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^{1}H and ^{13}C NMR of Virgin Olive Oil. An Overview

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The authentication and quality assessment of virgin olive oil were performed using high-resolution ^{1}H and ^{13}C NMR spectroscopy. An overview of the various determinations currently assessed is presented with emphasis on the detection of adulteration with foreign (seed) oils and esterified or refined olive and olive-pomace oils. Recent results on the NMR analysis of natural compounds (diacylglycerols, free fatty acids, aldehydes, polyphenols, etc.) related to the quality-freshness of virgin olive oil are also reported. The possible contribution of high-resolution NMR to the authentication of geographical origin of virgin olive oil is discussed. © **1997 John Wiley & Sons, Ltd.**

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INTRODUCTION

In the last few years, interest in virgin olive oil, traditionally consumed in the Mediterranean area, has been extended to other countries (Northern Europe, USA, Japan, etc.). The nutritional properties of virgin olive oil, the principal fatty ingredient in Mediterranean foods, arise from its chemical composition and have traditionally been attributed to the high content of monounsaturated acids (oleic acid). Recent studies focused on olive oil minor components have demonstrated how natural phenolic compounds present in virgin olive oil are responsible for its oxidation stability,[1–5] *in vivo* antioxidant action[6] and characteristic sensory attributes (bitter and pungent taste).[7–9] Phenolic compounds, however, are lost during solvent extraction (olive-pomace oil) or refining processes.[10]

Commercially, the low-grade refined olive oils and solvent-extracted olive-pomace oils must be blended with 'virgin' olive oil to produce 'olive oil' and 'olive-pomace oil.' The term 'virgin,' as established by the International Olive Oil Council and EU Regulations, defines the oil obtained from the olive (*Olea europaea sativa* H.) fruit only using mechanical and physical extraction systems (milling, centrifugation, pressing) without any thermal treatment or chemical manipulation and excluding solvent-extracted oils and esterified oils.[11,12] The higher sensory and nutritional quality of virgin olive oil and its higher commercial value, however, have led to its adulteration with low-grade foreign oils (seed oils), refined olive oils, olive-pomace oils and esterified oils.

Analytical methods to control the purity of olive oil and the addition of refined and solvent-extracted oils to virgin olive oil have recently been unified by the European Union.[12,13] At the same time, efforts are being made in the EU to develop new analytical methods in order to authenticate foods and, in particular, olive oils.[14,15]

This paper presents an overview of recent applications of high-resolution ^{1}H and ^{13}C NMR spectroscopy in the analysis of virgin olive oil.[16–26] New experimental results about the possible contribution of NMR to the authentication of geographical origin and quality assessment are also presented.

EXPERIMENTAL

Materials

Virgin olive oil samples were purchased from different extraction plants in southern and central Italy. Spanish and Greek virgin olive oil samples were furnished by the University of Milan (DISTAM) within an EC-FLAIR Project.[27] Refined olive oils and refined olive-pomace oils were purchased by commercial companies. Esterified oils were kindly supplied by the Stazione Sperimentale Oli e Grassi (Milan, Italy). Standard triacylglycerols, diacylglycerols, methyl esters and fatty acids were obtained from Fluka (Buchs, Switzerland), Larodan (Malmö, Sweden) and Nu-Chek-Prep (Elysian, MN, USA). Deuterated solvents were purchased from Aldrich Chemical (Milwaukee, WI, USA).

NMR spectroscopy

^{13}C NMR. Spectra were recorded on two high-resolution spectrometers (AC 270 and AM 400; Bruker,

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Karlsruhe, Germany) located at the CIMCF (University of Naples, Italy) operating at a carbon-13 frequency of 67.88 and 100.64 MHz (5.9 and 7.0 T), respectively, according to published experimental conditions.[16–26] Spectra were recorded at concentrations of 10–20% (w/v) (50–100 mg of oil in 0.5 ml of chloroform-*d*) using 5 mm NMR tubes at controlled temperatures of 30 ± 0.1 °C in the broadband proton decoupling mode. Full ^{13}C NMR spectra were obtained with the following acquisition parameters: 16K data points, spectral width 200 ppm, acquisition time 0.37 s, relaxation delay 5 s, pulse width 45° and 256–3000 scans. High-resolution carbonyl spectra were recorded with 16K data points, spectral width 10 ppm, acquisition time 12–20 s, relaxation delay 5 s and pulse width 45–90°. Free induction decays (FIDs) were transformed by zero filling up to 32K data points to yield a digital resolution of 0.05–0.08 Hz per point. All FIDs, prior to Fourier transformation (FT), were filtered using an exponential multiplication (0.2–0.4 Hz line broadening) for sensitivity enhancement. The peak intensities of the high-resolution ^{13}C NMR carbonyl spectra were accurately quantified using the Linesim (Bruker) curve resolution program. Spin–lattice relaxation times (T_1) were measured using the inversion–recovery (180–τ–90) pulse sequence.[28] The τ values were varied between 0.1 and 25 s, on the basis of the expected T_1 values. The peak intensities, measured for each signal in the inversion–recovery spectra recorded with different τ values, were fitted to an exponential curve via a three-parameter minimization in order to obtain the T_1 values.[29] Nuclear Overhauser effects (NOE) were determined by difference between the signal intensity of broadband decoupled spectra (with NOE) and inverse-gated decoupled spectra (without NOE) recorded with the same number of scans and transformed in the absolute intensity mode.[30] According to the NOE and T_1 values determined, the spectral conditions described above were selected to ensure the quantitative recovery of diagnostic carbon signals.

^{1}H NMR. Oil samples (20 μl) were placed in 5 mm NMR tubes and dissolved in chloroform-*d* (0.7 ml) and DMSO-d_6 (20 μl). One-dimensional spectra were recorded on Bruker AC270, AM400 and AMX600 instruments (CIMCF, University of Naples, and CNR, Rome) operating at 270, 400.13 and 600.13 MHz, respectively, and on a Varian (Palo Alto, CA, USA) Unity 400 spectrometer (Department of Chemistry, University of Naples) operating at 400.13 MHz. Spectra were acquired and processed in the phase-sensitive mode (TPPI) according to conditions described previously.[18,21–23]

RESULTS AND DISCUSSION

Figure 1 shows a typical ^{1}H NMR spectrum of a virgin olive oil with labeling of the major resonances (Table 1). A typical ^{13}C NMR spectrum is shown in Fig. 2 with

