A High-Field ^{1}H Nuclear Magnetic Resonance Study of the Minor Components in Virgin Olive Oils

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ABSTRACT: High-field (600 MHz) nuclear magnetic resonance (NMR) spectroscopy was applied to the direct analysis of virgin olive oil. Minor components were studied to assess oil quality and genuineness. Unsaturated and saturated aldehyde resonances, as well as those related to other volatile compounds, were identified in the low-field region of the spectrum by two-dimensional techniques. Unsaturated aldehydes can be related to the sensory quality of oils. Other unidentified peaks are due to volatile components, because they disappear after nitrogen fluxing. The statistical analysis performed on the intensity of these peaks in several oil samples, obtained from different olive varieties, allows clustering and identification of oils arising from the same olive variety. Diacylglycerols, linolenic acid, other volatile components, water, acetic acid, phenols, and sterols can be detected simultaneously, suggesting a useful application of high-field NMR in the authentication and quality assessment of virgin olive oil.
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KEY WORDS: Olive variety, quality, virgin olive oil.

The sensory and nutritional quality of virgin olive oil can be related to the presence of natural minor (volatile and nonvolatile) components arising from the olive drupe and present in virgin olive oils after the purely mechanical extraction from olives (1). The nonvolatile phenolic components play an important role in the nutritional characteristics and stability, in relation to their antioxidant activity (2), as well as for the sensory attributes of virgin olive oil, being responsible for the bitter and pungent throat-catching taste (3). However, the volatile components are the most important compounds in determining the sensory quality of virgin olive oils and their fruity flavor. Other minor components, related to oil degradation, arise from lipolysis (free fatty acids, partial glycerols, and linear alcohols) or from autooxidation (peroxides, aldehydes, etc.). These phenomena occur during harvesting and storage of olives, as well as during oil extraction and storage.

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The analytical definition of oil quality is actually based on the definition of lipid alterations, sensory profile, and detection of adulteration with foreign oils (seed oils and/or refined pomace and olive oils) (4). It has been shown recently that high-resolution nuclear magnetic resonance (NMR) spectroscopy (mainly ^{13}C at 50 and 100 MHz) is a technique able to detect virgin olive oil adulterations. In fact, virgin olive oils were distinguished from neutralized oils by NMR analysis of diacylglycerols (5). The determination of the content of saturated fatty acids in positions *sn*-1,3 and *sn*-2 was conducted under high digital resolution conditions while recording the spectra and led to the detection of synthetic esterified oils in mixtures with virgin olive oils (6). Additional information on virgin olive oil purity can be obtained by ^{13}C NMR analysis of the unsaponifiable fraction (7).

However, due its higher sensitivity, proton NMR at very high field (600 MHz) can be a more powerful technique for quality control of virgin olive oils. This is due both to its essential higher sensitivity as well as to its intrinsic linearity; as a consequence, much less analytical time is required and a higher precision is achieved. The purpose of this work is to show the potential of high-field proton NMR spectroscopy to furnish rapid information about minor components of virgin olive oil directly on the oil sample (i.e., without extraction or other concentration procedures). In particular, we focused on those compounds which serve as markers of virgin olive oil adulteration (linolenic acid, sterols, and partial glycerols) and on those related to oil quality and freshness (diacylglycerols, phenols, and aldehydic compounds).

EXPERIMENTAL PROCEDURES

Virgin olive oil samples were obtained from different olive varieties in different extraction plants located in Central Italy (Table 1). In this study, we used single variety oils produced from fruits picked from an eight-year-old olive orchard trained according to the IRO-CNR model (8) and located in Umbria, Italy. Drupes (25 kg) from 15 cultivars were picked when the fruit changed color and the flesh was still light-col-

TABLE 1
Origin, Variety of Cultivars, and/or Picking Day of the Drupes Whose Oil Has Been Studied

Origin	Variety of cultivars	Picking day	Sample
Fratta Todina	Dritta	Nov. 18	A
Fratta Todina	Frantoio	Dec. 16	B
Fratta Todina	N3	Nov. 18	C
Fratta Todina	S. Felice	Nov. 18	D
Fratta Todina	XII - 86	Nov. 18	E
Fratta Todina	Kalamata	Nov. 18	G
Fratta Todina	VI - 83	Nov. 18	H
Fratta Todina	Rayo	Nov. 18	I
Fratta Todina	Ascolana Semitenera	Nov. 18	M
Tuoro	Frantoio	Nov. 30	N
Tuoro	I - 77	Nov. 18	O
Tuoro	I - 77	Nov. 30	P
Tuoro	IV - 77	Nov. 18	Q
Tuoro	IV - 77	Nov. 30	R
Tuoro	FS 17	Nov. 18	U
Bettona	Toscanina	Nov. 18	S
Bettona	Coratina	Nov. 18	T

ored. In addition, samples from "I-77," "IV-77," and "Frantoio" were picked two weeks later at full ripening. Drupes were immediately processed for oil extraction in an experimental hammer mill, malaxed (30 min, 20°C), pressed at a maximum pressure of 300 bars, and the oil was separated by centrifugation. The selected cultivars were the following: (i) "Frantoio," "S. Felice," "Rajo," and "Dritta," typical cultivars from Central Italy that produce good quality oil; (ii) "FS-17" (Patent IRO CNR n.245 NV/88) and "N3," produced from breeding of "Frantoio"; (iii) "XII 86," "VI 83," and "IV 77," cultivars of unknown origin, which are considered ecotypes selected in several olive areas of Italy; their agronomical behavior is under study; (iv) "I-77" clone and "Toscanina," characterized by compact habit and high yield and used in a current study regarding high-density training systems in olive trees; (v) "Ascolana Semitenera" and "Kalamata," double purpose varieties; the latter is from Greece; (vi) "Coratina," typical cultivar from southern Italy, whose peculiar feature is a bitter-pungent taste and a marked oil flavor.

