Application of NMR to the study of olive oils

G. Vlahov*

Istituto Sperimentale per la Elaiotecnica, Contrada Fonte Umano 65013 Città S. Angelo, Pescara, Italy

Received 12 July 1999

Contents

1. Introduction .......................................................... 341
   1.1. Aims of the review ........................................... 341
   1.2. Proton and carbon-13 NMR spectroscopy ................. 342
2. 1H NMR spectroscopy of olive oil .............................. 343
   2.1. 1H NMR spectroscopy of glycerides of olive oil ........ 343
   2.2. 1H NMR spectroscopy of volatile compounds of olive oil 344
3. 13C NMR spectroscopy of olive oil ............................ 345
   3.1. 13C NMR spectroscopy of the triglyceride fraction of olive oil 345
       3.1.1. Carbonyl carbon region 172–174 ppm ............... 346
       3.1.2. Olefinic carbon region 124–134 ppm ............ 349
       3.1.3. Glycerol carbon region 60–72 ppm .......... 350
       3.1.4. Methylenic and methyl carbon region 10–35 ppm .... 352
       3.1.5. The whole 13C NMR spectrum 10–174 ppm of the triglyceride fraction of olive oil ... 353
   3.2. 13C NMR spectroscopy of the unsaponifiable matter of olive oil ... 354
References ............................................................ 356

Keywords: 1H; 13C; NMR; Olive; Oil

1. Introduction

1.1. Aims of the review

Virgin olive oil, according to the trade standard of International Olive Oil Council for olive oils and olive–pomace oils, is the oil obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions, particularly thermal, that do not lead to alterations in the oil, and which has not undergone any treatment other than washing, decanting, centrifuging and filtration [1].

Research efforts in the field of olive oil chemistry over the years, have been concerned with the establishment of analytical methodologies for detecting quality, adulteration and geographical origin of olive oils.

The traditional analytical chemistry of olive oil includes the determination of acidity and UV absorption to define olive oil quality, fatty acid and sterol compositions along with the determination of ECN 42 triglyceride class (the equivalent carbon number ECN of a triglyceride is defined as the carbon number...
minus 2 times the number of double bonds of the acyl chains) to detect adulteration with seed oils, saturated fatty acids in the 2-position of triglycerides to detect esterified olive oils, triterpene diols with wax esters to reveal the presence of olive–pomace oil and stigmastadiene in processed olive oils [2].

Spectroscopic methods combined with computer aided statistical and mathematical procedures are the emerging analytical techniques in the field of olive oil chemistry. A great deal of experimental work is in progress to check the availability of these new techniques for detecting adulteration and determining the geographical origin of olive oils, because they move away from the time-consuming procedures based on chemical and physical data [3].

Nuclear magnetic resonance spectroscopy has played an ever-increasing role in the study of properties of oils of vegetable origin [4–8] during the last 10 years, and this review is aimed at proposing the NMR spectroscopy as a new analytical tool for studying olive oil chemistry.

1.2. Proton and carbon-13 NMR spectroscopy

The NMR spectroscopy of proton nuclei, with nuclear spin quantum number $I = \frac{1}{2}$, has the advantages of operating with a “concentrated spin” given the high gyromagnetic ratio which is four times $(26.75 \times 10^7 \text{ rad T}^{-1} \text{ s}^{-1})$ that of carbon-13 $(6.73 \times 10^7 \text{ rad T}^{-1} \text{ s}^{-1})$, and the natural abundance of 99.985% [9]. These intrinsic characteristics reduce the signal averaging time to just that necessary to acquire a few scans.

The $^{13}$C nucleus with $I = \frac{1}{2}$, has become second
in importance to the proton in spite of its low gyromagnetic ratio and its very low natural abundance (1.1%) which make this nucleus a “diluted spin”. Signal averaging and Fourier transform methods have made natural abundance $^{13}$C experiments relatively routine. In the natural abundance spectrum, no $^{13}$C–$^{13}$C couplings complicate the spectrum appearance and the $^{13}$C–$^1$H couplings can be removed by proton decoupling thus making the $^{13}$C spectra easily assignable.

Moreover, the chemical shift range of the carbon-$^{13}$ nucleus is large compared to the proton range, ≈200 and ≈10 ppm, respectively. Chemical shifts are determined by a diamagnetic $\sigma_D$ and a paramagnetic $\sigma_P$ shielding contribution. The diamagnetic term of the order of few ppm, predominates for atoms such as hydrogen with only s electrons. These are distributed with spherical symmetry around the nucleus and under the influence of the applied field $B^0$, produce a resultant field acting in opposition to $B^0$.

The paramagnetic term predominates for nuclei such as carbon-$^{13}$ with the presence of p-electrons. They are not spherically distributed and under the influence of the $B^0$ field, their circulation generates high anisotropic magnetic fields, which reinforce the $B^0$ field. The paramagnetic shielding contribution which increases with a decreasing electronic excitation energy, [10] is larger than $\sigma_D$ and covers a range of hundreds of ppm according to the electronic configuration of different nuclei [11].

