, New Heaven, U.S.A.) coated with cyanopropyl methyl silicone (0.25 μm film thickness). Helium was used as the carrier gas and the flow rate was 1.8 ml min±1 at a split ratio of 1:60. The temperature of both the injector and detector was 250 °C. The initial oven temperature was set at 170 °C for 20 min; it was then programmed to increase at a rate of 10 °C min±1 until it reached 220 °C, maintained for 10 min. The samples (1 μl) were injected using an automatic injector Shimadzu AOC-20i (Shimadzu Italia, Milan, Italy). The individual FAMEs were identified by comparison with the retention times of pure standards (mixture Olive, Larodan, Malmoe, Sweden).

4.2.4 ^{1}H-NMR spectroscopy

The ^{1}H-NMR procedure reported by Sacchi *et al* (1998) and Mannina *et al*. (2001a), with some modification, was followed. Olive oils (6 mg) were placed into 5mm NMR tubes and dissolved in chloroform-*d* (600 μl). ^{1}H-NMR spectra were recorded on a Varian Unity INOVA 600 instrument operating at 600 MHz, with a cold probe. ^{1}H-NMR FIDs were recorded using the following acquisition parameters: acquired points 16K, processed points 16K, spectral width 8 ppm, relaxation delay 1s, acquisition time 1.2s, number of scans 128. The intensities of the selected resonances were compared to that of the methylene resonance at 1.602 ppm, the

intensity of which is set to 1000. The quantitative evaluation of peaks of interest was performed after a careful baseline correction.

4.3 RESULTS AND DISCUSSION

From NMR spectra at 600 Mhz have been integrated 5 resonances of minor compounds: *β-sitosterol* (0.68 ppm), *squalene* (1.63 ppm) and *terpenes* (4.59, 4.66 e 4.72 ppm) (**Figure 4.3.1**).
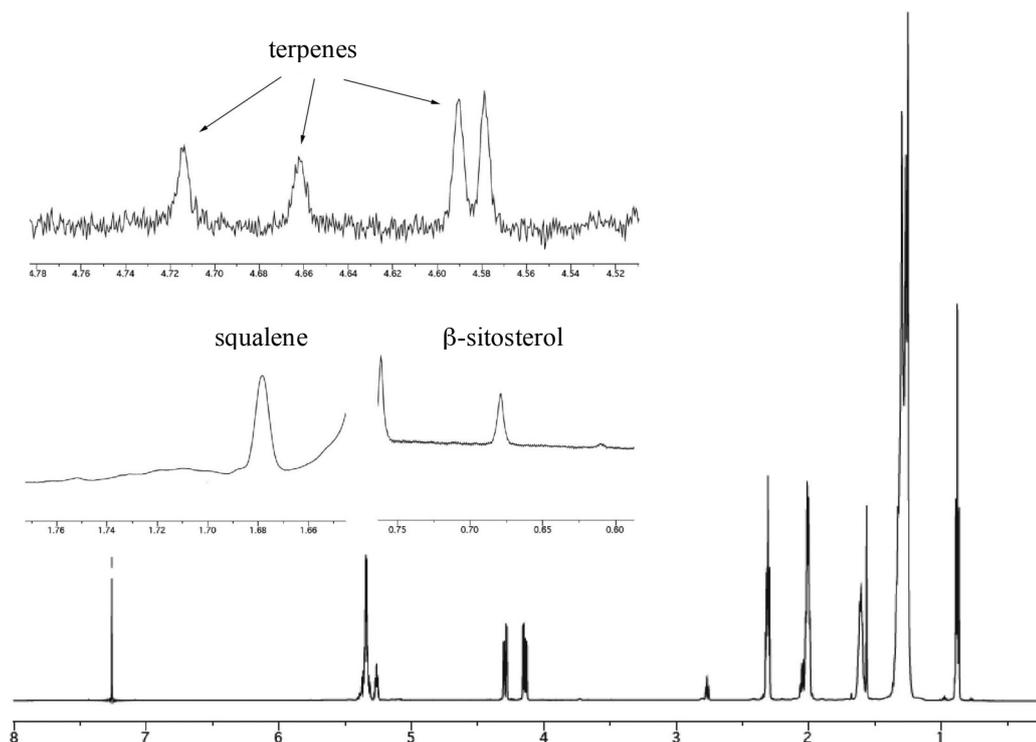
Figura 4.3.1. Virgin olive oil ^{1}H-NMR spectra at 600 MHz with expansion of the relative regions to the resonances of minor compounds.

Spectra, obtained with 128 scans in 6 minute of acquisition, have shown an good signal/noise relation allowing the quantitative analysis of these minor resonances. Before the quantitative evaluation of all peaks of interest, a careful baseline correction was performed. The intensities of the selected resonances (see **Figure 4.3.1**) were compared to that of the methylene resonance at 1.60 ppm, the intensity of which is set to 1000. This normalizing procedure gives for each resonance an index proportional to the molar ratio between each compound and the total amount of the fatty chains.

The data matrix obtained arranging NMR data with that of fatty acids composition, determined by gas chromatography, has been submitted to the statistical analysis.

In **Figure 4.3.2** are reported the *score plot* (samples distribution) and *loading plot* (correlations between variables) coming out from the principal components analysis (PCA) obtained from elaboration of NMR data (5 variable x 56 samples) and acidic composition (16 variables for 56 samples). The first two principal components explain approximately 46% of the variance.
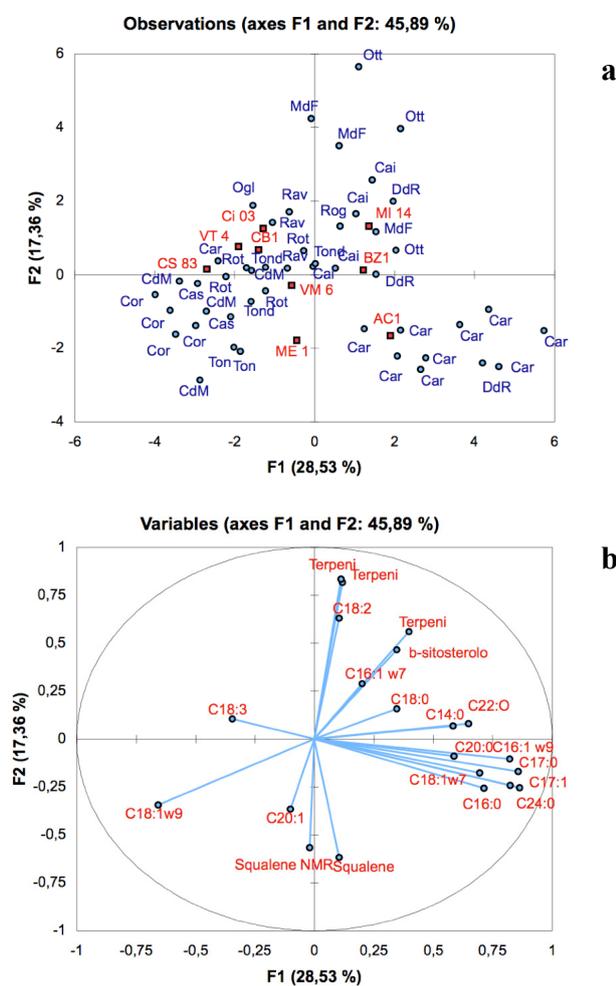
Figure 4.3.2. *Score plot* (**a**) and *Loading plot* (**b**) of Principal Components Analysis (PCA) carried out on NMR data and fatty acids composition (gas chromatographic data) of oils samples.

The PCA shows the distribution of oil samples along the first two principal components and their grouping in relation to the cultivars. In particular, the *score plot* (**Figure 4.3.2a**) shows a clear distinction of the *Carolea* (Car) and *Coratina* (Cor) cultivar along the first principal component (F1). NMR data, how shown in *loading plot* (**Figure 4.3.2b**), contribute to differentiate the samples along the second principal component (F2). The oil samples show a good dispersion, but not a clear discrimination between oils from the various cultivars.