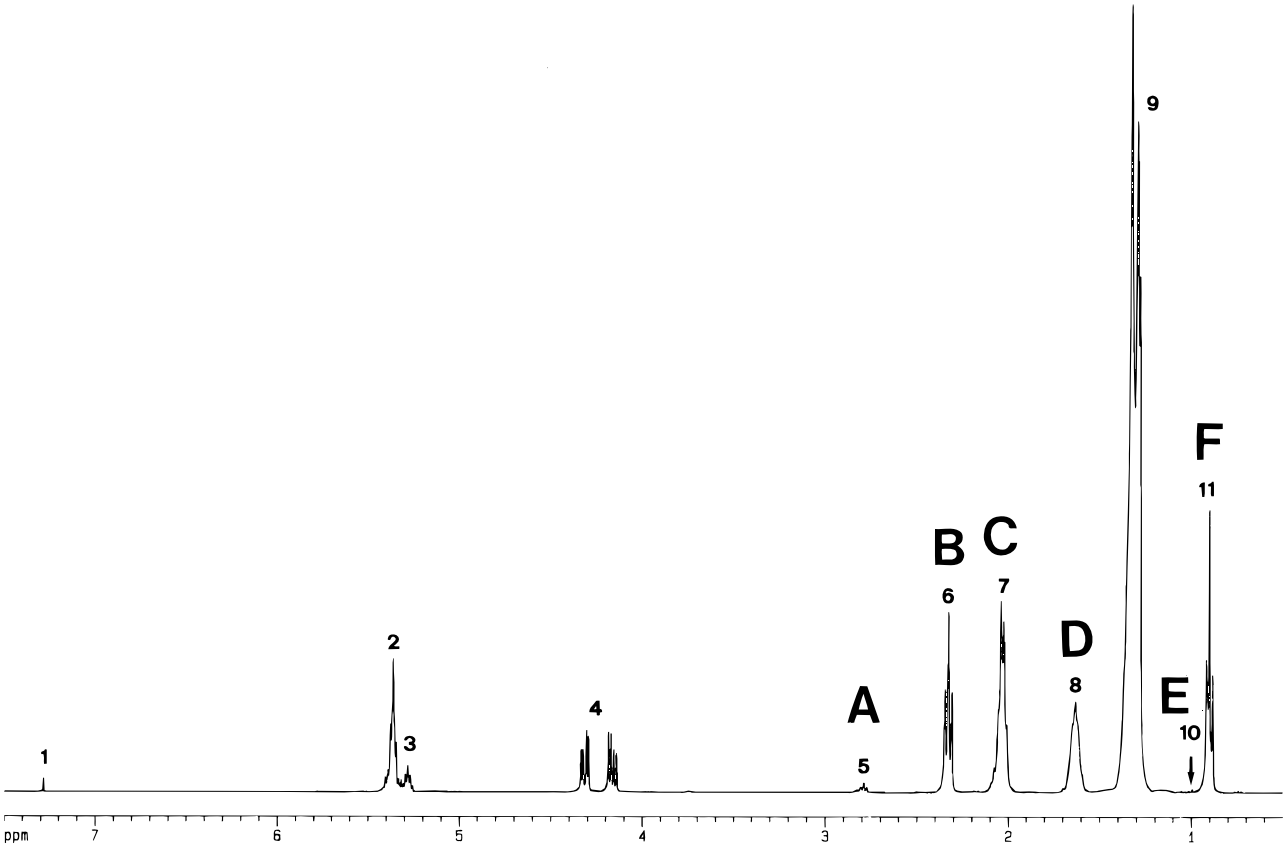


Figure 1. 400 MHz ^{1}H NMR spectrum of a virgin olive oil. Labeled resonances are assigned in Table 1.

Table 1. Chemical shifts (δ) and assignment of the main resonances in the ^{1}H NMR spectrum of virgin olive oil (see Fig. 1)

		Assignment	
Peak	δ (ppm)	Proton	Compound
1	7.26	$CHCl_3$	Chloroform (solvent)
2	5.29	C*H*=CH	All unsaturated fatty acids
3	5.15	CHOCOR	Glycerol (triacylglycerols)
4	4.19	C*H*$_2$OCOR	Glycerol (triacylglycerols)
5 (A)	2.76	CH=CHC*H*$_2$CH=CH	Linoleyl and linolenyl
6 (B)	2.2	C*H*$_2$COOH	All acyl chains
7 (C)	2.02	C*H*$_2$CH=CH	All unsaturated fatty acids
8 (D)	1.6	C*H*$_2$CH$_2$COOH	All acyl chains
9	1.2	$(CH_2)_n$	All acyl chains
10 (E)	0.95	CH$_2$CH$_2$CH$_2$C*H*$_3$	Linolenyl
11 (F)	0.85	CH=CHCH$_2$C*H*$_3$	All acids except linolenyl

indications of different spectral regions and labeling of some major resonances (Table 2).

Authentication

Determination of fatty acid composition: detection of adulteration with seed oils. NMR data on the major components of vegetable oils have been published by several groups and were recently reviewed by Gunstone[31] for ^{13}C NMR. Major components are known to belong to the class of fatty acids and their glycerol esters. In olive oil, the fatty acids that need to be considered are those based on the C_{16} and C_{18} saturated alkanes (mainly palmitic and stearic acids) and C_{18} alkenes, mono- (MUFA) and polyunsaturated (PUFA) (mostly oleic, linoleic and linolenic acids). In addition, several other minor components are present in virgin olive oil and they will be dealt with in the following sections.

Proton chemical shifts of the most abundant fatty acid components are summarized in Table 3. These data can be used for simple applications considering that signal overlaps in the spectral areas of interest such as those including olefinic and glyceridic regions do not allow the complete discrimination of single fatty acid components.

The first high-resolution proton NMR application in the field of oils and fats was the determination of global unsaturation (corresponding to the classical iodine number) made on the basis of the integral of olefinic protons at 5.3–5.4 ppm.[32] A good correlation between the iodine values and proton NMR data was also found

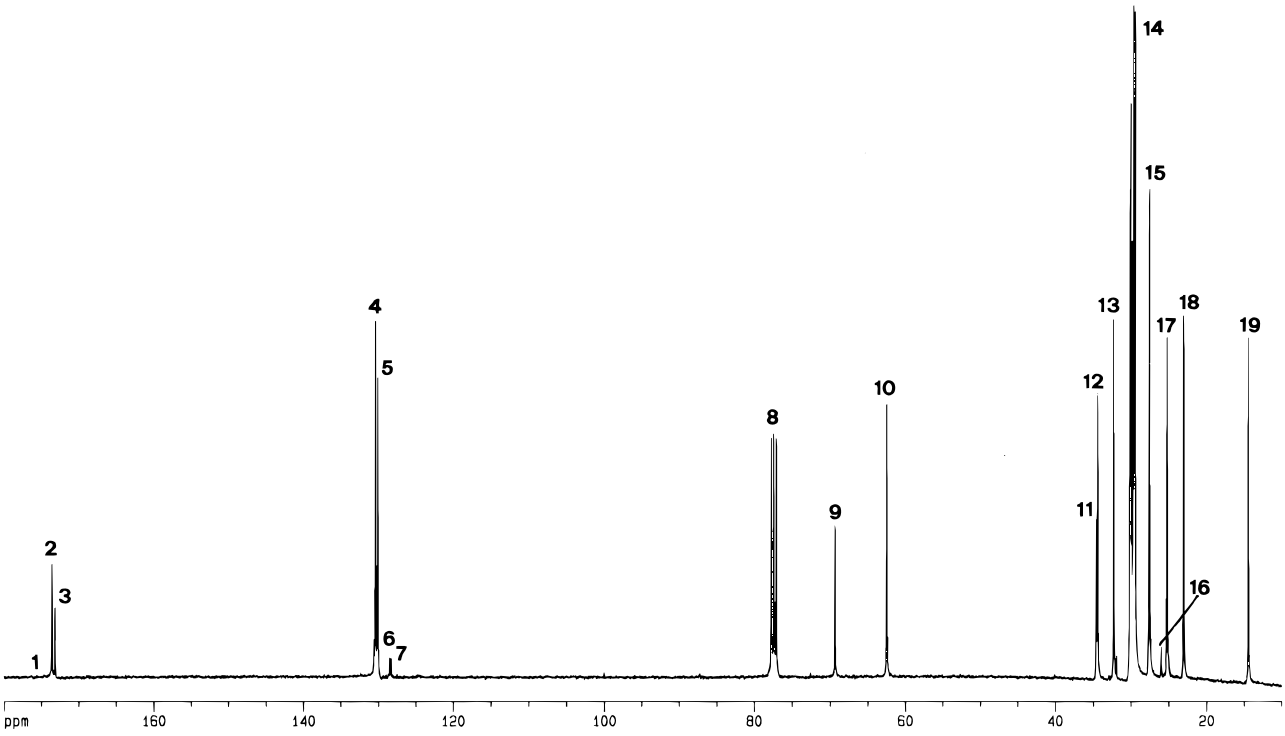


Figure 2. Typical 100 MHz ^{13}C NMR spectrum of a virgin olive oil. Labeled resonances are assigned in Table 2.