Oils (10 μL) were placed into 5-mm tubes and dissolved in a mixed solvent of chloroform-*d* (0.7 mL) and dimethyl sulfoxide-d6 (10 μL). This mixed solvent ensures perfect solubility of all minor oil components, even of those not soluble in pure chloroform. All NMR spectra were recorded on a Bruker (Karlsruhe, Germany) AMX600 spectrometer operating at 600.13 MHz. Two-dimensional totally correlated spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY) experiments were conducted to assign unknown resonances. Pulse sequences from the literature were used for the two-dimensional experiments (9,10): ^1H-^1H TOCSY: 512 × 512 data matrix size; number of scans (ns) = 64; dummy scans (ds) = 4; mixing time = 80 ms. NOESY: 512 × 512 data matrix; time domain 512 in F1 and 1024 in F2; rd = 2s; ns = 60; mixing time = 120 ms. Data were acquired and processed in the phase-sensitive mode (TPPI) (11).

Statistical data analysis was performed by the S-Plus statistical system (12). Multivariate data analysis included a principal components analysis and a variables selection method to select the independent variables most useful in differentiating the variety of oils, and a cluster analysis to reveal natural grouping of the samples. Because of a particular interest in volatile components, a few samples were fluxed with nitrogen; after this treatment, all resonances in the 8–10 ppm and 4.4–5 ppm disappeared.

RESULTS AND DISCUSSION

Figure 1 shows a 600.13 MHz ^1H NMR spectrum of a virgin olive oil; the main resonances have been assigned as shown in the figure and also in Table 2. For the same spectrum, some vertical expansions are also shown. This assignment was verified with a 2D-TOCSY experiment performed directly on the oil (Fig. 2). Different information available from this spectrum will be discussed separately.

Fatty acid composition. Fatty acid composition is the first feature for determining the purity of virgin olive oil. In fact, virgin olive oil contains a high amount (about 70–80%) of oleic acid (n-9, 18:1), a low level of linoleic acid (n-6, 18:2), and less than 1% linolenic acid (n-3, 18:3). A higher level of linolenic acid is considered one of the indices of seed oil addition, and this parameter is included in EEC regulation 2568/91 on olive oil classification (4). From the proton NMR spectrum, some information on fatty acid composition is available. One fact was defined by the basic work of Johnson and Shoolery (13). Operating at 60 MHz, measuring the olefinic proton integral, they determined the global unsaturation of oils and fats, which is a relevant parameter from a technological point of view. More recently, proton NMR spectrum has been used for quantitative determination of n-3 acids in fish oils (14).

In olive oil, the only n-3 acid present is linolenic; therefore, n-3 acids measured on the basis of the characteristic signal at 0.94 ppm (3H), labeled as E in Figure 1, furnish the level of linolenic acid directly, allowing direct detection of common seed oil addition (soybean, rapeseed, etc.). In olive

TABLE 2
Main Resonances in Olive Oil and Relative Assignment

Assignment	Attribution	^1H (ppm)
–CH=CH–	Triglycerides	5.30
–CH– glycerol	Triglycerides	5.22
–CH–	1,2 Diglycerides	5.07
$-CH_2-$ (α, α′) glycerol	Triglycerides	4.10; 4.25
$-CH_2-$ (α)	1,2 Diglycerides	4.29; 4.17
$-CH_2-$	1,3 Diglycerides	3.99
$-CH_2-$ (α′)	1,2 Diglycerides	3.66
–CH–	1,3 Diglycerides	4.07
–CH=CH–$\underline{CH_2}$–CH=CH–		2.73
–OCO$\underline{CH_2}$–		2.27
–$\underline{CH_2}$–CH=CH–$\underline{CH_2}$–		1.97
–OCOCH$_2\underline{CH_2}$–		1.57
$-CH_2-$		1.24
$-CH_3$	n-3 linolenic fatty chain	0.94
$-CH_3$		0.84