In **Figure 4.3.3** the PCA, obtained with only fatty acids composition, shows how the gas chromatographic data contribute mainly to the dispersion of samples in relation to cultivars.
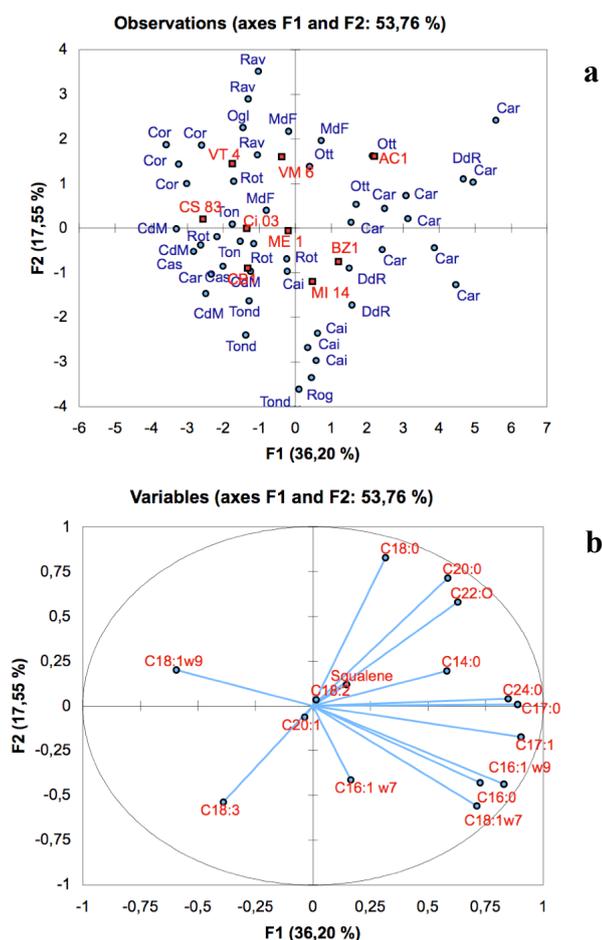
Figure 4.3.3. *Score plot* (**a**) and *Loading plot* (**b**) of Principal Components Analysis (PCA) carried out on fatty acids composition (gas chromatographic data) of oils samples.

Figure 4.3.3 shows the Tree clustering analysis (TCA) obtained on fatty acids composition (gas chromatographic data).
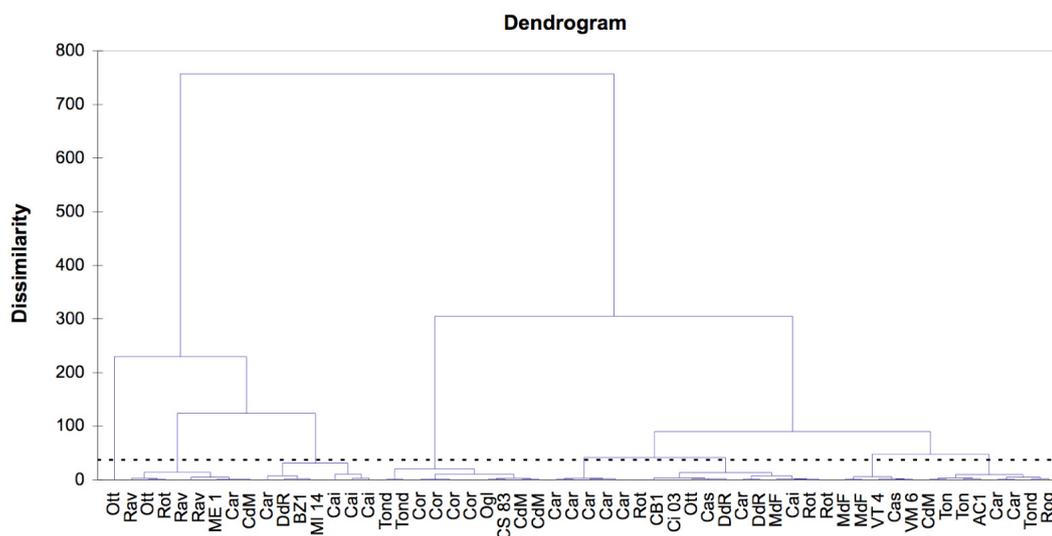
Figura 4.3.3. Tree clustering analysis (TCA) carried out on fatty acids composition (gas chromatographic data) of oil samples. Dissimilarity: Euclidean distance, Agglomeration method: Ward's method.

In this analysis (dendogram) the samples are grouped on their dissimilarity in a hierarchical diagram. Also using this analysis the *Carolea* (Car) and *Coratina* (Cor) cultivas show an appreciable grouping.

The dendogram obtained from NMR and GC data (**Figure 4.3.4** and **Table 4.3.1**) shows a subdivision in 10 groups statistically distinguished. The oil samples of *Carolea* (Car), *Coratina* (Cor), *Caiazzana* (Cai), *Cima di Melfi* (CdM), *Maiatica di Ferrandina* (MdF), *Tondina* (Tond) and *Tonda* (Ton) cultivars are distinguished in rather homogenous groups.
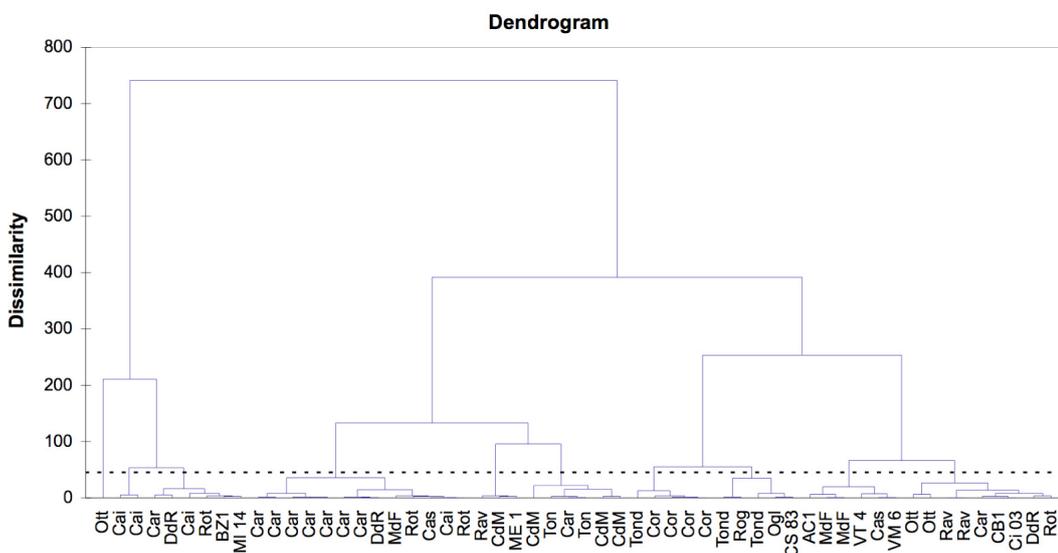
Figure 4.3.4. Tree clustering analysis (TCA) carried out on NMR data and fatty acids composition (gas chromatographic data) of oil samples. Dissimilarity: Chebychev distance, Agglomeration method: Ward's method.