Table 2. Chemical shifts (δ) and assignment of the main resonances in the ^{13}C NMR spectrum of virgin olive oil (see Fig. 2)

Peak	δ (ppm)	Assignment: Carbon	Assignment: Compound
1	174–176	C-1	Free fatty acids
2	173.26	C-1, *sn*-1,3	Triacylglycerols
3	172.81	C-1, *sn*-2	Triacylglycerols
4	129.98	C-10	Oleyl
5	129.67	C-9	Oleyl
6	128.06	C-10	Linoleyl
7	127.86	C-12	Linoleyl
8	77.01	$CDCl_3$	(Solvent)
9	68.92	CHO—, *sn*-2	Triacylglycerols
10	62.18	CH_2O—, *sn*-1,3	Triacylglycerols
11	34.18	C-2, *sn*-2	All acyl chains
12	34.02	C-2, *sn*-1,3	All acyl chains
13	31.88	$\omega 3$	Sat., $n-9$ and $n-6$ acids
14	29.1–29.8	$(CH_2)_n$	All acyl chains
15	27.16	Allylic: C-8–C-11 oleyl, C-8–C-14 linoleyl	
16	25.81	Diallylic: C-11 linoleyl, C-11–C-14 linolenyl	
17	24.84	C3	All acyl chains
18	22.65	$\omega 2$	All acyl chains
19	14.15	$\omega 1$ (—CH_3)	All acyl chains

for olive oils.[16] By using medium-field (200–400 MHz) modern NMR spectrometers, the good resolution of different methyl and methylene protons (0.8–3 ppm) allows one to extract satisfactory information also about the fatty acid composition of olive oil (Fig. 1).[21,23]

In fact, from the intensity of the methyl signal at 0.85 ppm (signal labeled F in Fig. 1) the sum of saturated (SFA), $n-9$ and $n-7$ monounsaturated (MUFA) and $n-6$ linoleic can be determined relative to the $n-3$ linolenic content measured on the basis of the characteristic methyl signal at 0.95 ppm (signal E in Fig. 1):

$$n-3 \text{ linolenic} = E/E + F \quad (1)$$

$$SFA + MUFA + n-6 \text{ linoleic} = F/E + F \quad (2)$$

The relative amount of MUFA and linoleic acids can be determined by referring the allylic protons centered at 2.02 ppm (signal C in Fig. 1) to all fatty chains as measured from the intensity of the C-2 protons around 2.27–2.3 ppm (signal B in Fig. 1):

$$MUFA + n-3 \text{ linolenic} + n-6 \text{ linoleic} = C/2B \quad (3)$$

$$MUFA + n-6 \text{ linoleic} = [C/2B] - [E/(E + F)] \quad (4)$$

Furthermore, the $n-6$ linoleic content can be determined by subtracting from the diallylic protons at 2.73 ppm (signal A) the relative amount of $n-3$ linoleic calculated from Eqn (1) as well as the monounsaturated fatty chains (MUFA):

$$n-6 \text{ linoleic} = 2A - [E/2(E + F)] \quad (5)$$

$$MUFA = \{[C/2B] - [E/(E + F)]\} - \{2A - [E/2(E + F)]\} \quad (6)$$

Saturated fatty acids can be finally obtained as follows:

$$SFA = 16:0 + 18:0 = [F/(E + F)] - [C/2B] - [E/(E + F)] \quad (7)$$

These data were found to be well correlated with gas chromatographic analyses and offer information which, although less detailed when compared with gas chromatography or ^{13}C NMR, is still sufficient for a rapid evaluation of the SFA, MUFA, linoleic and linolenic acid content and for a preliminary evaluation of olive oil purity. In particular, common adulterations with seed oils (soybean, peanut, maize, etc.) characterized by high contents of $n-3$ linolenic acid can be detected, taking into account that in olive oils $n-3$ linolenic acid cannot exceed 0.9%.[13]

Many of the interesting NMR applications in the field of lipid chemistry and technology are, however, obtained by exploiting ^{13}C NMR data. This is primarily due to the wider chemical shift spread of the ^{13}C NMR resonances, to the different carbon chemical environments and ultimately to the α–β position of fatty chains on the glycerol backbone. Many of the relevant chemical shift data have been reviewed by Gunstone[31] and they are briefly summarized in Tables 4 and 5 for both free fatty acids and their glyceridic esters.[13] Application of these data is straightforward. Olefinic, methylenic and carbonyl resonances are suitable for the direct and structure-specific analysis of the relative amounts of single fatty acids present in olive oil. Data obtained by using ^{13}C NMR have been compared with gas–liquid

Table 3. ^1H chemical shifts (δ ppm) of the major acyl chains present in olive oils

	Oleyl	Linoleyl	Linolenyl	Palmityl
CH_3	0.85	0.85	0.95	0.85
$(CH_2)_n$	1.2	1.2	1.2	1.2
$CH_2CH{=}CH$	2.02	2.02	2.02	—
$CH{=}CHCH_2CH{=}CH$	—	2.76	2.76	—
$CH{=}CH$	5.29	5.22	5.22	—
CH_2CH_2COOH	1.6	1.6	1.6	1.6
CH_2COOH	2.2	2.2	2.2	2.2

Table 4. ^{13}C chemical shifts (δ ppm) of the major acyl chains present in olive oils[a]

	Acyl chain				
Carbon	Oleyl	Linoleyl	Linolenyl	Stearoyl	Palmitoyl
C-1	180.54	180.54	180.56	180.60	180.58
C-2	34.20	34.15	34.14	34.24	34.26
C-3	24.80	24.70	24.64	24.81	24.84
C-4–C-7	29.4–29.9	29.1–29.7	29.0–29.6	29.2–29.9	29.2–29.9
C-8	27.31	27.22	27.63	29.2–29.9	29.2–29.9
C-9	132.40	130.02	130.24	29.2–29.9	29.2–29.9
C-10	132.40	128.12	127.80	29.2–29.9	29.2–29.9
C-11	27.31	25.67	25.65	29.2–29.9	29.2–29.9
C-12	29.4–29.9	127.95	128.30	29.2–29.9	29.2–29.9
C-13	29.4–29.9	130.21	128.27	29.2–29.9	29.2–29.9
C-14	29.4–29.9	27.25	25.56	29.2–29.9	32.11 (ω3)
C-15	29.4–29.9	29.19	127.16	29.2–29.9	22.82 (ω2)
C-16 (ω3)	32.06	31.58	131.95	32.07	14.13 (ω1)
C-17 (ω2)	22.80	22.61	20.58	22.79	
C-18 (ω1)	14.13	14.09	14.28	14.12	

[a] Data referred to free fatty acids (from Ref. 31).

chromatographic results and good agreement between the two methods was found.[16] Quantitative ^{13}C NMR data of the acyl profile have already been reported to be in good agreement with gas chromatographic data for other edible vegetable oils, fats and lipids,[33–36] and the standardization of the quantitative determination of fatty acid profiles in olive oils is currently in progress within an EC Project.[15]

Table 5. ^{13}C chemical shifts (δ) of glyceryl carbons in different glycerides (see Fig. 3)[a]

		Assignment	
Peak	δ (ppm)	Carbon	Compound
1	72.08	CH, *sn*-2	*sn*-1,2-Diacylglycerols
2	70.25	CH, *sn*-2	*sn*-1-Monoacylglycerols
3	68.84	CH, *sn*-2	Triacylglycerols
4	68.30	CH, *sn*-2	*sn*-1,3-Diacylglycerols
5	65.09	CH_2, *sn*-1	*sn*-1-Monoacylglycerols
6	64.99	CH_2, *sn*-1,3	*sn*-1,3-Diacylglycerols
7	63.34	CH_2, *sn*-3	*sn*-1-Monoacylglycerols
8	62.04	CH_2, *sn*-1	*sn*-1,2-Diacylglycerols
		CH_2, *sn*-1,3	Triacylglycerols
9	61.43	CH_2, *sn*-3	*sn*-1,2-Diacylglycerols

[a] From Refs 17 and 18.