2. $^1$H NMR spectroscopy of olive oil

2.1. $^1$H NMR spectroscopy of glycerides of olive oil

The $^1$H spectrum of an olive oil sample is shown in Fig. 1. The proton resonances of the triacylglycerol acyl chains (the structures of the major fatty acids of olive oil and the basic structure of triacylglycerols are shown in Fig. 2) were assigned according to the literature data [12,13]. The olefinic protons –$\text{CH}=\text{CH}$– of unsaturated fatty acids resonate at 5.2–5.5 ppm (m), the multiplet is not baseline resolved from the proton signal at 5.26 ppm (m) which was assigned to H-2 of the glycerol backbone. The H-1 and H-3 protons of glycerol resonate at 4.1 and 4.3 ppm (J = 6 Hz, dd), the assignments are interchangeable. The protons of bis-allylic and allylic methylenes of

\begin{align*}
(1) & \quad \text{CH}_3(CH_2)_n\text{COOH} \quad n = 14,16 \\
(2) & \quad \text{CH}_3(CH_2)_7-\text{CH}=\text{CH}-(CH_2)_7\text{COOH} \\
(3) & \quad \text{CH}_3(CH_2)_7-\text{CH}=\text{CH}-\text{CH}=\text{CH}-(CH_2)_7\text{COOH} \\
(4) & \quad \text{CH}_3CH_2-\text{CH}=\text{CH}-\text{CH}_2CH_2-\text{CH}=\text{CH}-(CH_2)_7\text{COOH}
\end{align*}
polyunsaturated and unsaturated acyl chains appear at 2.78 (m) and 2.05 ppm (m), respectively. The H-2 and H-3 protons of acyl moieties in triacylglycerols resonate at 2.30 and 1.6 ppm, respectively, and the protons of methylene envelope appear at 1.2 ppm. The methyl protons of n-3 polyunsaturated acids are shifted at higher frequency (0.91 ppm, J = 7 Hz, t) from the terminal methyl protons of saturated and unsaturated chains (0.88 ppm, t).

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 (δ = 1.25) and for a methyl group (δ = 0.88), the α- to ζ- substituent effects of the functional groups. A good correspondence was found between predicted and experimental values [14].

The configuration of the double bond protons could be determined by the coupling constant of methylene protons which is always larger for trans than for cis bonds, even if this is not possible in practice because the chemical shift differences of methylene protons of isolated double bonds are small and produce a signal envelope that is very difficult to analyse. The double bond configuration influences the absorptions of allylic methylene protons. In particular, the methylene protons adjacent to a cis double bond exhibit a resonance at 1.99 ppm sharper than that produced by a trans double bond at 1.94 ppm [15].

Moreover, 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. Only one signal appears for the glycerol protons in 1,3-diglycerides [16].

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′-CH2 of 1,3-diglycerides shift to higher frequency. As a consequence, they appear better resolved from the triacylglycerol resonances [17]. 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.

The fatty acid compositions of triacylglycerols of different vegetable oils comprising olive oil, have also been determined by 1H NMR spectroscopy. The integrated areas of the olefinic region of the 1H spectra of the oils being studied, were found to be linearly correlated with the iodine values which were obtained by the Wijs method, and NMR was proposed as a new method for determining the degree of unsaturation of vegetable oils in place of the iodine value of triglycerides. 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 [18].

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 [19]. 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 [19].

2.2. 1H NMR spectroscopy of volatile compounds of olive oil

The oxidation of unsaturated fatty acids by
Oxygen, generally known as autoxidation, is important in the development of rancidity and “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. They are important because, even if present in trace amounts, their strong flavours are responsible for the characteristic flavours, desirable and undesirable, of fat-containing foods [20].

The volatile compounds of virgin olive oils have been studied by high field (600 MHz) NMR spectroscopy [19]. 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 extra virgin olive oils, resonates at 9.46 ppm as a doublet \( J = 7.96 \text{ Hz} \). The singlet at 8.07 ppm was tentatively assigned to a volatile compound with an hemiacetalic group. The signals in the frequency range between 4.5 and 5.0 ppm were attributed to unsaturated alcohols. Statistical analysis of the intensities of proton resonances of volatile compounds proved that actual cultivar identification can be achieved and it also confirmed that the oil volatile compounds and the olive fruit ripening degree are closely correlated [19].

The \(^1\text{H}\) NMR spectroscopy at high field (600 MHz) was confirmed to be suitable to classify olive oils from different olive fruit cultivars grown in four Italian regions, by variety and geographical origin with a 96% of oils classified correctly. The study was carried out by the multivariate statistical analysis of the proton resonance intensities of \(\beta\)-sitosterol, \(n\)-alkanals, trans-2-alkenals and other volatile compounds [21].

3. \(^{13}\text{C}\) NMR spectroscopy of olive oil

3.1. \(^{13}\text{C}\) NMR spectroscopy of the triglyceride fraction of olive oil

The saponifiable matter is not less than 98% of olive oil. It is made up of very complex mixtures of triglycerides which, being esterified with different fatty acids distributed among the three glycerol positions, give rise to compositional, positional and optical triacylglycerol isomers (Fig. 2). Olive oil contains about 0.5–1.5% of unsaponifiable matter with nonoil components which consist mainly of the hydrocarbon squalene (0.1–0.7%), sterols (0.2%), tocopherols and other hydrocarbons and pigments [22]. \(^{13}\text{C}\) NMR
spectroscopy has been applied to study both, the saponifiable and unsaponifiable fractions of olive oil. The $^{13}$C NMR spectrum of an olive oil sample contains the resonances of carbons from the triglyceride fraction of olive oil, i.e. the fatty acid resonances. The carbon-13 resonances are grouped in four sets of signals, carbonyl carbons resonating from 172 to 174 ppm, unsaturated carbons in the range from 124 to 134 ppm, glycerol backbone carbons from 60 to 72 ppm and aliphatic carbons from 10 to 35 ppm.