Table 4.3.1. Results by class

Class	1	2	3	4	5	6	7	8	9	10
Objects	2	13	6	9	6	6	3	5	1	5
Sum of weights	2	13	6	9	6	6	3	5	1	5
Within-class variance	5,572	6,197	7,119	7,678	8,745	7,082	2,935	4,346	0,000	11,595
Minimum distance to centroid	1,669	1,065	1,148	0,958	1,469	0,991	1,366	0,682	0,000	2,435
Average distance to centroid	1,669	2,296	2,317	2,404	2,461	2,218	1,398	1,607	0,000	2,995
Maximum distance to centroid	1,669	3,431	3,108	4,127	4,295	3,856	1,461	3,185	0,000	3,999
	Cai	Cai	Cai	Car	Car	Cas	CdM	Cor	Ott	Tond
	Cai	Car	Car	DdR	CdM	MdF	Rav	Cor		Tond
		Car	DdR	Ott	CdM	MdF	ME 1	Cor		Ogl
		Car	Rot	Ott	CdM	AC1		Cor		Rog
		Car	BZ1	Rav	Ton	VM 6		Tond		CS 83
		Car	MI 14	Rav	Ton	VT 4				
		Car		Rot						
		Car		CB1						
		Cas		Ci 03						
		DdR								
		MdF								
		Rot								
		Rot								

The other cultivars do not appear sufficiently differentiated from each other. They show a fatty acids composition not much homogenous within the same cultivar probably because of a different olives ripeness degree or different agronomic and environment condition of harvesting. Furthermore the small samples number has not allowed to carry out other hypothesis on variability observed.

Carrying out a Discriminant Analysis (**Figure 4.3.5**) on the NMR and GC data matrix, a good differentiation of *Tondina* (Tond), *Ottobratica* (Ott), *Caiazzana* (Cai), *Ravece* (Rav), *Maiatica di Ferrandina* (MdF) and *Rotondella* (Rot) varieties is observed. The *Coratina* (Cor), *Carolea* (Car), *Cassanese* (Cas), *Cima di Mola* (CdM), *Tonda* (Ton) and *Dolce di Rossano* (DdR) cultivars even if shown a partial superimposition in the central zone of the plot, have a very characterized groups potentially useful in order to verify, *a posteriori*, the oils varietal origin. Data obtained from the determination of fatty acids and NMR, can contribute to an objective definition of variety and geographic origin of virgin olive oil.
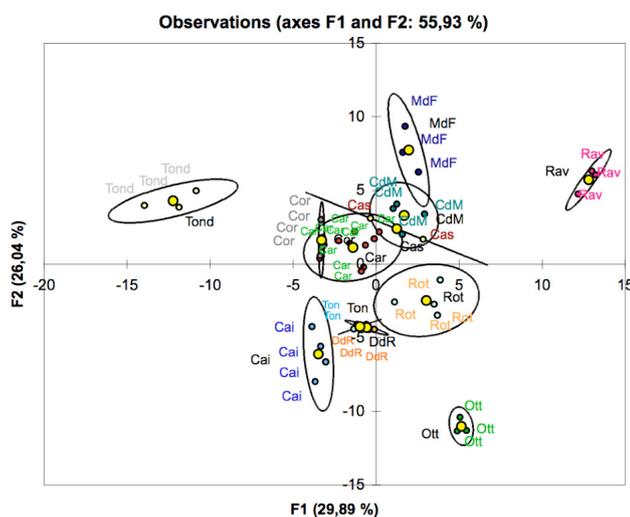
Figura 4.3.5. Discriminant analysis (DA) carried out on NMR data and fatty acids composition (gas chromatographic data) of oil samples.

4.4 CONCLUSIONS

The results obtained from the oil samples analysis confirm as a combination of data obtained from different analytical techniques and their multivariate statistic elaboration allow to characterize the varietal and geographic origin of virgin olive oils. The data obtained from fatty acids composition, arranged with those deriving from NMR analysis, can contribute to the objective definition of the cultivar and the geographic origin of virgin olive oils. Other information will be able also to derive from the use of other compositive data more correlated to varietal genetic profiling. Moreover it is of fundamental importance the choice of suitable statistical techniques for data elaboration to confirm the effectiveness of analytical results.

However, it must be considered which for being able analytically to estimate virgin olive oils, and to allow that such approach can be useful to the objective certification of the product, is necessary to refer to data-banks. Data-banks that collect the analytical results of many oil samples, for each crop year, relatively to the cultivars or geographical origins that have to be controlled. Certainly is more easy the traceability of monovarietal oils, above all for the more typical varieties of southern Italy regions, that of oils produced by olives mixture like PDO oils.

Anyway such approach, certainly complex and more expensive, will be able in future to support the control organisms of PDO certification, and to contribute to the "certification" and "traceability" of the olive oils productions. In any case a "typical oil" will go assessed mainly on sensory and aromatic characteristics, which are the key factors for the valuable productions of southern Italy.

5 NEW HORIZONS

The development of cryo-probes in combination with ultra-high field magnets (700-800 MHz) open new horizons with rispect the possibility of detec and quantify minor compounds of oils and fats directly on the sample and without any treatment or concetration.

Preliminary experiments carried out on a 700 MHz instrument (Facoltà di Farmacia, Univerità di Napoli Federico II) revealed the extraordinary information can be obtained by NMR analysis. **Figure 5.1** shows, as an example, the low field region of spectra recorded on extra virgin olive oils with different sensory profiles, in which directly by NMR analysis the aldehydes and phenolic compounds, related to bitter-pungent taste and fruity flavour, can be detected and assigned (**Table 5.1**).
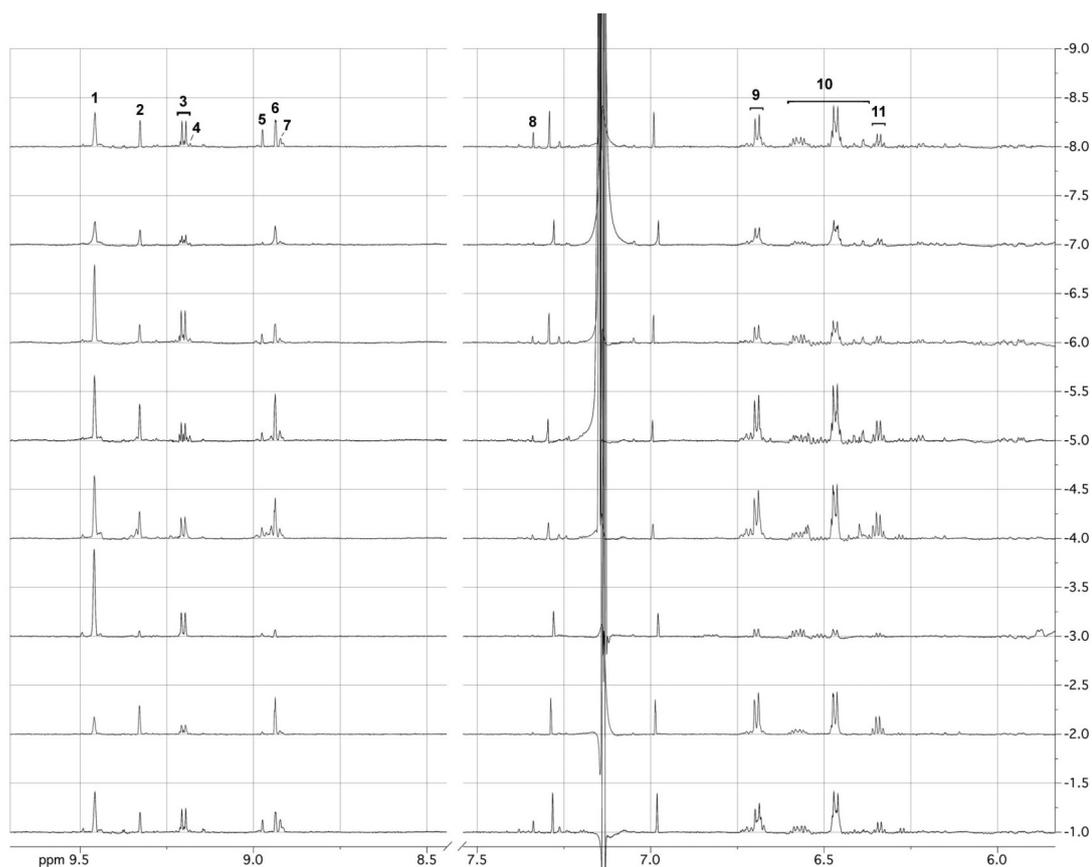
Figure 5.1. Low field regions of high resolution NMR spectra (700 MHz) of virgin olive oil samples.

Table 5.1. Chemical shifts of minor compounds identified in olive oil by high resolution ^{1}H NMR Spectroscopy (700 MHz).