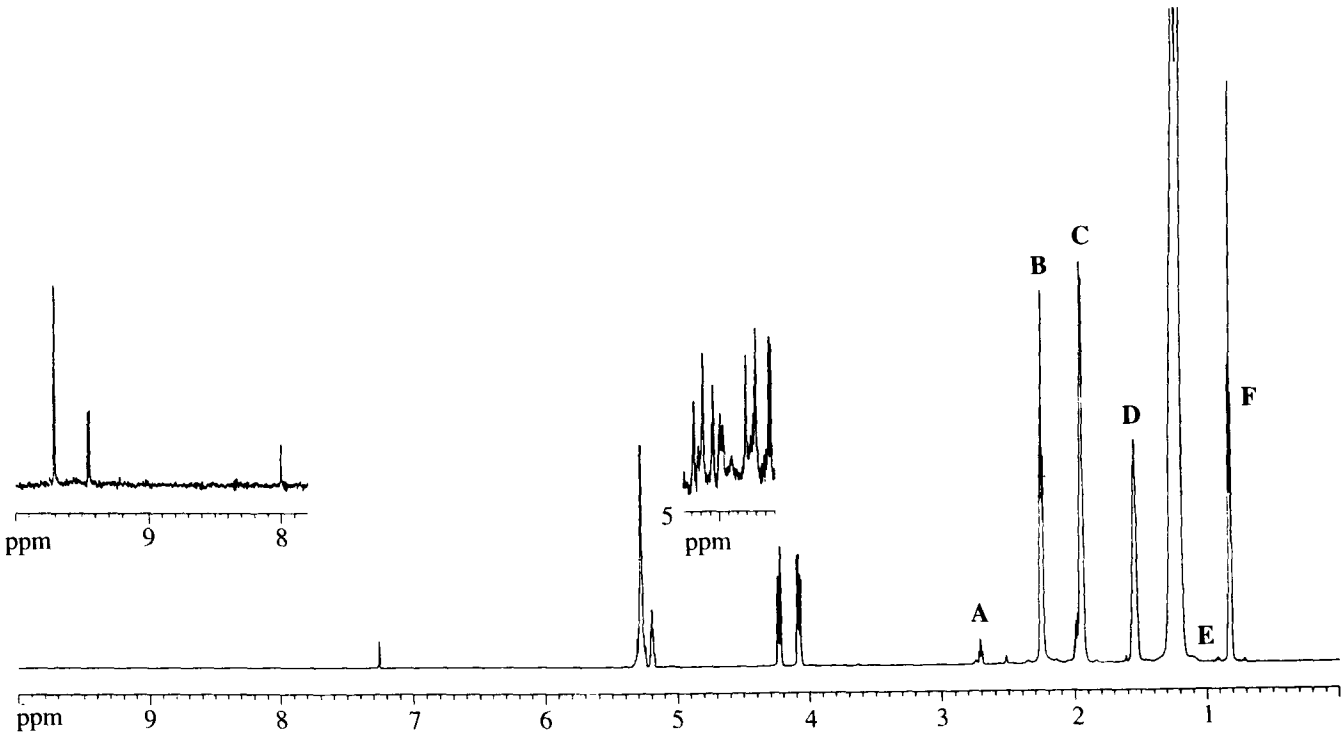

FIG. 1. 600.13-MHz ^{1}H nuclear magnetic resonance spectrum of virgin olive oil. Labeled peaks are identified as follows: A (diallylic protons); B (methylene protons bonded to C_2); C (allylic protons); D (methylene protons bonded to C_3); E (methyl protons of n-3 acids); F (methyl protons of fatty chain other than n-3). Vertical expansions show resonances of minor components.

oil, the signal at 0.94 ppm is quite low; the best way to perform a quantitative analysis by using this signal is to compare it with the nearby ^{13}C satellite of the main methyl resonance, labeled CF in Figure 3, whose amount is exactly 0.57% of the main methyl resonance, labeled F in Figure 3, i.e., 1.13% of ^{13}C natural abundance, split into a doublet with a ^{13}C-^{1}H one-bond coupling constant equal to 124.4 Hz. Thus, the total methyl F, necessary as normalization factor, can be obtained by the simple relation:

$$F = CF \times \frac{100}{0.57}$$

In this way, the amount of linolenic chains, expressed on a molar basis with respect to all fatty chains, can be obtained directly by integrating two resolved methyl resonances E and CF. In most of our samples, the content of linolenic fatty chains is rather constant, 0.51 ± 0.15, obtained by the relation:

$$\text{n-3 linolenic} = \frac{E}{E + F} \quad [1]$$

A detailed definition of the fatty acid profile can be obtained considering the relative intensities of methyl resonances (Fig. 1). In fact, from the intensity of methyl signal F at 0.84 ppm (3H) the global amount of saturated (STA) n-7 and n-9 monounsaturated acids (MUFA) and n-6 polyunsaturated (linoleic) fatty chains can be calculated (2):

$$\text{STA + MUFA + n-6 linoleic} = \frac{F}{E + F} \quad [2]$$

The relative level of MUFA and linoleic acids can then be determined by referring the allylic protons at 1.97 ppm (4H), labeled C in Figure 1, to all fatty acid chains measurable from the intensity of C_2 protons at 2.27 ppm (2H), labeled B in Figure 1:

$$\text{MUFA + n-3 linolenic + n-6 linoleic} = \frac{C}{2B} \quad [3]$$

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

The n-6 linoleic content can be determined by subtracting from the diallylic protons at 2.73 ppm (2H for n-6 linoleic and 4H for n-3 linolenic fatty chains) the relative amount of n-3 linolenic acid calculated from the methyl peaks as well as monounsaturated fatty chains (MUFA):

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

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

Saturated acids can be finally obtained as follows:

$$\text{Palmitic + stearic} = \frac{F}{E + F} - \left(\frac{C}{2B} - \frac{E}{E + F}\right) \quad [7]$$