Analysis of diacylglycerols and free fatty acids. ^{13}C chemical shift values of glycerol carbons in different glycerides allow the rapid and direct analysis of total diacylglycerols and of the relative amounts of *sn*-1,2- and *sn*-1,3-diacylglycerol forms in olive oil (Fig. 3, Table 5).[16–18,23] This information can also be obtained by using proton NMR spectral data (Fig. 4, Table 6). From the ^1H NMR spectra, *sn*-1,2-diacylglycerols can be easily quantified on the basis of resolved doublet at 3.66 ppm whilst overlaps occur in the glyceridic region between triacylglycerol and *sn*-1,3-diacylglycerol patterns (Fig. 4). In this case, a better resolution of *sn*-1,3-diacylglycerols can be obtained using an *in situ* derivatization with trichloroacetyl isocyanate.[18] Free glycerol hydroxyls are quantitatively converted into trichloroacetyl carbamates and *sn*-1,3-diacylglycerol methylene protons are low-field shifted and resolved from the triacylglycerol envelope (Fig. 4, Table 6).

Proton and/or carbon-13 NMR determination of diacylglycerols in virgin olive oils can be a useful index in detecting the presence of partially neutralized oils added to declared 'virgin' oils. In fact, diacylglycerols naturally

Table 6. ^1H chemical shifts (δ) of glyceryl protons in different glycerides and after *in situ* derivatization with trichloroacetyl isocyanate (see Fig. 4)[a]

	δ (ppm)		Assignment	
Peak	Underivatized	TAI derivatized	Proton	Compound
1	5.22	5.22	C*H*OCOR, *sn*-2	Triacylglycerols
2	5.07	5.32	C*H*OCOR, *sn*-2	*sn*-1,2-Diacylglycerols
3	4.29	4.48	CH_2OCOR, *sn*-1	*sn*-1,2-Diacylglycerols
4	4.25	4.25	CH_2OCOR, *sn*-1,3	Triacylglycerols
5	4.17	4.36	CH_2OCOR, *sn*-1	*sn*-1,2-Diacylglycerols
6	4.10	4.10	CH_2, *sn*-2	Triacylglycerols
7	4.07	4.24	C*H*OH, *sn*-2	*sn*-1,3-Diacylglycerols
8	4.03	4.21	CH_2OCOR, *sn*-1,3	*sn*-1,3-Diacylglycerols
9	3.66	4.36	CH_2OH, *sn*-3	*sn*-1,2-Diacylglycerols

[a] Chemical shifts measured on underivatized glycerides in chloroform-*d* solutions and after derivatization with trichloroacetyl isocyanate (TAI).[18]

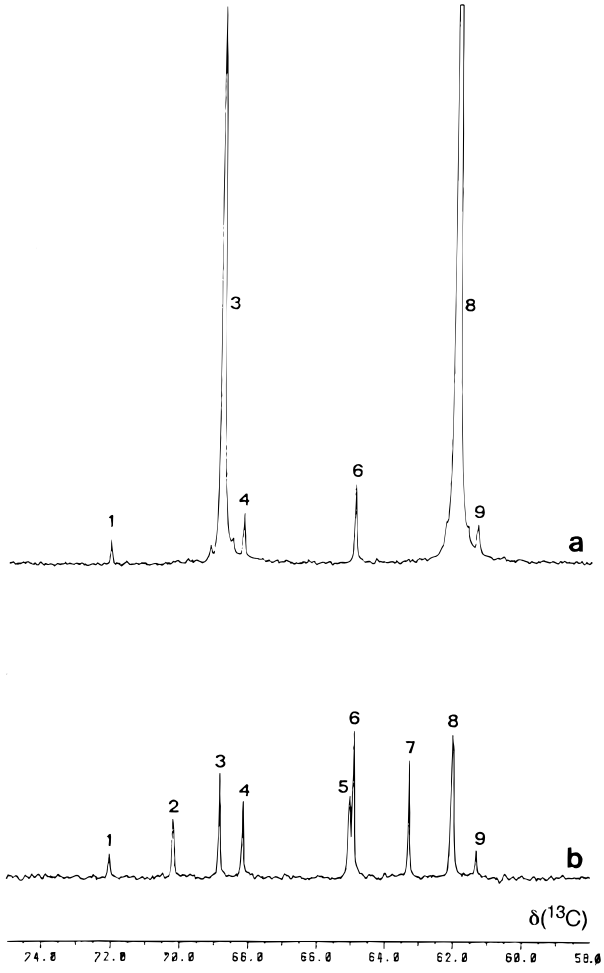


Figure 3. Glyceryl carbon resonances from the 67.88 ^{13}C NMR spectrum of a virgin olive oil (a) and of a standard mixture of glycerides (b). Signal assignment is shown in Table 5 (from Ref. 17).

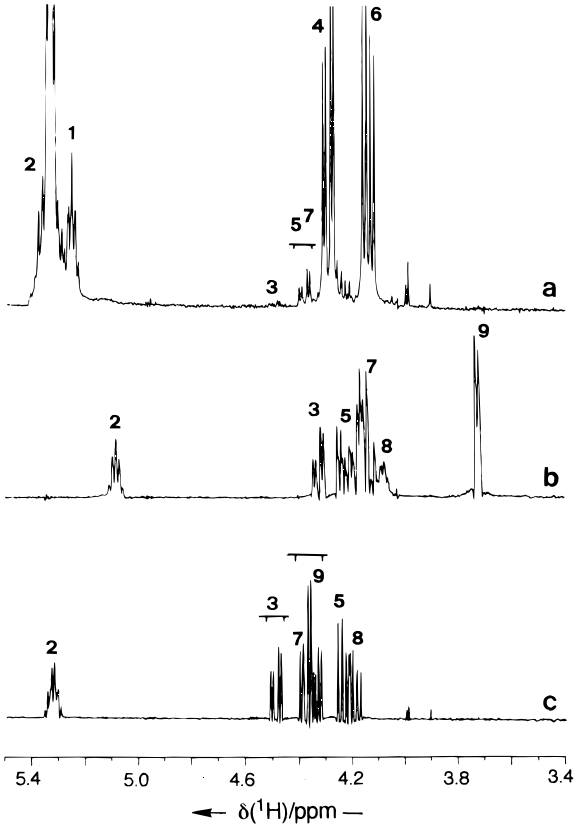


Figure 4. 270 MHz ^1H NMR spectra of a neutralized olive-pomace oil after addition of trichloroacetyl isocyanate (TAI) (a) and of a mixture of *sn*-1,2-diacylglycerols and *sn*-1,3-diacylglycerols underivatized (b) and after TAI addition (c). Spectra (b) and (c): resolution enhancement with a Gaussian multiplication function (from Ref. 18).

contained in virgin olive oil do not exceed 2–3% and they (mainly *sn*-1,2-diacylglycerols) arise either from incomplete triacylglycerol biosynthesis or from limited lipase action on triacylglycerols. Larger amounts of diacylglycerols (mainly *sn*-1,3-diacylglycerols) are found in neutralized olive oils ('refined olive oil' and 'refined olive-pomace oil') produced from a starting material (olives or olive-pomace) with a high level of free fatty acids. In fact, refining processes (involving treatments of chemical or physical neutralization, bleaching, deodorization and winterization) remove free fatty acids but cause only a partial loss of diacylglycerols (absorbed in the soapy phase during oil neutralization and washing). Therefore, the total content of diacylglycerols and the ratio of *sn*-1,3-diacylglycerols to total diacylglycerols (*sn*-1,2- + *sn*-1,3-diacylglycerols) can provide a good discrimination between virgin olive oils and refined oils ('olive oils' and 'olive-pomace oils') (Fig. 5).