Peak	Chemical shifts (ppm)	identification	References
1	9.54	*n*-alkanals	Sacchi *et al.*, 1996, 1998
2	9.49	Dialdehydic form of oleuropein and ligstroside	Dais and Christophoridou, 2008
3	9.33-9.32	*n*-2-alkenals	Sacchi *et al.*, 1996, 1998
4	9.30	n.i.	-
5	9.10	*cis*-3 esenale	simulation
6	9.06	Dialdehydic form of oleuropein and ligstroside	Dais and Christophoridou, 2008
7	9.04	Dialdehydic form (?)	-
8	7.46	Aldehydic form of oleuropein / ligstroside	Dais and Christophoridou, 2008
9	6.82-6.81	total tyrosol	Dais and Christophoridou, 2008
10	6.70-6.58	Pinoresinol - 1-Acetoxypinoresinol	Dais and Christophoridou, 2008
11	6.47-6.46	total hydroxytyrosol	Dais and Christophoridou, 2008

Further studies are now in progress to evaluate the correlation between NMR, sensory (panel test) and other instrumental analysis (HPLC of phenols, SPME-GC/MS of volatiles compounds) in order to validate this new "comprehensive" information on sensory and nutritional quality of extra virgin olive oils , available directly by proton NMR spectroscopy.

BIBLIOGRAPHY

Alais C. (2000). Scienza del latte, terza edizione, pubblicato da It. Tecniche Nuove, Milano.

Alberti E., Belton P.S. and Gil A.M. (2002) Application of NMR to Food Science. *Annual Reports on NMR spectroscop.* 47: 109-148.

Alemany L.B. (2002). Using simple ^{13}C NMR linewidth and relaxation measurements to make detailed chemical shift assignments in triacylglycerols and related compounds. *Chem. Phys. Lipids,* 120: 33-/44.

Alessandri, S., Cimato, A., Modi, G., Mattei, A., Crescenzi, A., Caselli S., Tracchi, S. (1997). Univariate models to classify Tuscan virgin olive oils by zone. *Riv. Ital. Sost. Grasse* 74, 155-163.

Andreotti G., Trivellone E., Lamanna R., Di Luccia A., and Motta A. (2000). Milk Identification of Different Species: ^{13}C-NMR Spectroscopy of Triacylglycerols from Cows and Buffaloes' Milks. *J Dairy Sci*, 83, 2432–2437

Andreotti G., Lamanna R., Trivellone E. and Motta A. *(2002).* ^{13}C NMR Spectra of TAG: An Easy Way to Distinguish Milks from Different Animal Species. *JAOCS*, 79, 123-127.

AOAC (2000). Official Method 982.27 – Polar Components In Frying Fats. AOAC International.

Aparicio, R.; Albi, T.; Cert, A.; Lanzon, A. (1988). SEXIA expert system: canonical equations to characterize Spanish olive oils by varieties. *Grasas Aceites*, 39, 219- 228.

Aparicio, R.; Ferreiro, L.; Cert, A.; Lanzon, A. (1990). Characterization of Andalusian Virgin olive oil. *Grasas Aceites*, 41, 23-39.

Aparicio, R.; Calvente, J. J.; Morales, M. T. (1996). Sensory authentication of European extra-virgin olive oil varieties by mathematical procedures. *J. Sci. Food Agric.*, 72, 435-439.

Aparicio R., Mcintyre P., Aursand M., Eveleigh L., Marighetto N., Rossell B., Sacchi R., Wilson R., Woolfe M. (1999). Oils & Fats, in *Food Authenticity: issues and methodologies*, Published by Eurofins Scientific, Nantes, France. pp. 209-269.

Aparicio R. (2002). "Chemometrics as an Aid in Authentication" in Oils and Fats Authentication. Edited by Michel Jee, published by Blackwell. 1st edition, pp 156-178.

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

Aursand M, Grasdalen H. (1992). Interpretation of the ^{13}C-NMR spectra of omega-3 fatty acids extracted from the white muscle of Atlantic salmon (Salmo salar). *Chem. Phys. Lipids*, 62: 239-251.

Aursand M., Rainuzzo J., Grasdalen H. (1993). Quantitative high-resolution ^{13}C and ^{1}H nuclear magnetic resonance of fatty acids from white muscle of atlantic salmon (*Salmo salar*). *J. Am. Oil Chem. Soc.,* 70: 971-981.

Aursand M., Jørgensen L., Grasdalen H. (1995). Positional distribution of ω-3 fatty acids in marine lipid triacylglycerols by high resolution ^{13}C Nuclear magnetic resonance spectroscopy, *J. Am. Oil Chem. Soc.,* 72 (3), 293-297.

Aursand M, Mabon F and Martin GJ. (2000). Characterization of farmed and wild Salmon (Salmo salar). *J. Am. Oil Chem. Soc.,* 77: 659-666.

Aursand M and Alexon DE. (2001). Origin recognition of wild and farmed salmon (Norway and Scotland) using ^{13}C-NMR spectroscopy in combination with pattern recognition techniques. In: Magnetic resonance in food science. A view to the future, Edited by Webb, G.A., Belton, B.S., Gil, A.M., and Delgadillo I. pp. 227-231, Royal Society of Chemistry, Cambridge (UK).

Bianchi G., Giansante L., Shaw A., Kell D.B. (2001). Chemometric criteria for the characterisation of Italian Protected Denomination of Origin (DOP) olive oils from their metabolic profiles *Eur. J. Lipid Sci. Technol.* 103:141-150.

Bianco A., Uccella N. (2000). Biophenolic components of olives. *Food Res. Int.* 33: 475-485.

Brenes M., Hidalgo F.J., Garcia A., Rios J.J., Garcia P., Zamora R., Garrido A., (2000). Pinoresinol and 1-acetoxypinoresinol, two new phenolic compounds identified in olive oil. *J. Am. Oil Chem. Soc.* 77: 715- 720.

Brescia M.A., Alviti G., Liuzzi V. and Sacco A. (2003). Chemometric Classification of Olive Cultivars Based on Compositional Data of Oils. *JAOCS,* 80: 945-950.

Brescia M.A. and Sacco A. (2008). High-Resolution ^{1}H Nuclear Magnetic Resonance in the Study of Oils. *Mod. Magn. Res.*, 1645–1650.

Brühl L. and Matthäus B. (2008). Short-chain fatty acids as marker for the degradation of frying fats and oils. *Lipid Tech.,* 20: 60-63.

Bonnet M., Denoyer C. and Renou J.P. (1990). High Resolution 13C NMR spectroscopy of rendered animal fats: degree of saturation of fatty acids chains and position on glycerol. Int. *J. Food Sci. Tech*., 25: 399-408.

Buchgraber M., Androni S. and Anklam E. (2007). Quantification of Milk Fat in Chocolate Fats by Triacylglycerol Analysis Using Gas-Liquid Chromatography**.** *J. Agric. Food Chem*., 55: 3275-3283.

Casale M., Armanino C., Casolino C., Forina M. (2007). Combining information from headspace mass spectrometry and visible spectroscopy in the classification of the Ligurian olive oils. *An. Chim. Acta*, 589: 89–95

Cengarle L, Pranzetti P, Carta A. (1992). Utilizzazione della spettrometria NMR nella determinazione di trans isomeri in margarine commerciali. *Riv. Ital. Sostanze Grasse* 69:159.

Christie W.W. (2003). Lipid analysis - Third Edition. The Oily Press Lipid Library.