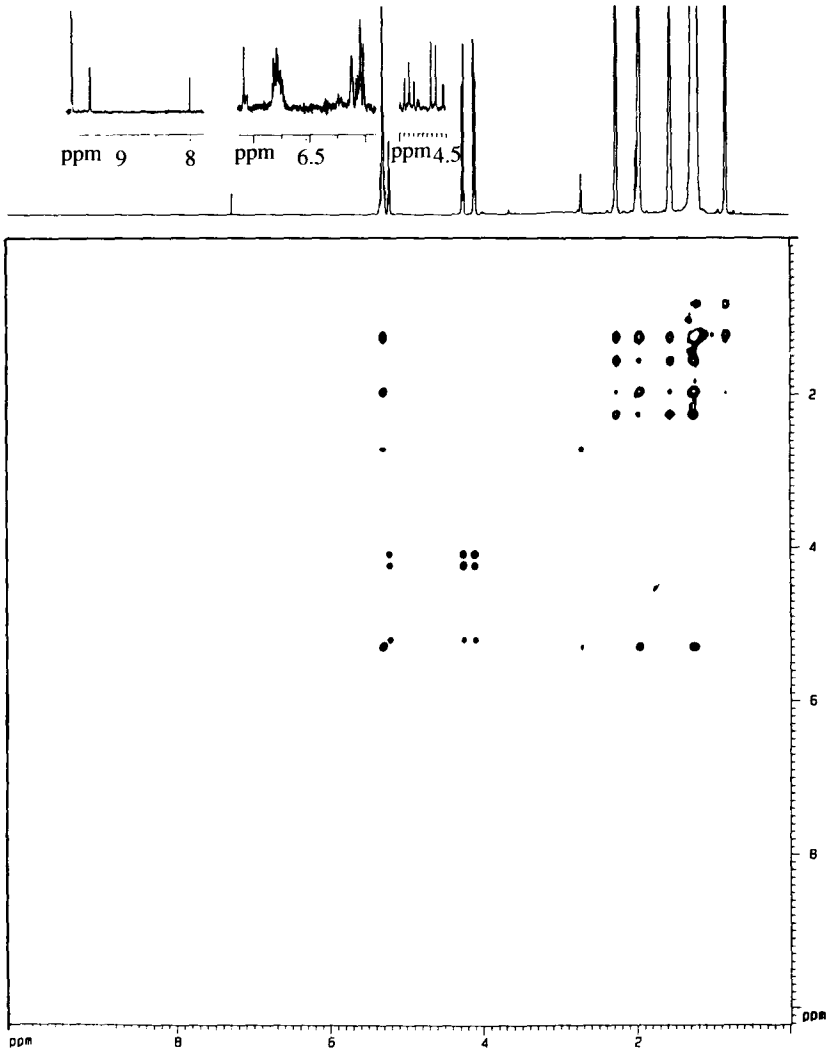

FIG. 2. Two-dimensional totally correlated spectroscopy spectrum (80 ms mixing time) of virgin olive oil. The one-dimensional spectrum at the top shows the resonances of some minor components: 8–10 ppm aldehydes; 5.8–7.2 ppm aldehydes and phenols; 4.5–5 ppm volatile compounds.

Sterols. Sterols can be quantified on the basis of the methyl resonance at 0.64 ppm (S) (15), which is the singlet resonating at higher field (Fig. 3). Again, a good quantitative analysis can be performed by direct comparison with ^{13}C satellites, i.e., with nearby signal CF. In our samples, the sytosterol content is rather constant, ranging from 0.06 to 0.14%.

Diacylglycerols and virgin olive oil quality. The amount of free fatty acids and the corresponding diglycerol profile can be used to define the degree of lipolytic alteration related to the quality of olives. Total diacylglycerols and the *sn*-1,2/1,3 diacylglycerol ratio can be determined by ^{13}C NMR (5). However, this measure is quite time-consuming due to long relaxation times of carbonyl resonances. The *sn*-1,2 and *sn*-1,3 indices studied in several virgin olive oil samples of different origins (Greece, Spain, and Italy) are influenced by the degree of olive ripening and oil storage (16). The 1,2/1,3 diacylglycerol ratio is strongly related to the quality-freshness of olive oils: young and good-quality olive oils contain mainly native *sn*-1,2 diacylglycerols and only small amounts of *sn*-1,3 diacylglycerols. The *sn*-1,3 diacylglycerols (of lipolytic origin) increased in the oils obtained from overripened olives or after several months' storage due to intramolecular transposition and/or lipolytic phenomena.

Medium-field proton NMR (200–400 MHz) has been used already to determine diacylglycerols by using an *in-situ* derivatization by trichloroacetylisocyanate (17). With a 600-MHz instrument, it was possible to resolve, without any derivatization, the glyceryl protons of *sn*-1,2 and *sn*-1,3 diacylglycerols, shown in Figure 4 and specified as follows:

CH_2–O–CO–R	4.29 ppm; 4.17 ppm
CH–O–CO–R	5.07 ppm
CH_2–OH	3.66 pm

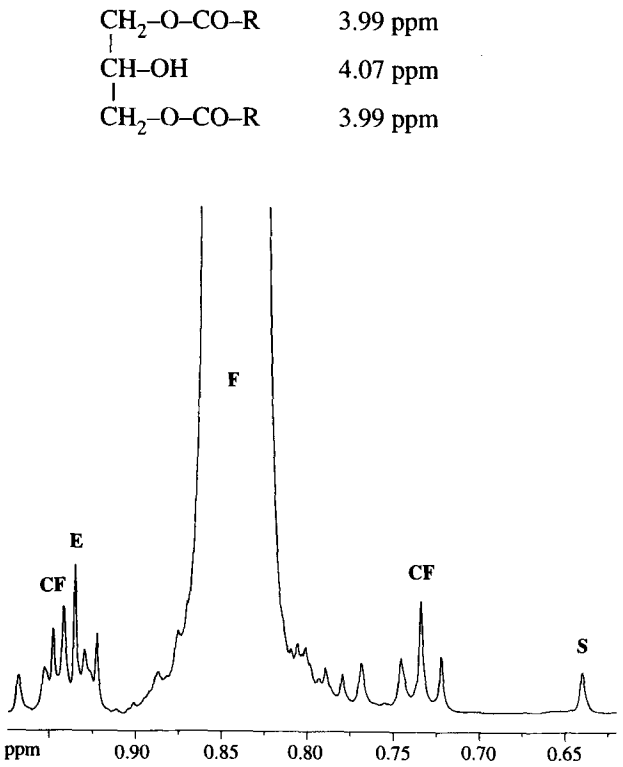

FIG. 3. Expansion of the methyl region in the 600.13-MHz ^{1}H nuclear magnetic resonance spectrum of virgin olive oil. Labeled peaks are identified as follows: E, n-3 polyunsaturated fatty acids; CF: ^{13}C satellites of main methyl resonance F; S: β-sytosterol.

This assignment was verified with a two-dimensional TOCSY experiment (Fig. 5). The differences in chemical shift with respect to previous literature (5,17) may be attributed to the solvent variation and also to the major resolution available in one-dimensional experiments compared with two-dimensional data. This finding suggests that the same information available from ^{13}C NMR can be obtained directly, rapidly and with higher precision from the 600-MHz proton spectrum.

Phenol compounds. Some minor resonances that can be assigned to phenol components of virgin olive oils have been singled out around 7 ppm (18). Bitter-pungent oil and sweet oil do in fact present major differences in the aromatic spectral region, near 7 ppm. A major difference in this spectral region was also observed in virgin olive oil compared with the same oil warmed up (200°C, for 5 min) (Fig. 6). Further work is in progress to characterize this region of the spectrum and to assign the observed resonances.

Volatile components: alcohols and aldehydes. Volatile components of olive oil have been the subject of several studies based on liquid and gas chromatography, followed by mass spectrometry analysis or olfactometry, to identify the relationship between identified compounds and sensory properties (19–21). *Trans*-2-hexenal, representing more than 50% of the headspace in extra virgin olive oils, has been associated with a positive sensory impact (herbaceous), which contributes to the olive fruity flavor. On the other hand, other unsaturated aldehydes, arising from the oxidation of unsaturated fatty acids, have been indicated as responsible for unpleasant

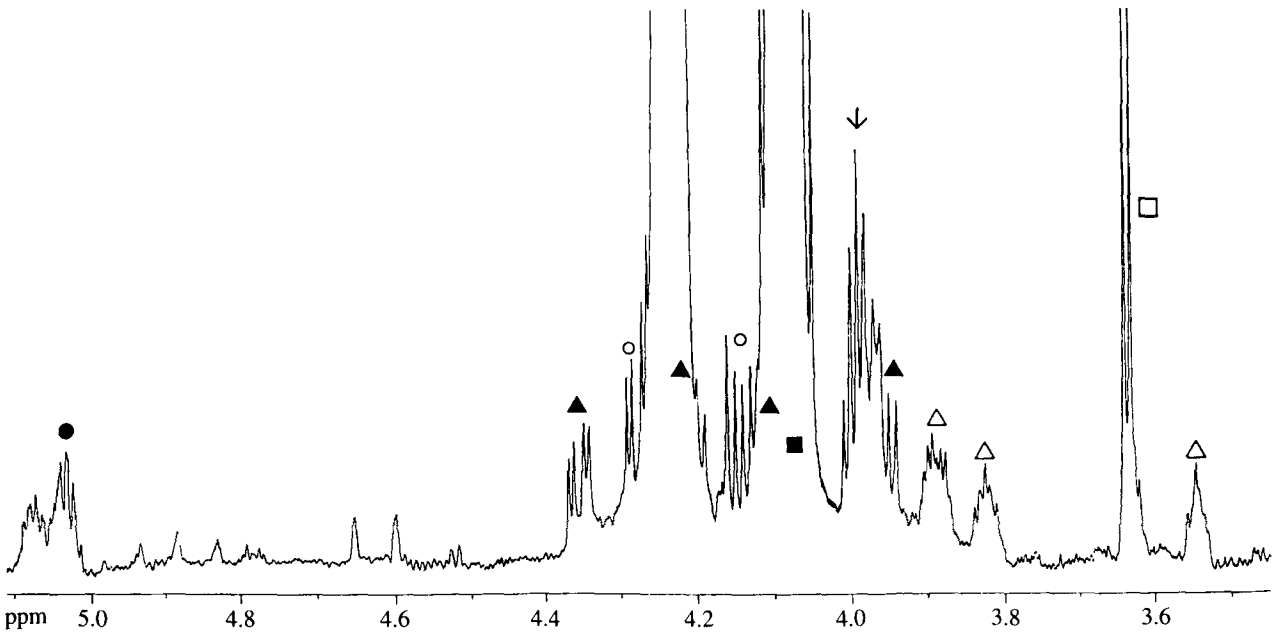

FIG. 4. Expansion of the 3.5–4.5 ppm region in the 600.13-MHz ^{1}H nuclear magnetic resonance spectrum of virgin olive oil. The strong resonances, out of scale, are due to α,α′ protons of triacylglycerols; their ^{13}C satellites are marked with an filled triangle. At 5.07 ppm, the CH of *sn*-1,2 diacylglycerols is marked with a filled circle. At 4.29 and 4.17 ppm, the α-CH_2 of *sn*-1,2 diacylglycerols are marked with an open circle. At 4.07 ppm, the CH of *sn*-1,3 diglycerols is marked with a filled square. At 3.99 ppm, the CH_2 of *sn*-1,3 diacylglycerols are marked with an arrow. At 3.66 ppm, the α′-CH_2 of *sn*-1,2 diacylglycerols are marked with an open square. Resonances marked with an open triangle are due to saturated alcohols present as minor components.