Determination of *trans*-fatty acids. The detection of *trans*-fatty acids in virgin olive oil is another interesting determination in which NMR spectroscopy can be used as an alternative to official gas chromatographic methods.[16,37] The absence of *trans*-isomers is considered as a purity index for virgin olive oils: refined (bleached and deodorized) olive and olive-pomace oils contain detectable levels (0.1–0.6%) of *trans*-isomers. For this purpose, good analytical sensitivity is required and standard capillary gas chromatographic methods are used in the European Union.[13] Using ^{13}C NMR and gas chromatography, 27 genuine samples of extra-virgin olive oils and seven samples of refined pomace oil have been studied.[37] As expected, no detectable *trans*-

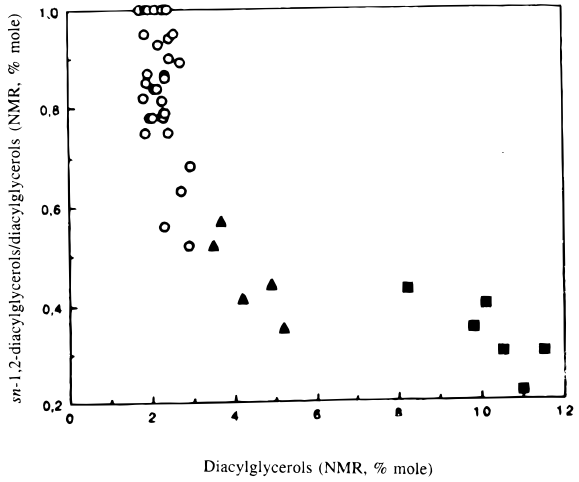


Figure 5. Plot of the total diacylglycerol content (DG) *vs.* the 1,2-DG/DG ratio. (○) Extra virgin olive oils; (▲) refined olive oils; (■) olive-pomace oils.

isomers were found in virgin olive oils whilst refined oils showed a variable level of *trans*-fatty acids (0.3–1%). For this analytical problem, the methylene region appears to be very useful, owing to the little dependence of the *trans*-allylic carbon chemical shift (32.5–32.7 ppm) on the double bond position.[31] Sensitivity tests were performed using standard triacylglycerol solutions containing 1, 0.5 and 0.1% trielaidate. At a ^{13}C frequency of 100 MHz, 0.1% *trans*-isomers were quantitatively detected from the spectral noise after an overnight accumulation (30 000 scans).

Olefinic carbons, in addition, can be useful in determining various isomers and to assess the positional distribution of *trans* fatty acids.[37] The advantage of using allylic carbons is the specific simultaneous detection of all-*trans*-isomers with an increase in sensitivity and specificity with respect to chromatographic techniques, where different isomers give rise to different small signals that are difficult to quantify accurately.[37]

Positional distribution of fatty acids: detection of esterified oils. The biosynthesis of triacylglycerols in vegetable oils involves a preferential esterification of unsaturated fatty acids in the *sn*-2 position of glycerol. In olive oils, the amount of oleic and linoleic acids in the *sn*-2 position represents 98–99% of the total fatty acid content while the saturated fatty acids cannot exceed 1.5%.[12,13] During the neutralization process of olive oils with a high free fatty acid content, such as the olive-pomace oils, large amounts of free fatty acids (FFA), with a fatty acid composition similar to that of olive glycerides, are recovered. These FFA mixtures can be industrially esterified into triacylglycerols by treatment with glycerol. In these synthetic oils, called esterified oils, fatty acids are randomly distributed on *sn*-glycerol so that about 15% by weight of saturated fatty acids are found in the *sn*-2-acyl position.[38]

The analysis of esterified oils (considered non-edible in the European Union) mixed with olive oils is currently carried out using standard methods based on the quantitative analysis of saturated fatty acids (stearic + palmitic) in the *sn*-2 position of triacylglycerols.[12,13] The EU official analytical procedure requires several steps based on (i) hydrolysis of triacylglycerols by pancreatic lipase, (ii) extraction of lipids with diethyl ether, (iii) thin-layer chromatographic fractionation of lipids and recovery of the 2-monoacylglycerol band and (iv) transmethylation of 2-monoacylglycerols and gas chromatographic analysis of the fatty acid composition. NMR making use of ^{13}C resonances yields immediate results, operating directly on the oil samples without any chemical manipulation, and it seems to represent the only direct instrumental method by which the positional distribution of fatty acids on glycerols can be specifically identified.[19] Carbonyls (C-1), C-2 and olefinic carbons of triacylglycerols, in fact, exhibit small chemical shift differences (Tables 7 and 8) in relation to (i) the positional distribution of fatty acids on glycerol *sn*-1,3 and *sn*-2 positions and (ii) the number and positions of the double bonds along the fatty chain.[19,31,33,39]

Table 7. ^{13}C chemical shifts (δ ppm) of carbonyls in standard triacyglycerols[a]

Triacylglycerol	*sn*-1	*sn*-2	*sn*-3
OOP	173.216	172.804	173.284
OPO	173.216	172.883	173.216
OOO	173.216	172.804	173.216
PPP	173.248	172.883	173.248
LLL	173.189	172.784	173.189

[a] OOP = *sn*-1,2-dioleyl-3-palmitoylglycerol; OPO = *sn*-1,3-dioleyl-2-palmitoylglycerol; OOO = triolein; PPP = tripalmitin; LLL = trilinolein. Data obtained in chloroform-*d* solutions at 30 °C (from Ref. 19).

When spectra of saturated/unsaturated triacylglycerols are obtained with a low digital resolution (0.6–12 Hz per point) at fields of 50–100 MHz, the carbonyl region shows only two resolved resonances corresponding to *sn*-1,3 and *sn*-2 positions. An enhancement of the spectral resolution (0.06 Hz per point), obtained by increasing the number of experimental data points and using longer acquisition times (10–20 s), leads to an α–β splitting of C-1, C-2 and olefinic carbons.[19,33]

Figure 6 shows a clear, although small, chemical shift difference between the carbonyl resonances of oleic (O) and palmitic (P) acids esterified in the *sn*-1,3 and *sn*-2 glycerol positions of standard mixed triacylglycerols (OPO and POO). When the same analysis is performed on a virgin olive oil [Fig. 7(a)], only one resonance is observed for the *sn*-2 glycerol position, corresponding to the unsaturated fatty acids (linoleyl and linolenyl resonances partially overlap oleyl resonances in spectra recorded at 9.4 T). In the *sn*-1,3 glycerol position saturated and unsaturated components can be separately observed and from their intensities the overall content of saturated fatty acids (33% mole faction) can be measured. When esterification occurs, a random distribution of fatty acids is observed with saturated fatty acids in both *sn*-1,3 and *sn*-2 glycerol positions [Fig. 7(b)].

Table 8. ^{13}C chemical shifts (δ ppm) of methylene (C-2) and olefinic carbons of major acyl components present in olive oil triacyglycerols (from Ref. 31)

	sn-1,3					*sn*-2				
	16:0	18:0	18:1	18:2	18:3	16:0	18:0	18:1	18:2	18:3
C-2	173.27	173.27	173.20	173.17	173.04	172.86	172.88	172.83	172.77	172.64
C-9	—	—	129.69	129.98	130.19	—	—	129.72	130.01	130.22
C-10	—	—	130.02	128.11	127.80	—	—	130.04	128.12	127.81
C-12	—	—	—	127.93	128.25	—	—	—	127.94	128.26
C-13	—	—	—	130.21	128.31	—	—	—	130.22	128.32
C-15	—	—	—	—	127.15	—	—	—	—	127.16
C-16	—	—	—	—	131.95	—	—	—	—	131.95

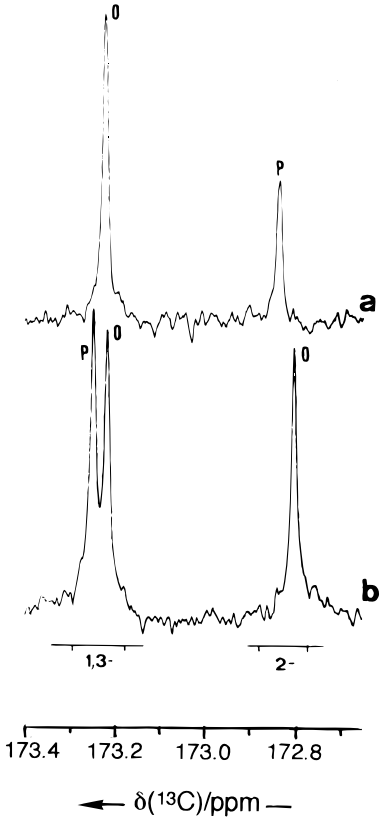


Figure 6. Expansion of the carbonyl region of 67.88 MHz ^{13}C NMR spectra of *sn*-1,3-dioleyl-2-palmitoylglycerol (a) and *sn*-1, 2-dioleyl-3-palmitoylglycerol (b). The carbonyl peaks are defined as attached to the *sn*-1,3 or *sn*-2 position. The acyl groups are palmityl (P) and oleyl (O) (from Ref. 19).