Claxson A.W.D., Hawkes G.E., Richardson D.P., Naughton D.P., Haywood R.M., Chander C.L., Atherton M., Lynch E.J., Grootveld M.C. (1994). Generation of lipid peroxidation products in culinaryoils and fats during episodes of thermal stressing: a high field ^{1}H NMR study. *FEBS Lett.* 355: 81-90

Council Regulation (EC) No 2991/94 of 5 December 1994 laying down standards for spreadable fats

Commission Regulation (EC) No 445/2007 of 23 April 2007 del 23 aprile 2007 laying down certain detailed rules for the application of Council Regulation (EC) No 2991/94 laying down standards for spreadable fats and of Council Regulation (EEC) No 1898/87 on the protection of designations used in the marketing of milk and milk products

Commission Regulation (EEC) No 4056/87 of 22 December 1987 laying down the methods of analysis and other technical provisions necessary for the implementation of Regulation (EEC) No 3035/80 laying down general rules for granting export refunds on certain agricultural products exported in the form of goods not covered by Annex II to the Treaty, and the criteria for fixing the amount of such refunds.

Commission Regulation (EEC) No 4154/87 of 22 December 1987 laying down the methods of analysis and other technical provisions necessary for the implementation of Regulation (EEC) No 3033/80 laying down the trade arrangements applicable to certain goods resulting from the processing of agricultural products.

Cordella C., Moussa I., Martel A.C., Sbirrazzuoli N. and Lizzani-Cuvelier L. (2002). Recent Developments in Food Characterization and Adulteration Detection: Technique-Oriented Perspectives. *J. Agric. Food Chem.,* 50: 1751-1764.

Cosio M.S., Ballabio D., Benedetti S., Gigliotti C. (2006). Geographical origin and authentication of extra virgin olive oils by an electronic nose in combination with artificial neural networks. *An. Chim. Acta,* 567: 202–210.

Dais P. and Christophoridou S. (2008). Detection and Quantification of Phenolic Compounds in Olive Oil by Employing [1]H High Resolution Nuclear Magnetic Resonance Spectroscopy. A proposal for on-line screening. 6th Euro Fed Lipid Congress, 07-10 September, Athens, Greece. Book of Abstracts, p. 129

Davis A.L., McNeill G.P., Caswell D.C. (1999). Analysis of conjugated linoleic acid isomers by ^{13}C NMR spectroscopy. *Chem. Phys. Lipids*, 97: 155-165.

Dobarganes M.C., Velasco J., And Dieffenbacher A. (2000). Determination of polar compounds, polymerized and oxidized triacylglycerols, and diacylglycerols in oils and fats Results of Collaborative Studies and the Standardized Method (Technical Report). *Pure Appl. Chem.*, 72: 1563-1575.

Dobarganes M. C., Márquez-Ruiz G. (2003). Oxidised fats in foods. *Curr. Opin. Clin. Nutr. Metab. Care*, 6: 157–163.

Di Bella G., Maisano R., La Pera L., Lo Turco V., Salvo F. and G. Dugo (2007). Statistical Characterization of Sicilian Olive Oils from the Peloritana and Maghrebian Zones According to the Fatty Acid Profile. *J. Agric. Food Chem.*. 55: 6568-6574

European Comunity. Regulation (1992). 2081/92. Official J. Eur. Communities

European Community Regulation No. 2568/91. EC Official J. September 5th, L248 (1991).

Esterbauer H. (1993). Cytotoxicity and genotoxicity of lipid-oxidation products. *Am. J. Clinic Nutr.*, 57: 779-786.

Falch E. (2006). Lipids from residual fish raw materials - Quality assessment by advanced analytical methods. PhD Thesis - The Norwegian University of Science and Technology, NTNU, Department of Biotechnology.

Fan, T.W.M. (1996). Metabolite profiling by one- and two-dimensional NMR analysis of complex mixtures. *Progr. Nuclear Mag. Res. Spectr..*, *28*, 161-219.

Fauhl C., Reniero F., Guillou C. (2000). ^{1}H-NMR as a tool for the analysis of mixtures of virgin olive oil with oils of different botanical origin. *Magn. Reson. Chem*. 38: 436-443.

Fontanazza, G.; Patumi, M.; Solinas, M.; Serraiocco, A. (1993). Influence of cultivars of the composition and quality of olive oil. *Acta Hortic.*. 356: 358-361.

Forina, M.; Tiscornia, E. (1982). Pattern recognition methods in the prediction of Italian olive oil origin by their fatty acid content. *Ann. Chim*. 72, 143-155.

Forina, M., Boggia R., Casale M. (2007). The information content of visible spectra of extra virgin olive oil in the characterization of its origin. *Annali di Chimica*, 97: 615-633.

Frankel E.N. (1987). Secondary products of lipid oxidation. *Chem. Phys. Lipids*. 44: 73-85.

Frankel E.N. (1991). Recent advances in lipid oxidation. *J. Sci. Food Agric*. 54, 495-511.

Frankel E.N. (1998). Lipid oxidation. The Oily Press, Dundee, Scotland.

Galtier O., Dupuy N., Le Dréau Y., Ollivier D., Pinatel C., kister J., Artaud J. (2007). Geographic origins and compositions of virgin olive oils determinated by chemometric analysis of NIR spectra. *An. Chim. Acta*, 595: 136-144.

Glaeser H. (2002). Nota breve. Determination of the milk fat content of fat mixtures. *Grasas y Aceites*, 53: 357-358

Guillén M.D, Ruiz A (2003a). Edible oils: discrimination by ^{1}H nuclear magnetic resonance. *J. Sci. Food Agric.,* 83: 338-346.

Guillén M.D, Ruiz A (2003b). Rapid simultaneous determination by proton NMR of unsaturation and composition of acyl groups in vegetable oils. *Eur. J. Lipid Sci. Technol.* 105: 688-696.

Guillén M.D, Ruiz A (2005a). Monitoring the oxidation of unsaturated oils and formation of oxygenated aldehydes by proton NMR. *Eur. J. Lipid Sci. Technol.* 107: 36-47.

Guillén M.D, Ruiz A (2005b). Oxidation process of oils with high content of linoleic acyl groups and formation of toxic hydroperoxy- and hydroxyalkenals. A study by ^{1}H nuclear magnetic resonance. *J. Sci. Food Agric.* 85: 2416-2420.

Guillén M.D, Ruiz A (2006). Study by means of ^{1}H nuclear magnetic resonance of the oxidation process undergone by edible oils of different natures submitted to microwave action. *Food Chemistry* 96: 665–674.

Guillén M.D., and Goicoechea E. (2007). Detection of Primary and Secondary Oxidation Products by Fourier Transform Infrared Spectroscopy (FTIR) and [1]H Nuclear Magnetic Resonance (NMR) in Sunflower Oil during Storage. .*J Agr. Food Chem.* 55: 10729-10736.

Guillén M.D., Ruiz A (2008). Monitoring of heat-induced degradation of edible oils by proton NMR. *Eur. J. Lipid Sci. Technol*., 110: 52–60.

Gunstone F.D. (199la). The composition of hydrogenated fats determined by high resolution 13C-NMR spectroscopy. *Chemistry & Industry* 19:802.

Gunstone F.D. (1991b). ^{13}C-NMR studies of mono-, di- and tri-acylglycerols leading to qualitative and semiquantitative information about mixtures of these glycerols esters. *Chem. Phys. Lipids* 58:219.

Gunstone F.D. (1993a). Information on the composition of fats from their high resolution [13]C Nuclear Magnetic Resonance spectra. *J. Am. Oil Chem. Soc.* 70:361.

Gunstone F.D. (1993b). The composition of hydrogenated fats by high resolution [13]C Nuclear Magnetic Resonance spectroscopy. *J. Am. Oil Chem. Soc.* 70:965.

Gunstone F.D. (1994). High resolution ^{13}C NMR spectroscopy of lipids. In Advances in lipid methodology-Two, edited by W.W. Christie, The Oily Press, Dundee, Scotland, pp.1-68.

Gunstone F.D., Harwood J.L. and Dijkstra A.J. (2007). The Lipid Handbook - Third Edition CRC Press Taylor & Francis Group.

Guth H., Grosch W. (1991). A comparative study of the potent odorantes of different virgin olive oils. *Fat. Sci. Technol*. 93: 335-339.