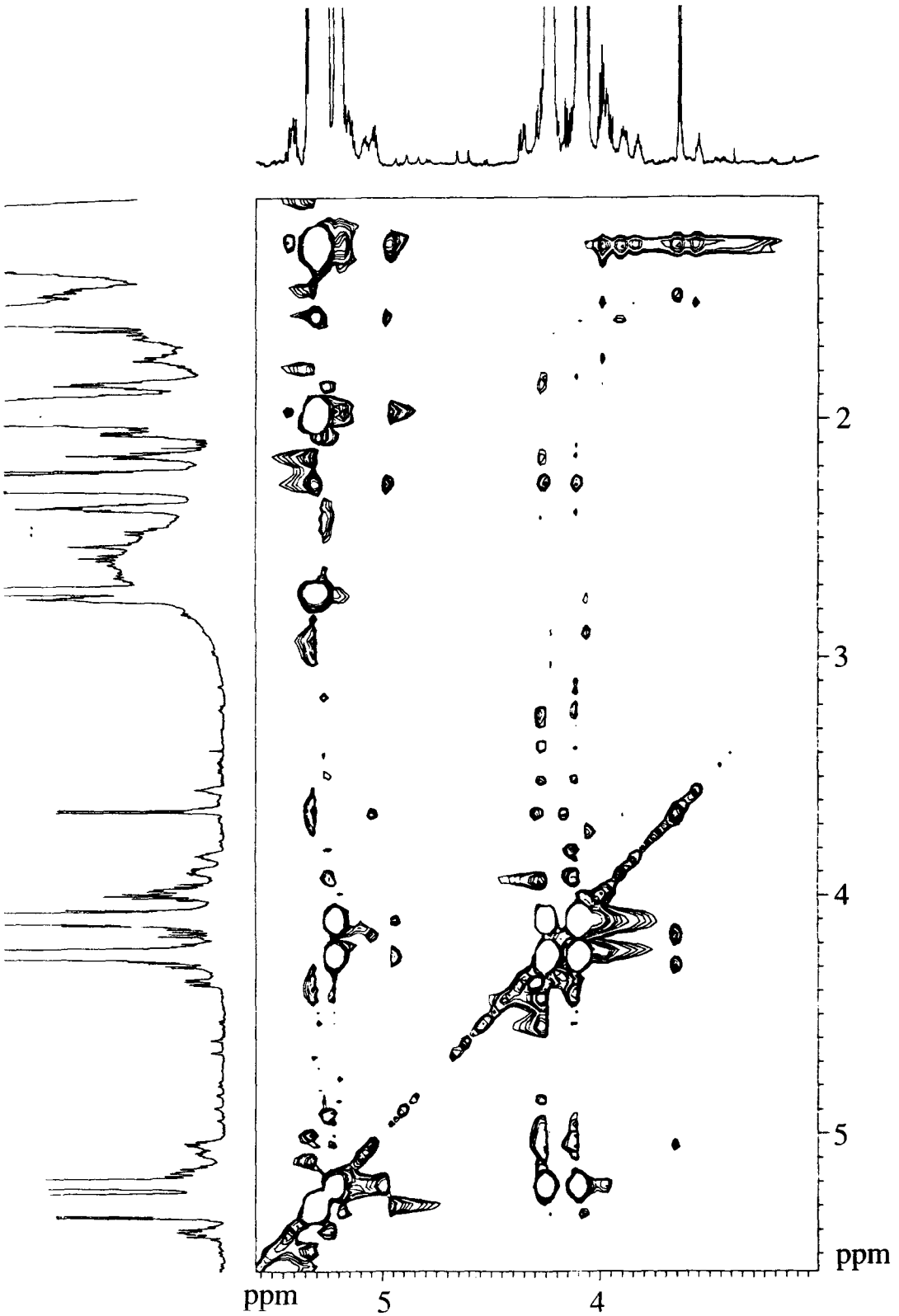

FIG. 5. Slice of the 3.3–5.2 ppm region in the totally correlated spectroscopy experiment. Cross peaks, due to *sn*-1,3 and *sn*-1,2 diacylglycerols and saturated alcohols, are observable.

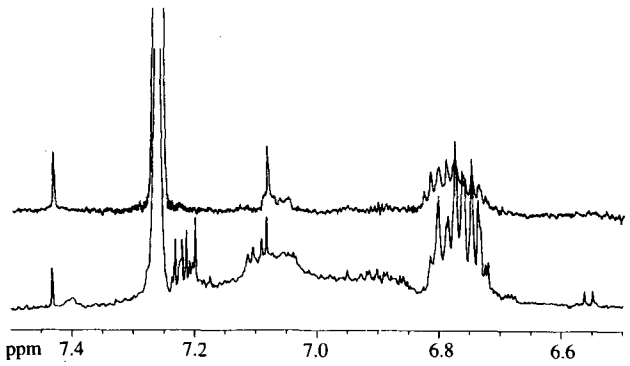

FIG. 6. Spectral region of aromatic compounds in the 600.13-MHz ^{1}H nuclear magnetic resonance spectrum of virgin olive oil. Upper trace refers to a virgin olive oil, lower trace to an oil warmed up for 5 min at 200°C.