### 3.1.1. Carbonyl carbon region 172–174 ppm

The carbonyl carbons of fatty acids of olive oil triglycerides appear as two sets of resonances, the high frequency set includes the chains esterified at 1(3)-glycerol positions, whereas the low frequency set includes the 2-glycerol position chains (Fig. 3). In particular, the signals from carbonyl carbons of 1(3)-chains are shifted by about 0.42 ppm at higher frequency from those of carbonyls of 2-chains. This shift difference, which is consistently detected for all chains, was explained by noting that C=O groups of 2-position chains experience two $\gamma$–gauche interactions against just one interaction intervening for carbonyls of 1(3)-chains [23].

Within each set of signals, the saturated, oleyl and linoleyl chains appear from higher to lower frequency in that order, according to the assignments based on synthetic models of triacylglycerols [24,25]. Signals from linoleyl and linolenyl chains overlap in both sets of resonances.

The carbonyl carbon resonances are resolved on the basis of the number of unsaturation centres. They were explained by noting that the effect of a multiple bond dipole in non-conjugated polyenoic acids is transmitted, according to the $\sigma$-inductive theory, through the C–C bonds up to the polarisation of the $\pi$ electrons of C=O group [23]. Therefore, no chain differentiation can be achieved on the basis of the sole carbon number. All the saturated chains of olive oil, of which palmitic (C16:0) and stearic (C18:0) chains are the major components with 7.0–17.0 and 1.5–4.0%, respectively [26], are comprised under the label “saturated”.

High resolution $^{13}$C NMR spectroscopy of triglyceride carbonyl carbons has enabled the analysis of the positional distribution of fatty acids in triacylglycerols. It was introduced as a new analytical method to detect the adulteration of virgin olive oils with synthetic esterified oils [27]. In these oils, which show a random distribution of fatty acids among glycerol positions (a random distribution predicts that 33.3% of each fatty acid enters the 2-position), the percentages of saturated chains at the 2-position are higher than the maximum value of 1.3% found for an extra virgin olive oil [26] where the saturated chains are esterified predominantly at the 1(3)-positions [28].

The carbonyl carbon resonances were used to study the molecular structure of triglycerides of monovarietal olive oils [29]. The integrated carbonyl resonances of saturated, oleyl and linoleyl chains were used to carry out the regiospecific analysis of triglycerides by calculating the chain composition of the total triglyceride, the chain distribution between the 1(3)- and 2-glycerol positions and the chain 2-position specificity.

In order to validate carbon-13 NMR spectroscopy as a rigorously quantitative methodology, the spectra have to be measured without signal intensity distortions. Signal intensities are directly proportional to nucleus concentrations and the proportionality constant is the same for all the resonances [30]. However, this constant can be made different for the lines by signal saturation that occurs when repetition time of the pulse sequence is shorter than five times the longest longitudinal relaxation time $T_1$ and by nuclear Overhauser enhancement which, occurring upon proton decoupling of carbon-13 nuclei through the mechanism of dipole–dipole relaxation, can affect the intensities of carbon-13 resonances to different extents. The spectra were measured with suppressed intensity distortions.

<table>
<thead>
<tr>
<th>Carbon atom</th>
<th>$\delta$ (ppm)$^a$</th>
<th>NOE (1 + $\eta$)</th>
<th>$T_1$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C$_1$ sn-1(3)-</td>
<td>173.10</td>
<td>1.77</td>
<td>5.6</td>
</tr>
<tr>
<td>Tripalmitin</td>
<td>173.07</td>
<td>1.78</td>
<td>5.6</td>
</tr>
<tr>
<td>Tripalmolin</td>
<td>173.06</td>
<td>1.73</td>
<td>5.4</td>
</tr>
<tr>
<td>C$_1$ sn-2-</td>
<td>172.70</td>
<td>1.74</td>
<td>3.9</td>
</tr>
<tr>
<td>Triolein</td>
<td>172.67</td>
<td>1.67</td>
<td>4.5</td>
</tr>
<tr>
<td>Trilinolein</td>
<td>172.66</td>
<td>1.68</td>
<td>4.5</td>
</tr>
</tbody>
</table>

$^a$ Chemical shift are referenced to TMS = 0 ppm.
NOE by using the inverse-gated proton-decoupled sequence, which gated the decoupler on only for the acquisition time [31]. As a result, the actions of increasing repetition time and suppressing NOE decrease the signal-to-noise ratio per unit time.

However, the spectrum sensitivity was improved by measuring the proton-decoupled spectra with full NOE after demonstrating that proton decoupling affected the carbonyl carbon intensities to the same extent [32]. The $^{13}\text{C} \{^1\text{H}\}$ NOE factors $(1 + \eta)$ were
calculated from the ratio of the integrated intensities of carbonyl resonances measured with full NOE, with the decoupler turned on before and during acquisition, and with suppressed NOE, with the decoupler turned on only during acquisition. The results are reported in Table 1. The spectrum digitisation of 0.034 Hz/point, which was obtained by acquiring the frequency range of carbonyl carbons only (2200 Hz) using 64 K points zero-filled to 128 K points, assured the integration accuracy. The carbonyl resonances of saturated and oleyl chains were determined with a coefficient of variation less than 2%, which increased up to 3.5% for the linoleyl chain producing a signal of lower intensity.