Haywood R.M., Claxson W.D., Hawkes G.E., Richardson D.P., Naughton D.P., Coumbarides G., Hawkes J., Lynch E.J. And Grootveld M.C., (1995). Detection of aldehydes and their conjugated hydroperoxydiene precursors in thermally-stressed culinary oils and fats: investigations using high resolution proton NMR spectroscopy. *Free Radic. Res*. 22: 441-482.

Hidalgo F.J. and Zamora R. (2003). Edible oil analysis by high-resolution nuclear magnetic resonance spectroscopy: recent advances and future perspectives. *Trends in Food Science &* Technology 14: 499–506.

Hopkins C.Y., Bernstein H.J. (1959). Applications of proton magnetic resonance spectra in fatty acid chemistry. *Can. J. Chem*. 37: 775-782.

Hu F., Furihata K., Ito-Ishida M., Kaminogawa S. and Tanokura M. (2004). Nondestructive Observation of Bovine Milk by NMR Spectroscopy: Analysis of Existing States of Compounds and Detection of New Compounds. *J. Agric. Food Chem.,* 52, 4969-4974.

Hu F., Furihata K., Kato Y. and Tanokura M. (2007). Nondestructive Quantification of Organic Compounds in Whole Milk without Pretreatment by Two-Dimensional NMR Spectroscopy. *J. Agric. Food Chem.,* 55, 4307-4311.

Hunter, C.A.; Packer, M.J.; Zonta, C. (2005). From structure to chemical shift and vice-versa. *Progr. Nuclear Magnetic Res. Spectr.***,** 47: 27-39.

Ichihara K., Shibahara A., Yamamoto K. and Nakayama T. (1996). An Improved Method for Rapid Analysis of the Fatty Acids of Glycerolipids. Lipids, 31: 535-539.

Igarashi T., Aursand M., Hirata Y., Gribbestad I.S., Wada S., Nonaka M. (2000). Nondestructive quantitative determination of docosahexaenoic acid and n-3 fatty acids in fish oils by high-resolution [1]H-nuclear magnetic resonance spectroscopy. *J. Am. Oil Chem. Soc*. 77: 737-748.

Igarashi T., Aursand M., Sacchi R., Paolillo L., Nonaka M., Wada S. (2002). Determination of docosahexanenoic acid and n-3 fatty acids in refined fish oils by ^{1}H-NMR spectroscopy: IUPAC Interlaboratory Study. *J. AOAC Internat*. 85: 1341-1354.

Jacobsen N.E. (2007). NMR Spectroscopy Explained. Simplified Theory, Applications and Examples for Organic Chemistry and Structural Biology. Published by John Wiley & Sons, Inc., Hoboken, New Jersey.

Johnsthon L.F., Shoolery J.N., (1962). Determination of unsaturation and average molecular weight of natural fats by Nuclear Magnetic Resonance. *Anal. Chem*. 34: 1136-1139.

Kalo P, Kempinnen A. and Kilpelainen I. (1996). Determination of positinal distribution of butyril groups in milkfat tiacylglycerols mixtures, and isolated positional isomers of triacylglycerols by gas chromatography and ^{1}H nuclear magnetic resonance. *Lipids,* 31:331-336.

Kamm W., Dionisi F., Hischenhuber C., Engel K.H., (2001). Authenticity assessment of fats and oils. *Food Rev. Int*. 17: 249-290.

Kamm W. (2002). Authenticity control of edible fats and oils by analysis of minor constituents via on-line liquid chromatography-gas chromatography. PhD thesis, Technischen Universität München.

Katsuta I., Shimizu M., Yamaguchi T., Nakajima Y. (2008). Emission of Volatile Aldehydes from DAG-Rich and TAG-Rich Oils with Different Degrees of Unsaturation During Deep-Frying. *J. Am. Oil Chem. Soc*., 85: 513-519.

Knothe G., Lie Ken Jie M.S.F., Lam C.C., Bagby M.O. (1995). Short communication. Evaluation of the 3C-NMR signals of the unsaturated carbons of triacylglycerols. *Chem. Phys. Lipids,* 77: 187-191.

Knothe G. and Kenar J.A. (2004). Determination of the fatty acid profile by ^{1}H-NMR spectroscopy. *Eur. J. Lipid Sci. Technol.,* 106: 88–96

Lachenmeier D.W., Frank W., Humpfer E., Schäfer H., Keller S., Mörtter M., Spraul M. (2005). Quality control of beer using high-resolution nuclear magnetic resonance spectroscopy and multivariate analysis. *Eur Food Res Technol* 220:215-221.

Lambert J.B. and Mazzola E.P. (2003). Nuclear Magnetic Resonance Spectroscopy. An Introduction to Principles, applications and experimental methods. Publisher: Prentice Hall, Pearson Education Inc., New Jersey.

Lanza C.M., Russo C. and Tomaselli F. (1998). Relationship between geographical origin and fatty acid composition of extra-virgin olive oils produced in three areas of eastern sicily. *Ital. J. Food Sci*. 10: 359-366.

Lanzetta R. (2002). "La spettroscopia di Risonanza Magnetica Nucleare" in La chimica organica in laboratorio. Piccin Editore, pp. 246-283.

López-Feria S., Cárdenas S., García-Mesa J.A., Valcárcel M. (2007). Usefulness of the direct coupling headspace–mass spectrometry for sensory quality characterization of virgin olive oil samples. *An. Chim. Acta* 583: 411–417.

Lopez-Varela S., Sánchez-Muniz F.J. and Cuesta C. (1995). Decreased food efficiency ratio, growth retardation and changes in liver fatty acid composition in rats consuming thermally oxidized and polimerized sunflower oil used for frying. *Fd. Chem. Toxic.*, 33: 181-189.

Lie Ken M.S.F. Jie and Mustafa J. (1997). High-Resolution Nuclear Magnetic Resonance Spectroscopy. Applications to Fatty Acids and Triacylglycerols. *Lipids,* 32, 1019-1034.

Lie Ken Jie M.S.F., Pasha M.K., and Alam M.S. (1997). Synthesis and Nuclear Magnetic Resonance Properties of All Geometrical Isomers of Conjugated Linoleic Acids. *Lipids*, 32: 1041-1044.

Mannina, L., Luchinat, C., Emanuele, M.C., and Segre, A. (1999). Acyl positional distribution of glycerol tri-esters in vegetable oils: a ^{13}C NMR study. *Chem. Phys. Lipids*, 103: 47-55.

Mannina L., Luchinat C., Patumi M., Emanuele M.C., Rossi E. and Segre A. (2000). Concentration dependence of ^{13}C NMR spectra of triglycerides: implications for the NMR analysis of olive oils. *Magn. Reson. Chem.,* 38: 886-890.

Mannina L., Patumi M., Proietti N., Bassi D. and Segre A.L. (2001a). Geographical Characterization of Italian Extra Virgin Olive Oils Using High-Field ^{1}H NMR Spectroscopy. *J. Agr. Food Chem.* 49, 2687-2696.

Mannina L., Fontanazza G., Patumi M., Ansanelli G. and Segre A.L. (2001b). Italian and Argentine olive oils: a NMR and gas chromatographic study. *Grasas y Aceites*, 52: 380-388.

Mannina, L. and Segre, A. (2002) High resolution nuclear magnetic resonance: from chemical structure to foodauthenticity. *Grasas y Aceites*, 53: 22-33.

Mannina L, Calcagni C, Rossi E, Segre A (2003a) Review about olive oil characterization using high-field nuclear magnetic resonance. *Annali di Chimica* by Società Chimica Italiana 93: 97-103

Mannina L., Sobolev A. and Segre A. (2003b). Olive oil as seen by NMR and chemometrics. *Spectroscopy Europe* 15: 6-14

Mannina L, D'Imperio M., Lava R., Schievano E. and Mammi S. (2005). Caratterizzazione NMR e analisi statistica di oli di oliva DOP veneti. *Riv. Ital. Sostanze Grasse*. 82, 59-63.