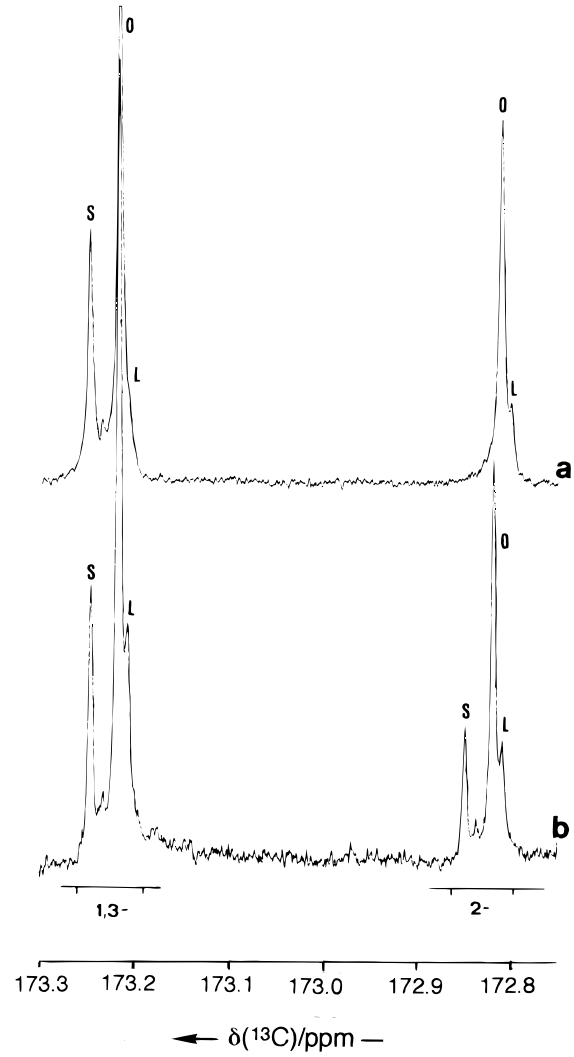


Figure 7. 100 MHz ^{13}C NMR carbonyl spectra of a virgin olive oil (a) and of an esterified oil (b). The carbonyl peaks are defined as attached to the *sn*-1,3- or *sn*-2 position. The acyl groups are saturated (S), oleyl (O) and linoleyl (L) (from Ref. 19).

Determinations made on virgin olive oils spiked with known amount of esterified oils showed a good quantitative accuracy of the NMR method.[19] From the experimental data, it was observed that the method above outlined can be easily applied to olive oils containing a level of 2–3% of saturated components in the *sn*-2 position. This limit can be lowered by using higher magnetic fields (11–14 T) or by increasing the spectral line intensities with longer accumulations. In order to obtain the best resolution, good probe temperature control during accumulation is required and appropriate proton decoupling.[40] Complementary information can be obtained from the olefinic region in which the α–β splitting of C-9 and C-10 carbons allows a better study of the positional distribution of linoleyl and linolenyl (not shown).[33]

Analysis of the unsaponifiable fraction. Further information about virgin olive oil purity can be obtained from the analysis of the unsaponifiable fraction which, in virgin olive oil, is constituted mainly of squalene, β-sitosterol and aliphatic alcohols. The ^{13}C and ^{1}H NMR spectra of these compounds have been reported.[41–43] ^{13}C NMR analysis of the unsaponifiable matter has recently been used in combination with multivariate statistical analysis for the discrimination of virgin olive oil from olive-pomace oil and refined olive oils.[44] Recently, the spectra of steroidal hydrocarbons arising from oil refining (stigmastadienes) have been intrepreted and can also be used in detecting refined oils in mixtures with virgin oils.[45]

^{1}H NMR geographical characterization of virgin olive oil. Recent results, obtained by multivariate statistical analysis of high-field proton NMR data obtained from different extra-virgin olive oil samples from four Italian regions, indicate a possible contribution of NMR also in the authentication of the geographical origin of virgin olive oils.[26,46] Apart from the obvious need to collect reference spectra for each year of production (sampling from the representative varieties of each region and databank preparation), the application of multivariate statistics to NMR data offers an interesting developing area for the certification of the geographical origin of extra-virgin olive oils.

Virgin olive oil quality assessment

Virgin olive oil quality can be related to its oxidation stability, sensory properties and nutritional aspects.

Both ^{13}C and ^{1}H NMR spectra offer complementary information on olive oil quality which can be obtained simultaneously (Figs 1 and 2). They will be discussed separately in the following sections.

Determination of free acidity. The first quality requirement for classifying virgin olive oils (together with peroxide values, UV analysis and sensory assessments made by a panel of tasters) is the determination of its free fatty acid content. By comparing the signal intensity of free carbonyls resonating at 176–178 ppm with the esterified carbonyls (171–174 ppm envelope) a quantitative measure of free acidity (% mole fraction) can be easily obtained by ^{13}C NMR.[16,17]

Diacylglycerols and virgin olive oil quality-freshness. The amount of free fatty acids and the corresponding diacylglycerol profile can also be used to define the degree of lipolytic alteration which is correlated with the quality of olives from which virgin olive oil has been produced. As shown, total diacylglycerols and the *sn*-1,2-/*sn*-1,3-diacylglycerol ratio can be easily determined using either ^{13}C or ^{1}H NMR. These parameters were studied in several virgin olive oil samples of different origins (Greece, Spain, Italy) and they were related to variety, olive ripeness and oil storage (Fig. 8).[20] It was noted that the ratio between *sn*-1,2- and *sn*-1,3-diacylglycerols can be correlated with the quality-freshness of virgin olive oils: recently extracted oils from normal ripe olives contained mainly the native *sn*-1,2-diacylglycerols and only small amounts of *sn*-1,3-diacylglycerols. The *sn*-1,3-diacylglycerols increase in oils obtained from over-ripe olives or after several months of oil storage due to lipolysis and 1,2–1,3 isomerization.[20]

Evaluation of oxidation products. The oxidation stability of virgin olive oil is one of its main quality attributes. The degree of oxidative alteration of virgin olive oil, and also other oils and fats, can be successfully evaluated using proton NMR spectroscopy.[21–23,47] In fact, both primary oxidation products (hydroperoxidienes) and aldehydes arising from their degradation can be simultaneously observed and quantified in the low-field region of the ^{1}H NMR spectrum (Fig. 9, Table 9). Using ^{13}C NMR, the diagnostic signals for several oxygenated compounds can also be found in rancid or thermally stressed olive oils. The diagnostic ^{13}C chemical shifts of oxo and hydroxy acids have recently been reviewed by Gunstone.[31] In the spectra of oxidized virgin olive oils (thermal stressing at 180 °C for 90 min), some resonances with chemical shifts corresponding to the chemical shifts of hydroxy and oxo acids can be singled out (Fig. 10, Table 10). Further applications of NMR in this area are currently being investigated.