characteristics (rancidity) (19–21). Other compounds, such as alcohols, are also involved in the formation of olive oil flavor (21). In the two-dimensional TOCSY experiment shown in Figure 5, some minor peaks present in Figure 4 show characteristic cross peaks only with resonances in the 1.2–1.5 ppm region. All of these minor signals can be identified as saturated alcohols (see peaks labeled with an empty triangle in Fig. 4). Aldehydic protons, in turn, resonate in the low-field region (Fig. 7 and Table 2). Figure 7 shows the expansion of the aldehyde spectral region (8–10 pm) of a virgin olive oil sample. Here, the lower-field broad singlet at 9.74 ppm is due to saturated aldehydes, such as hexanal and heptanal. The triplet with a small splitting observable in pure compounds is not resolved due to small chemical shift differences between linear aldehydes from butanal on. In the lower field of this main resonance, small peaks can be observed and attributed to propanal and saturated ramified aldehydes (Table 2, data from pure aldehydes). The doublet (J = 7.96 Hz) centred at 9.46 ppm is due to *trans*-2-hexenal and other related unsaturated aldehydes. This was confirmed by direct comparison with the standard compound data and a two-dimensional TOCSY experiment (Fig. 8).

Other minor unassigned peaks appear in the one-dimensional spectrum. Among these is the strong singlet at 8.07 ppm (Fig. 7). To identify this singlet, a NOESY experiment

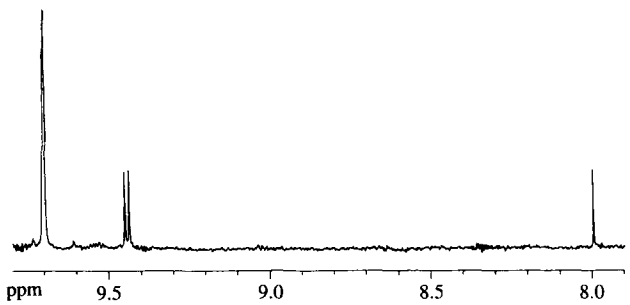

FIG. 7. Expansion of the spectral range 7.8–10 ppm in the 600.13-MHz ^{1}H nuclear magnetic resonance spectrum of virgin olive oil. Resonances due to aldehydic protons are clearly observable.

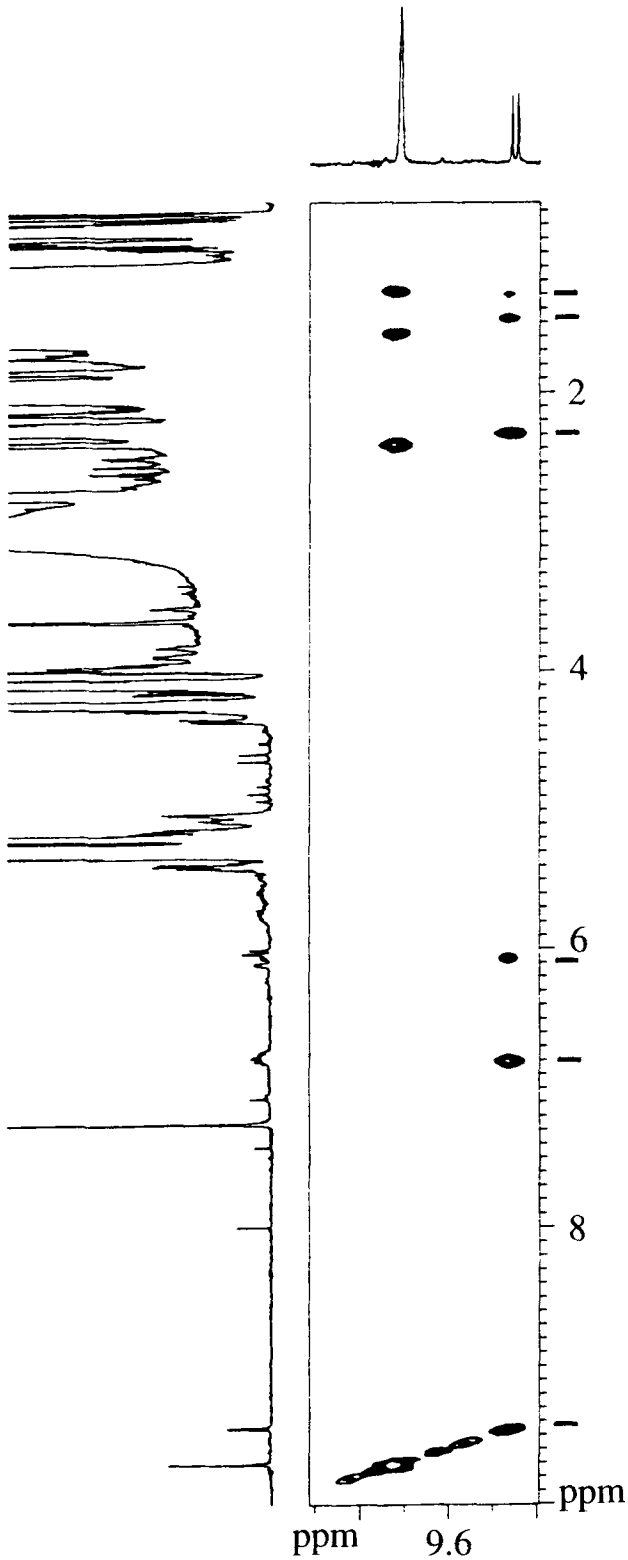

FIG. 8. Slice of the aldehydic region in the totally correlated spectroscopy experiment. Cross peaks marked with a bar are due to *trans*-2-hexenal and other linear unsaturated aldehydes.