This methodology was used to carry out the regio-specific analysis of triacylglycerols of the oils extracted from mesocarp and seed parts of olive fruits of different cultivars [33]. The saturated chains was found to be confined to 1(3)-positions, thus confirming that two different pools of fatty acids entered the 1(3)- and 2-positions in triacylgllycerols. The distribution of oleyl and linoleyl chains between 1(3)- and 2-glycerol positions appeared to be regulated by the chain concentration of the whole triglyceride.

The 2-position specificities of oleyl and linoleyl chains were higher than 33.3% value that is expected if the chains are distributed randomly among the 1,3- and 2-glycerol positions. However, the averaged values which were for oleyl chain in mesocarp and seed oils 38 and 36%, respectively, and 48 and 44% for the linoleyl chain demonstrated that the oleyl chain moved away from a pure random distribution model less than the linoleyl chain.

Fig. 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.
The trends observed for 1(3)- and 2-distribution data of oleyl and linoleyl chains which were linearly correlated with the chain concentration of the whole triglyceride, and for 2-position specificities which appeared to be a characteristic of the chain, are shared with other vegetable oils. The oils containing both C-18 unsaturated fatty acids and saturated chains at levels lower than 25%, were selected. Fig. 4 shows the regression lines of 2-distribution data of oleyl and linoleyl chains against the chain compositions of the whole triglyceride of 7 olive oils from different cultivars, of 5 monovarietal olive oils, and of 24 vegetable oils from different sources.

3.1.2. Olefinic carbon region 124–134 ppm

The olefinic carbon region was studied in several edible vegetable oils and in high oleic sunflower oil whose fatty acid composition is close to that of olive oil [24]. The unsaturated carbon resonances, which were assigned according to chemical shifts of standard triacylglycerols, spread over a range of frequencies wider than that of carbonyl carbons. The C=C spectral region of olive oil along with the shift assignments, is reported in Fig. 5. The olive oil was enriched with soybean oil in order to show the resonances of linolenic acid that is present at undetectable levels in the olive oil.

The unsaturated carbons were resolved according to double bond number and chain position on glycerol backbone thus confirming, as for C=O carbons, the usefulness of $^{13}$C NMR for carrying out the regio-specific analysis of triacylglycerol mixtures. Considering that the C=O shift range is the sole region which enables the detection of saturated chain, and that linoleyl and linolenyl chains can be easily detected only in the C=C region, the carbon resonances of both C=O and C=C frequency ranges allows the full regiospecific analysis of natural triacylglycerols.

The nonequivalence of chemical shifts of olefinic carbons has been studied in a series of monounsaturated fatty acids with different number of C–C bonds separating the ester group and the double bond. The relevant feature of these chemical shifts is that the carbons of a double bond pair are shifted in opposite directions, i.e. the unsaturated carbon nearest to the chain ester group is shifted to lower frequency from the unsaturated carbon closest to the chain methyl end. The chemical shift differences which increase as the double bond moves towards the carboxy group, were proved to arise from a linear electric field effect [34]. The σ-inductive theory of transmission of the dipolar effect of a C=O bond and of one C=C bond upon another C=C bond operating through bonds in non-conjugated polyenoic acids, replaced the model with dipolar electric fields acting through space [23,35,36]. The theory allows the shift differences of a double bond pair to be predicted as a function of number of C–C bonds intervening between the dipolar group and the C=C bond.

Each unsaturated carbon is split according to the chain glycerol position. The carbon of a double bond which is closer to the carbonyl C=O is shifted, in a 2-position chain, at lower frequency from that of a 1(3)-position chain. The opposite trend is observed for the carbon of a double bond further from the carbonyl C=O. Moreover, the shift differences between the unsaturated carbons of 1(3)- and 2-position chains, respectively, become smaller on moving towards the methyl end. They decrease from +0.025 ppm for C-9 to −0.015 for C-10 of oleyl and linoleyl chains, to +0.012 for C-12 and to −0.08 for C-13 of the linoleyl chain. The shift differences of a double bond carbon pair are the same for different acyl chains, but no definitive explanation was given for this observation [37].

The unsaturated fatty acid compositions of different olive oil grades, i.e. virgin-olive oil–refined olive oil–olive–pomace oil, have been determined by $^{13}$C NMR spectroscopy. The data are in agreement with those obtained by gas-chromatography of fatty acid methyl esters except for the linoleic acid content which is higher than that obtained by GLC. This result was explained by noting that polyunsaturated fatty acids of triglycerides, which are removed by neutralization of olive oil sample before GLC, are more easily hydrolized as compared to saturated and mono-unsaturated chains [38].

The correspondence of the two techniques, i.e. $^{13}$C NMR and GLC, with differences of 1–2 mol% of fatty acids, was confirmed for fatty acids of virgin olive oils produced in different geographic areas of Greece [39].

The adulteration of olive oil with soybean oil was studied by a new quantitative $^{13}$C NMR methodology which applies DEPT (distortionless enhancement by
polarization transfer) pulse sequence. The DEPT sequence used was:

$$1^H: \gamma_1^{H} \times -1/2J_{CH} -180^\circ -1/2J_{CH} -45_{\pm x}^\circ$$

$$13^C: \gamma_1^{C} \times 90^\circ_{x} 180^\circ$$

The $^{13}$C spectrum has the protonated carbons CH, CH$_2$ and CH$_3$ of triglyceride fatty acids all as positive signals. The delay for polarization transfer, defined by $1/2J_{C-H}$, was optimized to 144 Hz which is the average C–H coupling measured experimentally [37].