Marini F., Balestrieri F., Bucci R., Magrì A.D., Magrì A.L., Marini D. (2004). Supervised pattern recognition to authenticate Italian extra virgin olive oil varieties. *Chemometrics and Intelligent Laboratory Systems*. 73, 85– 93

Márquez-Ruiz G., Tasioula-Margari M., Dobarganes M.C. (1995). Quantitation and distribution of altered fatty acids in frying fats. J. *Am. Oil Chem. Soc*. 72, 1171-1176.

Marquez-Ruiz G., Dobarganes M.C. (1996). Short-chain fatty acid formation during thermoxidation and frying. *J. Sci. Food Agric.*, 70: 120–126.

Medina I., Sacchi R. (1994). Acyl stereospecific analysis of tuna phospholipids via high resolution 13C-NMR spectroscopy. *Chem. Phys. Lipids*, 70: 53-61.

Montedoro, G.F.; Servili, M. (1992). I parametri di qualita` dell'olio di oliva ed i fattori agronomici e tecnologici che li condizionano. *Riv. Ital. Sostanze Grasse*. 69, 563-568.

Montedoro G.F., Servili M., Baldioli M., Selvaggini R., Miniati E., Macchioni A. (1993). Simple and hydrolyzable phenolic compounds in olive oil. 3. Spectroscopic characterizations of the secoiridoid derivatives. *J. Agric. Food Chem.* 41: 2228-2234.

Molkentin J. and Precht D. (1997). Representative determination of the butyric acid content in European milk fats. *Milchwissenschaft*, 52**:** 82-85.

Molkentin J. and Precht D. (1998). Comparison of gas chromatographic methods for analysis of butyric acid in milk fat and fats containing milk fat. *Z Lebensm Unters Forsch A* , 206: 213-216

Molkentin J. and Precht D. (2000). Validation of a gas-chromatographic method for the determination of milk fat contents in mixed fats by butyric acid analysis. *Eur. J. Lipid Sci. Technol.,* 194–201.

Muuse, B. and Martens, R. (1993). Mixtures of milk fat with non-milk fat - Determination of the milk fat content. *Bulletin of the IDF*, 285: 65-69

Neff W.E., Frankel E.N., Fujimoto K. (1988). Autoxidative dimerization of methyl linolenate and its monohydroperoxides, hydroperoxy epidioxides and dihydroperoxides. *J. Am. Oil Chem. Soc.* 65: 616-623.

Ng S (1983). High resolution ^{13}C NMR spectra of the carbonyl carbons of the triglycerides of palm oil. *J. Chem. Soc. Chem. Commun*. 179.

Ng, S., & Ng, W.L. (1983). ^{13}C NMR spectroscopic analysis of the fatty acid composition of palm oil. *J. Am. Oil Chem. Soc*. 60: 1266-1268.

Ng S (1985). Analysis of positional distribution of fatty acids in palm oil by ^{13}C-NMR spectroscopy. *Lipids* 20: 778.

Ng S (2000). Quantitative Analysis of Partial Acylglycerols and Free Fatty Acids in Palm Oil by ^{13}C Nuclear Magnetic Resonance Spectroscopy. *J. Am. Oil Chem. Soc.*, 77: 749-755

Ögütçü M., Mendes M. ˛ Yilmaz E. (2008). Sensorial and Physico-Chemical Characterization of Virgin Olive Oils Produced in Çanakkale. *J Am Oil Chem Soc.*, 85:441–456.

Petrakis P.V., Agiomyrgianaki A., Christophoridou S., Spyros A. and Dais P. (2008). Geographical Characterization of Greek Virgin Olive Oils (Cv. Koroneiki) Using ^{1}H and ^{31}P NMR Fingerprinting with Canonical Discriminant Analysis and Classification Binary Trees. *J. Agric. Food Chem.*, 56: 3200-3207.

Pfeffer P.E., Luddy F.E. and Unruh J. (1977a). Analytical ^{13}C-NMR: A rapid, nondestructive method for determining the *cis*, *trans* composition of catalytically treated unsaturated lipid mixtures. *J. Am. Oil Chem. Soc.* 54:380.

Pfeffer, P.E., Sampugna, J., Schwartz, D. P., & Shoolery, J. N. (1977b). Analytical ^{13}C NMR: detection, quantitation, and positional analysis of butyrate in butter oil. *Lipids*, 12: 869-871.

Pocklington W. D. and Hautfenne A. (1986). Determination of butyric acid in fats containing butterfat. Results of a collaborative study and the standardised method. *Pure & AppL Chem.*, 58: 1419-1428.

Reid L.M., O'Donnell C.P. and Downey G. (2006). Recent technological advances for the determination of food authenticity. *Trends in Food Sci. & Tech*. 17: 344–353.

Rezzi S., Axelson D.E., Héberger K., Reniero F., Mariani C., Guillou C. (2005). Classification of olive oils using high throughput flow ^{1}H NMR fingerprinting with principal component analysis, linear discriminant analysis and probabilistic neural networks. *Ana. Chim. Acta*, 552: 13-24.

Sacchi R., Addeo F., Giudicianni I., Paolillo L. (1989). La spettroscopia di risonanza magnetica nucleare nell'analisi degli oli di oliva. *Riv. Ital. Sost. Grasse,* 56: 171-178.

Sacchi R., Addeo F., Giudicianni I., Paolillo L. (1990). Applicazione della spettroscopia ^{13}C-NMR alla determinazione di mono-digliceridi ed acidi grassi liberi nell'olio di oliva di pressione e nei rettificati. *Riv. It. Sost. Grasse,* 57: 245-252.

Sacchi R., Paolillo L., Giudicianni I., Addeo F. (1991). Rapid ^{1}H-NMR determination of 1,2 and 1,3 diglycerides in virgin olive oils. *Italian Journal of Food Science* 3 (4), 253-262.

Sacchi R., Addeo F, Giudicianni I and Paolillo L. (1992). Analysis of the positional distribution of fatty acids in olive oil triacylglycerols by high resolution ^{13}C-NMR of the carbonyl region. *Ital. J. Food Sci*. 4: 117-123.

Sacchi R., Medina I., Aubourg S.P., Addeo F., Paolillo L. (1993a). Proton nuclear magnetic resonance rapid and structurespecific determination of ω-3 polyunsaturated fatty acids in fish lipids. *J. Am Oil Chem Soc.,* 70: 225-228.

Sacchi R, Medina I, Aubourg S, Addeo F, Giudicianni I and Paolillo L. (1993b). Quantitative high resolution ^{13}C-NMR analysis of lipids extracted from the white muscle of Atlantic tuna. *J. Agr. Food Chem*. 41: 1247-1251.

Sacchi R, Medina I, Aubourg S, Paolillo L. Addeo F (1994). High-resolution ^{13}C-NMR olefinic spectra of DHA and EPA acids, methyl esters and triacylglycerols. *Chem. Phys. Lipids*, 69: 65-73.

Sacchi R, Addeo F, Spagna Musso S, Paolillo L and Giudicianni I (1995). High resolution ^{13}C-NMR study of vegetable margarines. *Italian J. of Food Sci*. 7: 27-36.

Sacchi R, Patumi M, Fontanazza G, Barone P, Fiordiponti P, Mannina L, Rossi E and Segre AL (1996). A high field [1]H-Nuclear Magnetic Resonance study of the minor components in virgin olive oils. *J. Am. Oil Chem. Soc*., 73: 747-758.

Sacchi R, Addeo F, Paolillo L (1997). ^{1}H and ^{13}C-NMR of virgin olive oil. An Overview. *Magn. Res. Chem.,* 35: 133-145

Sacchi R, Mannina L, Fiordiponti P. Barone P. Paolillo L. Patumi M and Segre AL (1998). Characterization of Italian Extra Virgin Olive Oils using ^{1}H-NMR spectroscopy. *J. Agr. Food Chem*. 46: 3947-3951.

Sacchi R., Paduano A., Fiore F., Della Medaglia D., Ambrosino Ml., Medina I. (2002). Partition behavior of virgin olive oil phenolic compounds in oil-brine mixtures during thermal processing for fish canning. *J. Agric. Food Chem*. 50: 2830-2835.