Analysis of the phenolic fraction. Phenolic compounds play an important role in the nutritional characteristics and oxidation stability of virgin olive oils. They are in fact natural antioxidants and the level of phenolic compounds has been found to be correlated with the oxidative resistance of virgin olive oil.[1–3] Another aspect related to phenols is their role in determining the sensory response (bitter, astringent, pungent taste) and consumer acceptability (oils that are too bitter are unacceptable to the usual consumers).[7–9,48]

Tyrosol (Ty) and hydroxytyrosol (OHTy) are the main simple phenols present in olive oil and they were found in virgin olive oil mainly as esterified compounds recently identified using NMR spectroscopy.[49,50] These

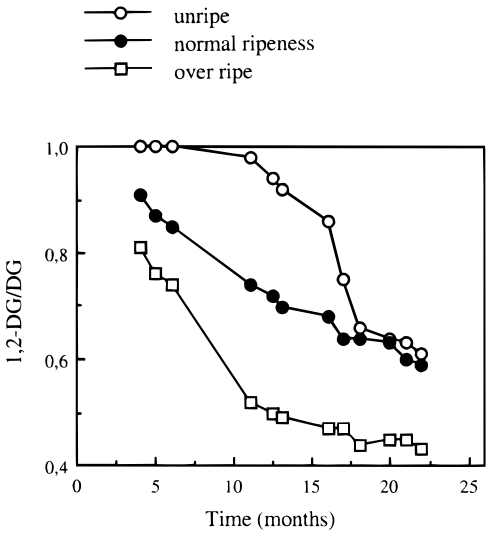


Figure 8. Evolution of the *sn*-1,2-diacylglycerols/total diacylglycerols ratio as measured by NMR during the storage (22 months) of virgin olive oils obtained from olives at different ripening degrees (from Ref. 20).

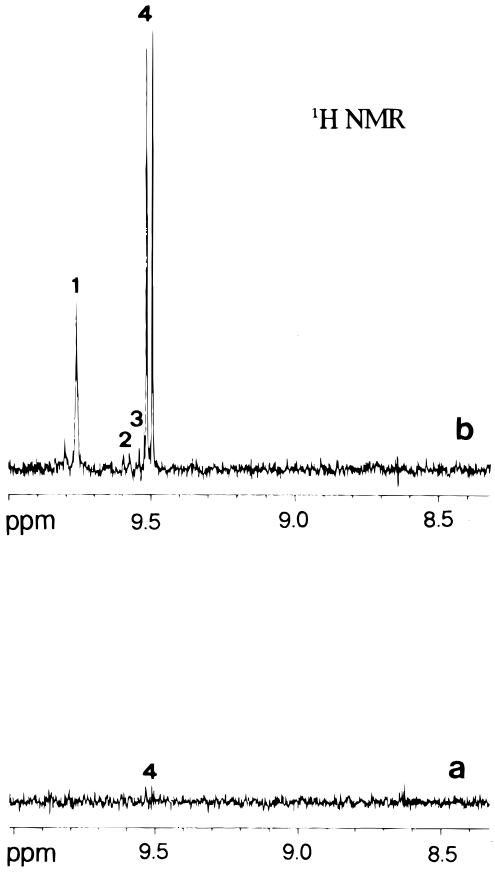


Figure 9. Expansion of the aldehydic region of the 400 MHz proton NMR spectra of a virgin olive oil before (a) and after 90 min thermal stressing at 180 °C (b). Labeled resonances are assigned in Table 9.

Table 9. ^{1}H chemical shifts (δ) of aldehydes (see Fig. 9) and hydroperoxydienes found in autoxidized (prolonged storage in open bottles) or thermally stressed (180 °C) virgin olive oils[a]

Peak	δ (ppm)	Assignment: Proton	Assignment: Compound
1	9.74	—C*H*O	*n*-Alkanals (esanal)
2	9.63	—C*H*O	4-Hydroxy-*trans*-alk-2-enals
3	9.52	—C*H*O	Alka-2,4-dienals
4	9.48	—C*H*O	*trans*-Alk-2-enals (*trans*-hex-2-enal)
	8.44–8.53	—CHOO*H*	Hydroperoxydienes
	6.85	—CH=C*H*CHO	*trans*-Alk-2-enals
	6.53–6.55	—CHOOHCH=C*H*CH=	*cis,trans*-Hydroperoxydienes
	6.15	—C*H*=CHCHO	*trans*-Alk-2-enals
	5.94	CHOOHCH=CHC*H*=	*cis,trans*-Hydroperoxydienes
	5.74	CHOOHCH=CHC*H*=	*trans,trans*-Hydroperoxydienes

[a] Assignments made according to data from Refs 21–23 and 47.

complex phenols (aglycones from oleuropein and other olive glycosides) have a strong antioxidant and bitter activity. Recent studies, however, have demonstrated how Ty and Ty derivatives have only a small influence on oil stability; in contrast, the amounts of OHTy and OHTy derivatives (oleuropein aglycones) are strongly correlated with resistance to autoxidation.[2,4,5,51] Therefore, the ^{13}C NMR spectra of the polar fraction extracted from virgin olive oil (without any preliminary separation of components) can yield two types of information related to oil quality (Fig. 11).[21,24] First, the ratio between free and esterified Ty + OHTy in phenol extracts can be determined directly via ^{13}C NMR by comparing the intensity of the C-1′ carbon of free Ty + OHTy (signal D at 64.5 ppm) with the C-1′ signal of the corresponding esterified compounds (signal C at 66.8 ppm).[49] This simple measurement can contribute to the sensory quality evaluation (the esters of Ty and OHTy with elenolic acid have a strong pungent–bitter

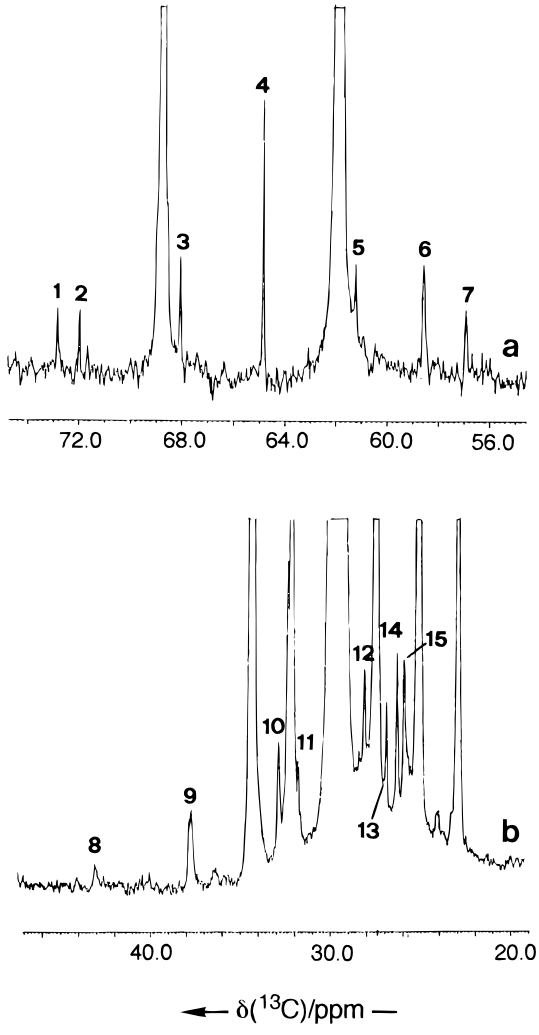
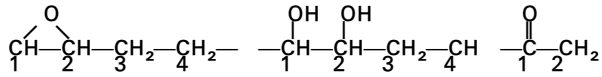


Figure 10. 100 MHz ^{13}C NMR spectrum of a virgin olive oil after 2 h thermal stressing at 180 °C. Labeled resonances are assigned in Table 10; unlabeled major peaks correspond to glycerol and methylene C-1–C-3 and ω1–ω3 carbons reported in Table 2.