The experiments of polarization transfer such as DEPT, enhance the intensities of carbon-13 resonances by transferring populations from proton nuclei to the lower sensitivity carbon-13 nuclei provided that the hydrogen and carbon nuclei are $J$-coupled. Moreover, the pulse repetition rate is determined from the relaxation times of proton nuclei which are substantially lower, the longest $T_1(1.51\, s)$ being that of methyl protons (Table 2). As a result, the sensitivity of the carbon-13 resonances increases and spectra can be obtained in considerably shorter experiment times. The sensitivity increased by a factor proportional to $\gamma_1^{H}/\gamma_1^{C}$, where $\gamma_1^{H}$ and $\gamma_1^{C}$ are the gyromagnetic ratios of proton and carbon-13 nuclei, respectively [40]. The DEPT pulse sequence which does not detect unprotonated carbons, i.e. carbonyl carbons (polarization transfer through weak long-range C–H couplings being more difficult to achieve) [31], enables the regiospecific analysis of the sole unsaturated chains.

This methodology has been employed to detect the adulteration of olive oil with soybean oil, having a high linoleic acid content. The resonance intensities of unsaturated carbons C-9 of oleyl chain, C-10 of linoleyl and linolenyl chains, at 1(3)- and 2-glycerol positions, were used to calculate the calibration graphs based on a linear relationship between resonance intensities and soybean oil concentration in olive oil. The limits of detection of soybean oil in olive oil are 6.9 and 8.3% for C-9 of oleyl chain at 1(3) and 2-position respectively, 5.9–6.8 and 10.2–20.2% for C-10 of linoleyl and linolenyl chains at 1(3) and 2-position, respectively. The results highlight the reliability of the DEPT pulse sequence as a quantitative method for detecting olive oil adulteration by a mixture with vegetable oils of different fatty acid profiles.

3.1.3. Glycerol carbon region 60–72 ppm

Glycerol esters have been studied by $^{13}$C NMR spectroscopy of glycerol carbons for a qualitative and semiquantitative estimation of their mixtures [41].

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. The 1(3)-glycerol carbons, CH$_2$OH and CH$_2$OCOR in asymmetric glycerol esters were not definitively assigned and may be interchanged.

The use of the DEPT pulse sequence which provides a means for selection of carbon-13 multiplicity [40], demonstrated that the resonances of the glycerol C-2 shifted in all glycerides to higher frequencies from those of the glycerol 1(3)-carbons.
The spectral region of glycerol carbons of a standard mixture of glycerides, is reported in Fig. 6 and the chemical shift assignments in Table 3. The shift data demonstrate that the length and degree of unsaturation of the acyl chains does not influence the chemical shifts of glycerol carbons.

The $^{13}$C NMR methodology based on glycerol carbon resonances, was applied to studies of mono and diacylglycerols in virgin and refined olive oils [42]. Quantitative measurements of the resonance intensities were assured by using appropriate relaxation delays and pulse widths which, depending on $T_1$ longitudinal relaxation times, enable the

**Table 3**

<table>
<thead>
<tr>
<th>Glycerol ester</th>
<th>1 CH$_2$</th>
<th>2 CH</th>
<th>3 CH$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Monopalmityl</td>
<td>63.34</td>
<td>70.26</td>
<td>65.11</td>
</tr>
<tr>
<td>1-Monooleyl</td>
<td>63.35</td>
<td>70.26</td>
<td>65.13</td>
</tr>
<tr>
<td>1,2-Dipalmityl</td>
<td>61.54</td>
<td>72.11</td>
<td>62.02</td>
</tr>
<tr>
<td>1,2-Oleoyl</td>
<td>61.56</td>
<td>72.12</td>
<td>62.00</td>
</tr>
<tr>
<td>1,3-Dipalmityl</td>
<td>65.04</td>
<td>68.38</td>
<td>65.04</td>
</tr>
<tr>
<td>1,3-Oleoyl</td>
<td>65.03</td>
<td>68.34</td>
<td>65.03</td>
</tr>
<tr>
<td>1,2,1,2,3-Tripalmitin</td>
<td>62.10</td>
<td>68.87</td>
<td>62.10</td>
</tr>
<tr>
<td>1,2,3-Trilinolein</td>
<td>62.10</td>
<td>68.88</td>
<td>62.10</td>
</tr>
<tr>
<td>1,2,3-Triolein</td>
<td>62.08</td>
<td>68.87</td>
<td>62.08</td>
</tr>
</tbody>
</table>
full recovery of magnetisation between subsequent pulses. The longitudinal relaxation times $T_1$ of 2- and 1(3)-glycerol carbons which were measured by the inversion-recovery pulse sequence, were found to be 0.27 and 0.30 s, respectively [42]. These results are in agreement with the $T_1$ data determined for dipalmityllecithin whose carbon nuclei relax increasingly slowly as their position increases from the central glycerol moiety ($T_1$ less than 0.2 s) to the methyl end ($T_1 = 3.0$ s). This $T_1$ pattern can be attributed to a less efficient relaxation process attributed to the chain mobility which constantly increases from the glycerol backbone along the chain [43].