Sacchi R., Falcigno L., Paduano A., Ambrosino M.L., Savarese M., De Giulio B., Addeo F. and Paolillo L. (2006a). Quantitative evaluation of the aldehydes formed in heated

vegetable oils using high resolution proton-NMR spectroscopy. *Riv. Ital. Sost. Grasse*. 83: 257-263.

Sacchi R, Savarese M, Falcigno L, Giudicianni I, Paolillo L. (2006b). Proton NMR of fish oils and lipids. In Handbook of Modern magnetic Resonance, marine Science, Volume II. Edited by G.H. Webb, Kluwer Academic Publishers (UK).

Sacchi R. and Paolillo L. (2007). NMR for food quality and Traceability. In Advances in Food Diagnostics, Edited by Leo M.L. Nollet and Fidel Toldrá, Blackwell Publishing. 1st edition, pp 101-118.

Sacco A., Brescia M.A., Liuzzi V., Reniero F., Guillou C., Ghelli S., van der Meer P (2000). Characterization of Italian olive oils based on analytical and nuclear magnetic resonance determinations. *J. Am. Oil Chem. Soc*. 77: 619-625.

Scano P., Rosa A., Marincola F.C., Locci E., Melis M.P., Dessì M.A., Lai A. (2008). ^{13}C NMR, GC and HPLC characterization of lipid components of the salted and dried mullet (*Mugil cephalus*) roe "bottarga". *Chem. Phys. Lipids*, 151: 69-76.

Scarpa P., Ambrosino M.L., Paduano, Della Medaglia D., Amato S., Sacchi R. (2005). Contributo della composizione in acidi grassi alla determinazione dell'origine geografica degli oli extravergini di oliva DOP italiani. *Annali della Facoltà di Agraria della Università di Napoli Federico II*, Serie quinta, 1: 115-125.

Selke E., Rohwedder W. K. and Dutton H. J. (1977). Volatile components from triolein heated in air. *J. Am. Oil Chem. Soc*. 54:62.

Selke E., Rohwedder W. K. and Dutton H. J. (1980). Volatile components from trilinolein in air. *J. Am. Oil Chem. Soc*. 57:25.

Shoolery J.N. (1977). Some quantitative applications of ^{13}C NMR spectroscopy. *Progress NMR Spec.*, 11:79-93.

Silwood C.J.L. and Grootveld M. (1999). Application of High-Resolution, Two-Dimensional ^{1}H and [13]C Nuclear Magnetic Resonance Techniques to the Characterization of Lipid Oxidation Products in Autoxidized Linoleoyl/Linolenoylglycerols. *Lipids*, 34: 741-756.

Simova S., Ivanova G., Spassov S.L. (2003). Alternative NMR method for quantitative determination of acyl positional distribution in triacylglycerols and related compounds. *Chem. Phys. Lipids*, 126: 167-176.

Sobolev A.P., Segre AL and Lamanna R. (2003). Proton high-field NMR study of tomato juice. *Magn. Reson. Chem*. 41: 237–245

Solinas, M. (1987). HRGC analysis of virgin olive oil phenolic compounds in relation to olive ripening degree and variety. *Riv. Ital. Sostanze Grasse*. 64: 255-262.

Solinas M.; Marsilio V.; Angerosa F. (1987). Behaviour of some components of virgin olive oil flavour in relation to olive ripening. *Riv. Ital. Sostanze Grasse*. 64: 475-480.

Solinas M., Angerosa F., Camera L. (1988). Evoluzione ossidativa di oli vegetali durante la frittura: determinazione dei componenti volatili mediante HRGC e HPLC. *Riv. Ital. Sostanze Grasse*. 65: 567-574.

Spitzer V. (1999). Reviews. Screening analysis of unknown seed oils. *Fett/Lipid,* 101: 2-19.

Tapp H.S., Defernez M. and Kemsley E.K. (2003). FTIR Spectroscopy and Multivariate Analysis Can Distinguish the Geographic Origin of Extra Virgin Olive Oils. *J. Agric. Food Chem.,* 51: 6110-6115.

Tonolo G., Marzo S. (1989). Determinazione della vitamina E aggiunta e dei tocoferoli naturali negli oli di semi dietetici via HPLC. *Riv. Ital. Sostanze Grasse* 66: 3-6

Tsimidou, M.; Macrae, R.; Wilson, I. (1987). Authentication of virgin olive oils using principal components analysis of triglyceride and fatty acid profiles: part 1. Classification of Greek olive oils. *Food Chem.,* 25, 227-239.

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

Tyl C.E., Brecker L., Wagner K.-H. (2008). Research Paper. ^{1}H NMR spectroscopy as tool to follow changes in the fatty acids of fish oils. *Eur. J. Lipid Sci. Technol.,* 110: 141-148.

Ulberth F. (1995). Quantitation of foreign fat in foreign fat/milkfat mixtures by multivariate regression analysis of fatty acis data. *J. Agric. Food Chem.* 43: 1556-1560.

Ulberth F. (1998). A rapid headspace gas chromatographic method for the determination of the butyric acid content in edible fats. *Z Lebensm Unters Forsch A,* 206: 305-307.

Van Calsteren, M.R., Barr C., Angers P. and Arul J. (1996). ^{13}C NMR of triglycerydes. *Bull. Magn. Reson.* 18:175-177.

Vatèle J.-M., Fenet B., Eynard T. (1998). Complete ^{13}C assignments and structural elucidation of n-3 polyunsaturated fatty acids by the use of a new 2D NMR technique: SAPHIR-HSQC. *Chem. Phys. Lipids,* 94: 239-250.

Veazey R.L. (1986). Rapid analysis of dimer acid by HPLC/FID. *J. Am. Oil Chem. Soc.* 63: 1043-1046.

Vichi S., Pizzale L., Conte L.S., Buxaderas S. and López-Tamames E. (2003). Solid-Phase Microextraction in the Analysis of Virgin Olive Oil Volatile Fraction: Characterization of Virgin Olive Oils from wo Distinct Geographical Areas of Northern Italy. *J. Agric. Food Chem.,* 51: 6572-6577

Vlahov G. (1999). Application of NMR to the study of olive oils. *Progr. Nuclear Magn. Res. Spectr.,* 35:341–357.

Vlahov, G., Shaw, A.D., and Kell, D.B. (1999). Use of 13C nuclear magnetic resonance distortionless enhancement by polatization tranfer pulse sequence and multivariate analysis to discriminate olive oil cultivars. *J. Am. Oil Chem. Soc.,* 76: 1223-1231.

Vlahov G, Schiavone C and Simone N (2001). Quantitative ^{13}CNMR method using the DEPT pulse sequence for the determination of the geographical origin (DOP) of olive oils. *Magn. Reson. Chem.* 39: 689–695.

Vlahov G. (2006). Determination of the 1,3- and 2-positioinal distribution of fatty acids in olive oil triacylglycerols by ^{13}C Nuclear Magnetic Resonances Spectroscopy. *J. AOAC Int.,* 89: 1071-1076.

Walking Al., Wessels M. (1981). Chromatografic separation of polar and non-polar components of frying fats. *J. Assoc. Off. Anal. Chem.*, 62: 1329-1330.

Warner K. (2002). Chemistry of Frying Oils. In: Food Lipids - Chemistry, Nutrition, and Biotechnology. Press by Marcel Dekker, Inc., New York (US).

White P.J., Wang Y. (1986). A high performance size-exclusion chromatographic method for evaluating heated oils. *J. Am. Oil Chem. Soc.* 63: 914-920.

Wollenberg K.F. (1990). Quantitative high resolution ^{13}C nuclear magnetic resonance of the olefinic and carbonyl carbons of edible vegetable oils. *J. Am. Oil Chem. Soc*. 67: 487-494.

Zhanga G., Nia Y., Churchillc J. and Kokotc S. (2006) Authentication of vegetable oils on the basis of their physico-chemical properties with the aid of chemometrics. *Talanta*, 70: 293-300.