Table 10. ^{13}C chemical shifts (δ) and tentative assignments of newly formed signals in the spectrum of virgin olive oil after thermal stressing at 200 °C for 6 h (see Fig. 10)[a]

Peak	δ (ppm)	Assignment: Carbon[b]	Assignment: Compound
1	72.84	C-1, C-2	Hydroxy acids
2	72.08	CH, *sn*-2	*sn*-1,2-Diacylglycerols
3	68.3	CH, *sn*-2	*sn*-1,3-Diacylglycerols
4	65.0	CH_2, *sn*-1,3	*sn*-1,3-Diacylglycerols
5	61.4	CH_2OH, *sn*-3	*sn*-1,2-Diacylglycerols
6	58.73	C-1, C-2	*trans*-Epoxides
7	56.82	C-1, C-2	*cis*-Epoxides
8	43.0	C-2	Oxo acids
9	37.6	C-3	Dihydroxy acids
10	32.7	$CH=CHCH_2$	all-*trans*-fatty acids
11	31.2	C-3	*trans*-Epoxides
12	28.1	C-3	*cis*-Epoxides
13	26.8	C-4	Dihydroxy acids
14	26.2	C-4	*cis*-Epoxides
15	25.8	C-4	*trans*-Epoxides and
		C-11 (ω9)	linoleyl

[a] Assignment made by comparison with data obtained on model compounds (from Ref. 31).
[b] Carbons are identified according to the following labeling:



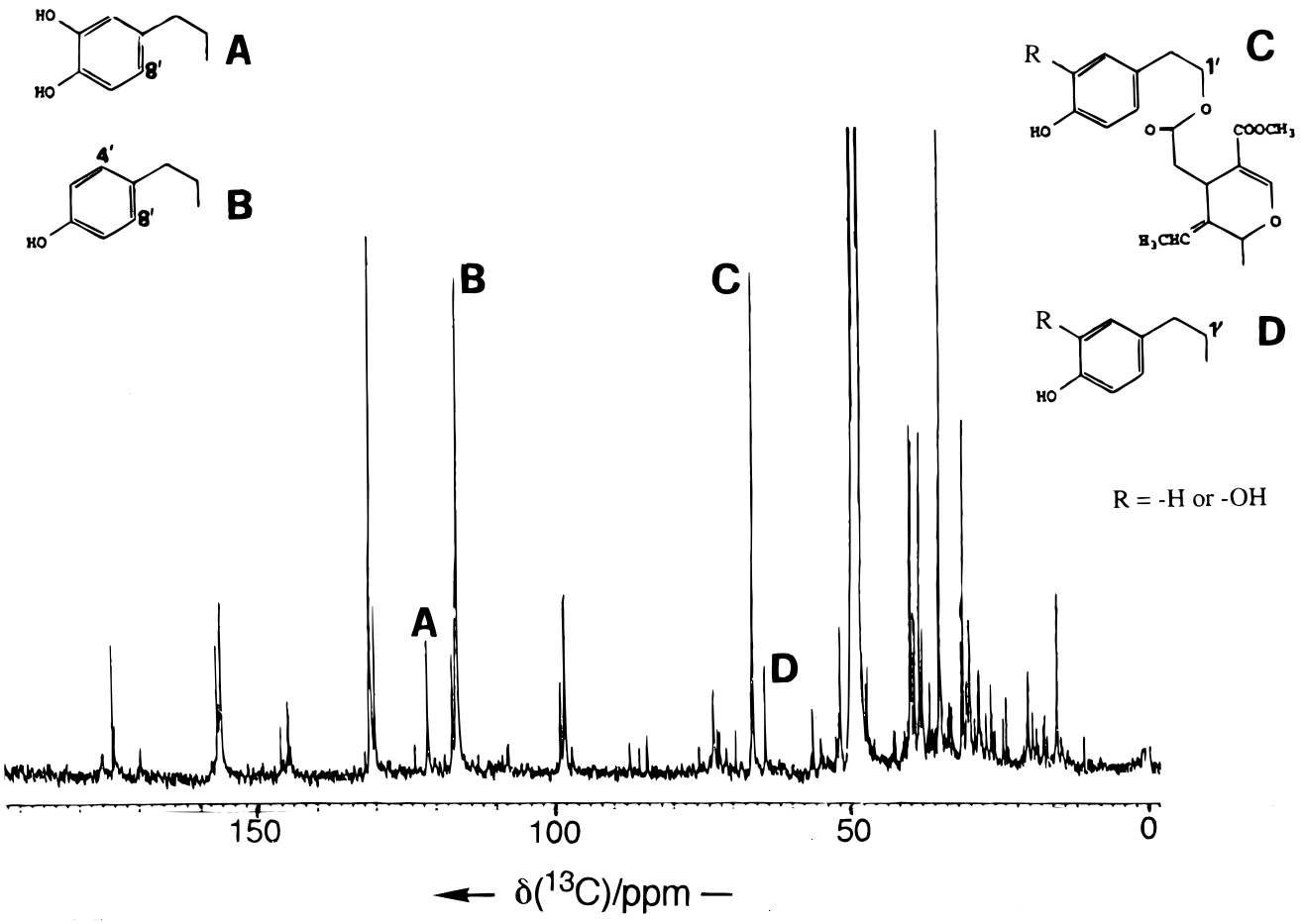


Figure 11. 100 MHz ^{13}C NMR spectrum of virgin olive oil polar fraction (phenolic compounds). Labeled resonances correspond to carbons indicated in the structures: A, B, C and D (assignment according to Ref. 49).

activity, not exhibited by free Ty and OHTy) of virgin olive oil and to the study of its evolution during oil shelf-life.[21]

Second, the molar ratio between total (free + esterified) *o*-diphenols (OHTy) and Ty can be calculated by comparing the intensities of aromatic signals at 121.3 ppm (signal A corresponding to C-8′ of OHTy) and at 116.1 ppm (signal B corresponding to C-4′ and C-8′ of Ty) (Fig. 11). This ratio is referred to the total (free or esterified) OHTy and Ty present in the polar fraction, the chemical shifts of these carbons being only slightly influenced by the ester bond or the hydroxyl group on C-1′.[49] The amount of total OHTy (determined by NMR and/or high-performance liquid chromatography) has been found to be correlated with the oil stability measured using accelerated oxidation tests and can be useful in predicting virgin oil shelf-life.[24]

Direct analysis of the main volatile components. The composition of volatile components in virgin olive oils is one of the most important aspects in defining virgin olive oil sensory quality. The green herbaceous flavour of extra-virgin olive oil is in fact attributed to several volatile compounds identified by gas chromatography–mass spectrometry, where the major role seems to be played by *trans*-hex-2-enal and hexanal.[52,53] Other aldehydes have been found to be strongly related to a deep-fried and rancid smell (alka-2,4-dienals).[53] A higher level of alcohols in virgin olive oils obtained by traditional pressing plants has also been found and related to the off-flavours due to fermentation occurring during olive storage and/or extraction.[54]

The very low concentration of volatile compounds in virgin olive oil makes their direct determination by ^{13}C NMR difficult. High-field (400–600 MHz) ^{1}H NMR, in contrast, can be a useful tool for evaluating the major volatile compounds (aldehydes, alcohols, acetic acid) directly in the oil solution.[23] The higher sensitivity of modern high-field spectrometers allows the determination of *n*-alkanals (hexanal, octanal, etc.), *trans*-alk-2-enals (*trans*-hex-2-enal, major natural volatile component of good quality virgin olive oils) and alka-2,4-dienals.[21,23,47] Acetic acid, arising from the olive fermentation and one of the compounds responsible for the wine-vinegar off-flavour, can be easily detected, and in addition to some other unidentified alcohol resonances can be observed in oils obtained by traditional pressing.[23]

CONCLUSIONS

Both ^{1}H and ^{13}C NMR spectroscopy offer new opportunities for assessing virgin olive oil quality and genuineness. The advantages of using high-resolution

^{1}H and ^{13}C NMR spectroscopy in virgin olive oil analysis have been briefly discussed. A large number of components can be simultaneously determined via NMR and the straightforward analysis of the major components has now been enhanced by the identification of some minor components related to virgin olive oil genuineness, sensory–nutritional quality and geographical origin. Other nuclei can also be used for this last purpose; as in the case of wine classification, deuterium should provide further information also in the field of virgin olive oil authentication.[55,56]

The results described in this review show the enormous potential of NMR techniques in olive oil analysis. The structure-specific analysis of different components indicating the genuineness and quality of the product is by itself a rapid and reliable methodology to be used in an official method in conjunction with other more traditional analytical techniques. In addition, the problem of quantitative response can be fully exploited after a more detailed assessment and standardization of the described procedures.

A more promising application which is just in the first stages concerns the problem of product geographical classification, in particular for oils with Protected Origin Denomination. For this purpose great efforts are required from different laboratories in different countries in order to built up reliable data sets for statistical evaluations.

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