Refined olive and olive–pomace oils can be clearly differentiated from extra virgin olive oils by a level of diacylglycerols higher than 7% (molar fraction) whereas the maximum content of diacylglycerols in virgin olive oils is lower than 3% (molar fraction) [42].

The glyceride fraction of olive oils of superior grade was studied by the quantitative $^{13}$C NMR methodology based on a linear relationship between glyceride concentrations and corresponding areas of glycerol C-2 resonance. The limits of detection which were determined after calculating the best straight lines by the least square method, were found to be 1.34, 2.21, 0.87 and 31.96 mg/0.5ml for 1-monooleyl, 1,2-dioleyl, 1,3-dioleyl and triolein, respectively [44].

The one-way analysis of variance (ANOVA) of the total diacylglycerol data of olive oils extracted from different cultivars, showed that significant ($P = 0.05$) differences occurred among the cultivars. In particular, the cultivars with lower total diacylglycerol contents can be classified as late cultivars whereas early maturing fruits produce higher levels of total diglycerides [45]. The ratios of 1,2- to 1,3-diacylglycerols vary considerably among cultivars, but do not exceed the range 0.5–1.6. Considering the Kennedy pathway which hypothesizes that plant tissues synthesize their triacylglycerols by acylation of diacylglycerols at position sn-3 [46], the presence of 1(3)-diacylglycerols may be the results of artifacts because of different factors such as olive storage time and extraction and purification methods, even if a possibility exists that some of the 1(3)-diacylglycerols may form during fruit maturation.

3.1.4. Methylene and methyl carbon region 10–35 ppm

The resonances of methylene and methyl carbons of saturated and unsaturated acyl chains are reported in Fig. 7.

The carboxylic group of fatty acids, whose electro-negative effect falls off rapidly with increasing chain length [47] shifts the resonances of α(C-2) and β(C-3) methylenes by $22 \pm 1$ and $3 \pm 1$ ppm, respectively. The effect of substituents such as the carboxylic group was evaluated by subtracting the shifts of the carbons of the hydrocarbons from the corresponding carbons of the substituted hydrocarbon, i.e. the fatty acid [10].

The C-2 and C-3 methylenes centred at 34.06 and 24.84 ppm, respectively, are resolved according to different chains and to glycerol position. The saturated, oleyl and linoleyl chains at C-2 of glycerol resonate in this order, at higher frequency from the same set of chains at 1(3)-positions. The resonances were assigned on the basis of $T_1$ measurements assuming that 2-chains have shorter $T_1$ value because of a greater steric restriction on their motion [3]. As previously reported for common C-18 acyl chains [48], the C-2 and C-3 resonances of 2- and 1(3)-position chains were confirmed to differ by 0.16 and 0.04 ppm, respectively.

The C-16–C-18 carbons, which were assigned using the additivity relationship which predicts the $^{13}$C chemical shifts of n-alkanes [49], appear at chemical shifts centred at 31.73, 22.63 and 14.06 ppm, respectively. Within each set of signals, saturated, oleyl and linoleyl chains resonate from higher to lower frequency in this order. However, an influence of the chain position on glycerol backbone was not detected.

The carbons of the methylene envelope, C-4–C-7 and C-12–C-15, were assigned by means of peak integrals and consideration of the trend of carbon-13 longitudinal relaxation times $T_1$ which, in long chain fatty acids, were found to increase regularly from the glycerol backbone up to methyl chain end [50].

The allylic methylene carbons of natural unsaturated fatty acids which are cis-isomers, resonate about 2.5 ppm to lower frequency of the unperturbed shift of central chain carbons (29 ppm) because they have a strong γ-steric interaction with each other, whereas the allylic methylenes of elaidic acid, the C18:1 trans-isomer, are shifted by 5.3 ppm to higher
frequency of those of cis-isomer [51]. However, two steric interactions with C-14 and C-8 carbons, cause the bis-allylic methylene C-11 of linoleyl chain to shift to lower frequency (25 ppm) [4].

The absence of trans-isomers is a property of extra virgin olive oils whereas olive and olive-pomace oils contain from 0.1 to 0.6% of trans-isomers as a consequence of the refining processes of bleaching and deodorizing. The methylene envelope region was used to check the ability of $^{13}$C NMR spectroscopy to detect trans-isomers of fatty acids in olive oil. The resonances of trans-allylic methylenes were measured on standard triacylglycerol solutions containing 1, 0.5 and 0.1% of trielaidate. The results demonstrated that 0.1% of trans-isomers can be detected at a $^{13}$C frequency of 100 MHz, after overnight signal averaging [52].

3.1.5. The whole $^{13}$C NMR spectrum 10–174 ppm of the triglyceride fraction of olive oil

Spectroscopic methodologies coupled with statistical methods of multivariate analysis are the emerging tools for detecting adulteration of olive oils with oils of different botanical origin and for discriminating oils by cultivars and geographical origins.

$^{13}$C nuclear magnetic resonance spectroscopy in combination with multivariate analysis has been successfully applied to differentiate olive oils by cultivar and geographical origin [53].

The whole carbon-13 spectrum of the triglyceride fraction of olive oil was measured. Carbon-13 nucleus is the nucleus of choice because of its electronic configuration which makes the chemical shifts spread over a wide range of frequencies, thus producing

Fig. 7. The methylenic and methyl carbon region 10–35 ppm of the 300 MHz $^{13}$C 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.
high-resolution spectra with the number of resonances considerably higher than that of $^1$H nucleus. Moreover, the low gyromagnetic ratio of $^{13}$C and its low natural abundance of 1.1%, suggested that the spectra of the most abundant fraction of olive oil, i.e. the triglyceride fraction, be measured to reduce the signal averaging time and produce a signal-to-noise ratio suitable for the integration accuracy.

This approach is based first on the assumption that all olive oil samples are composed of a common set of structural units which are the long-chain acids of triglycerides. As a result, the peaks appearing at the same chemical shifts in all the olive oil spectra were measured. Secondly, the oils differ only in relative amounts of components and the corresponding absolute resonance intensities are the quantitative parameters for the correlations of oils from the same cultivar and/or from the same geographical area. The conventional $^{13}$C NMR technique which carries out signal averaging to improve signal-to-noise ratio by using the basic one-pulse sequence, was used to measure the spectra of extra virgin olive oils from different cultivars sampled in different Italian regions [53].

As resonance intensity comparisons of different spectra are demanded, care was taken to eliminate any intensity distortions because of differences in nuclear Overhauser enhancements and signal saturation by using, respectively, the inverse-gated proton-decoupled pulse sequence and repetition rates not lower than five times the longest longitudinal relaxation time $T_1$.

The DEPT experiment was applied to obtain $^{13}$C NMR quantitative spectra over the whole carbon-13 frequency range [54] with the purpose of producing $^{13}$C NMR resonance intensity patterns for statistical interpretation which are more accurate and precise (considering that the DEPT pulse sequence had been validated as a quantitative methodology for studying the unsaturated carbon profile of triglyceride acyl chains) [37].

As a result, the spectrum sensitivity was substantially improved in an experiment time reduced by 50% as compared with that needed by the $^{13}$C NMR conventional methodology. The only drawback was the loss of carbonyl carbons resonances which are not detected by the DEPT sequence [31].

As the integration accuracy strongly depends on the definition of the spectrum baseline [31], it was necessary to divide the frequency ranges, keeping them constant in all olive oil samples, in order to carry out accurate phase adjustments. Moreover, as inadequate spectrum digitisation makes the integral calculation inaccurate, the digital resolution, i.e. the frequency spacing between data points, was improved by zero-filling the data before Fourier transformation. The spectra were resolution enhanced by narrowing the lines artificially. The Lorentz-to-Gauss transformation was applied to the FID considering that a Lorentzian line is five times wider at 1% of the peak amplitude than a Gaussian line with the same half-height linewidth [40].

The intensities of 35 resonances (Table 4) were measured using the software provided with the spectrometer. Whenever signals were poorly resolved, the strategy of integrating a group of resonances rather than single resonances was adopted. The resonance intensities from different olive oil spectra constitute the data set to be analyzed by statistical methods of multivariate analysis.

Multivariate calibration methods derive their power from the simultaneous use of multiple intensities, i.e. multiple variables, in each spectrum. The Principal Component Analysis (PCA), Principal Component Regression (PCR) and Partial Least Squares (PLS) were applied to quantitative data produced by conventional $^{13}$C NMR spectroscopy of six olive oil cultivars sampled in different Italian regions. The Variable Selection was performed by the methods of Fisher Ratio and the Ratio of Inner Variance to Outer Variance which improved the PCA clustering and the prediction results of PCR and PLS.

PCR2 and PLS2 predicted the variety or region with a 70% certainty whereas multiple PLS1 gave over 90% certainty [53].

Three olive oil cultivars were predicted with a 100% certainty using the $^{13}$C NMR methodology which applies the DEPT sequence [54] thus confirming along with the previous results obtained by conventional $^{13}$C NMR technique, that carbon-13 spectroscopy can be used to produce resonance intensity measurements accurate and precise enough to guarantee that Statistical Analysis is successful.

3.2. $^{13}$C NMR spectroscopy of the unsaponifiable matter of olive oil

Quantitative analysis of components of the
Table 4

<table>
<thead>
<tr>
<th>Resonance</th>
<th>Acyl chain&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Glycerol position&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Chemical shift (ppm)</th>
<th>Resonance</th>
<th>Acyl chain carbon</th>
<th>Glycerol position&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Chemical shift (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R 1</td>
<td>13 L</td>
<td>α + β</td>
<td>130.10</td>
<td>R 20</td>
<td>14 O</td>
<td>α + β</td>
<td>29.53</td>
</tr>
<tr>
<td>R 2</td>
<td>10 O</td>
<td>α</td>
<td>129.95</td>
<td>R 21</td>
<td>6 S</td>
<td>α</td>
<td>29.47</td>
</tr>
<tr>
<td>R 3</td>
<td>10 O</td>
<td>α</td>
<td>129.93</td>
<td>R 22</td>
<td>15 S</td>
<td>α</td>
<td>29.37</td>
</tr>
<tr>
<td>R 4</td>
<td>9 L</td>
<td>α</td>
<td>129.90</td>
<td>R 23</td>
<td>15 L</td>
<td>α + β</td>
<td>29.34</td>
</tr>
<tr>
<td>R 5</td>
<td>9 L</td>
<td>α</td>
<td>129.87</td>
<td>R 24</td>
<td>13 + 15 O</td>
<td>α + β</td>
<td>29.32</td>
</tr>
<tr>
<td>R 6</td>
<td>9 O</td>
<td>α</td>
<td>129.64</td>
<td>R 25</td>
<td>5 S</td>
<td>α</td>
<td>29.27</td>
</tr>
<tr>
<td>R 7</td>
<td>9 O</td>
<td>α</td>
<td>129.41</td>
<td>R 26</td>
<td>5 O + L</td>
<td>α</td>
<td>29.18</td>
</tr>
<tr>
<td>R 8</td>
<td>10 L</td>
<td>α</td>
<td>128.05</td>
<td>R 27</td>
<td>5 O + L</td>
<td>α</td>
<td>29.16</td>
</tr>
<tr>
<td>R 9</td>
<td>10 L</td>
<td>α</td>
<td>128.04</td>
<td>R 28</td>
<td>Unresolved</td>
<td>–</td>
<td>29.10&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>R 10</td>
<td>12 L</td>
<td>α</td>
<td>127.88</td>
<td>R 29</td>
<td>4 O + L</td>
<td>α</td>
<td>29.07</td>
</tr>
<tr>
<td>R 11</td>
<td>12 L</td>
<td>α</td>
<td>127.87</td>
<td>R 30</td>
<td>4 O + L</td>
<td>α</td>
<td>29.03</td>
</tr>
<tr>
<td>R 12</td>
<td>Glycerol</td>
<td>α</td>
<td>68.89</td>
<td>R 31</td>
<td>8 + 11 O</td>
<td>α + β</td>
<td>27.17&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>R 13</td>
<td>Glycerol</td>
<td>α (β&lt;sup&gt;l&lt;/sup&gt;)</td>
<td>62.05</td>
<td>R 32</td>
<td>8 + 14 L</td>
<td>α + β</td>
<td>25.61&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>R 14</td>
<td>2 S</td>
<td>α</td>
<td>34.06&lt;sup&gt;c&lt;/sup&gt;</td>
<td>R 33</td>
<td>3 S</td>
<td>α</td>
<td>24.84</td>
</tr>
<tr>
<td>R 15</td>
<td>2 O + L</td>
<td>α + β</td>
<td>31.73&lt;sup&gt;c&lt;/sup&gt;</td>
<td>R 34</td>
<td>3 O + L</td>
<td>α + β</td>
<td>22.63</td>
</tr>
<tr>
<td>R 16</td>
<td>16 O + L</td>
<td>α + β</td>
<td>29.76</td>
<td>R 35</td>
<td>17 S</td>
<td>α</td>
<td>14.06</td>
</tr>
<tr>
<td>R 17</td>
<td>Unresolved</td>
<td>–</td>
<td>29.70&lt;sup&gt;c&lt;/sup&gt;</td>
<td>R 36</td>
<td>18 O + L</td>
<td>α + β</td>
<td>14.06</td>
</tr>
<tr>
<td>R 18</td>
<td>Unknown</td>
<td>–</td>
<td>29.66</td>
<td>R 37</td>
<td>18 O + L</td>
<td>α + β</td>
<td>14.06</td>
</tr>
<tr>
<td>R 19</td>
<td>Unresolved</td>
<td>–</td>
<td>29.61&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Acyl chain: S, saturated; O, oleyl; L, linoleyl.

<sup>b</sup> α and β indicate the 1,3- and 2- glycerol positions, respectively.

<sup>c</sup> Chemical shifts correspond to the central frequency of the unresolved group of resonances.
unsaponifiable fraction of olive oils by means of chromatographic techniques, is the main tool for detecting olive oil authenticity and adulteration with olive pomace oils. Nevertheless, the unsaponifiable matter of three grades of olive oils, virgin, refined and pure, and one grade of olive–pomace oil, refined olive–pomace oil, have been studied by high resolution $^{13}$C NMR spectroscopy in order to check the suitability of $^{13}$C NMR for determining olive oil grades [55].

The spectra which were measured with proton-decoupling, using a $45^\circ$ excitation pulse and a $2$ s relaxation delay, were the result of $14,000$ scans.

The high frequency range from $120$ to $145$ ppm comprised the resonances of the double bond carbons of squalene, at $135.1$, $134.9$, $131.3$, $124.4$, $124.3$ ppm, squalene being the major component (up to $50\%$) of the unsaponifiable matter of virgin olive oils. The heights of these resonances are reduced during refining as it was proved by examining the spectra of refined olive oil and refined olive–pomace oils. The unsaturated carbons of sterols, which represent $20$–$30\%$ of unsaponifiable matter, resonate at $140.7$ (C-5) and $121.8$ (C-6) ppm, whereas the resonances of saturated carbons of $\beta$-sitosterol were reported in the low frequency range from $71.9$ to $11.9$ ppm. The peaks at $130.0$ and $129.8$ ppm which were not definitively assigned, are likely to be related both to triterpenic alcohols as well to long-chain monounsaturated alcohols. Difficulties were met in assigning the resonances of the components present at low concentration.

Eighty-five peaks at the same chemical shift positions were selected and the peak heights were analysed by Stepwise Discriminant Analysis. Significant discrimination was achieved among olive oil grades, virgin, pure and refined, and refined olive–pomace oil. These findings suggest that intensity patterns of compounds that constitute the unsaponifiable matter determined by $^{13}$C NMR spectroscopy can be really useful in determining different grades of virgin olive oils.

References