was performed. As shown in Figure 9, the 8.07 ppm resonance presents a strong negative cross peak with a resonance at 4.96 ppm. While attributing these resonances reliably is

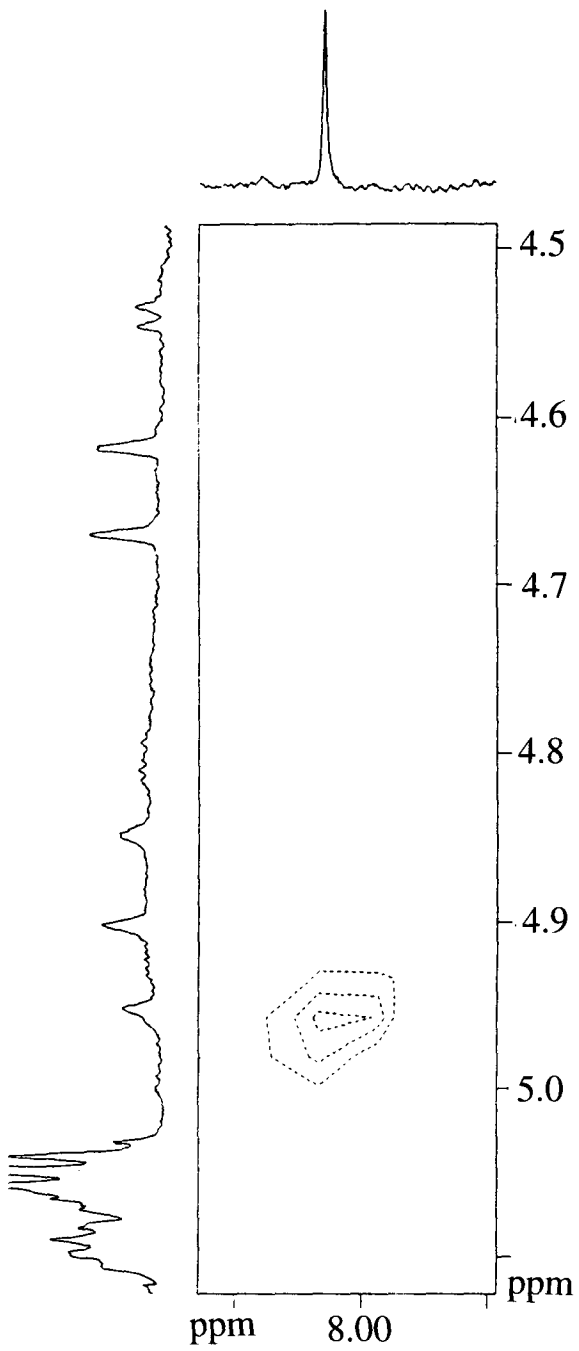

FIG. 9. Nuclear Overhauser effect spectroscopy (NOESY) experiment: slice relative to the 8.07-ppm unknown resonance. Statistical analysis proved the interdependence of the 8.07 and 4.96 ppm signals. The NOESY negative cross peak confirms that these resonances belong to the same molecule.

still impossible, a volatile compound, maybe with an hemiacetalic group, can be imagined, related in some way to a particular taste or flavor of the oil. Between 4.5 and 5 ppm, a spectral region can be found that is clear of any resonance related to the main glyceridic components of oil. Here, probably due to unsaturated alcohols, few resonances can be observed, with different intensities in different samples, shown in the histograms of Figure 10. The volatile nature of compounds resonating in the 9–10 ppm range, as well as in the 4.5–5 ppm range, has been established by fluxing virgin olive oil with nitrogen at room temperature. All of these signals disappeared after only 5 min of nitrogen.

Because the fruity sensation of virgin olive oil can be related to a series of different volatile compounds (21), it was thought interesting to evaluate quantitatively the amount of volatile compounds, even if the sensory impact and threshold for each compound can be significantly different. To this purpose, a careful base-line correction was performed in proton NMR spectra, followed by a quantitative evaluation of all peaks, compared with the methylene resonance at 1.26 ppm

TABLE 3
Chemical Shifts and Relative Assignment of Aldehydes[a]

Aldehyde	Cnr.	Type	^{1}H (ppm)	Aldehyde	Cnr.	Type	^{1}H (ppm)
Propanal	1	COH	9.77	Endecanal	1	COH	9.74
	2	CH_2	2.44		2	CH_2	2.39
	3	CH_3	1.06		3	CH_2	1.60
					4–10	$(CH_2)_7$	1.24
					11	CH_3	0.86
Butanal	1	COH	9.74	Dodecanal	1	COH	9.74
	2	CH_2	2.39		2	CH_2	2.39
	3	CH_2	1.63		3	CH_2	1.60
	4	CH_3	0.95		4–11	$(CH_2)_8$	1.25
					12	CH_3	0.86
Pentanal	1	COH	9.74	Tridecanal	1	COH	9.74
	2	CH_2	2.40		2	CH_2	2.39
	3	CH_2	1.60		3	CH_2	1.59
	4	CH_2	1.34		4–12	$(CH_2)_9$	1.23
	5	CH_3	0.91		13	CH_3	0.86
Hexanal	1	COH	9.74	Tetradecanal	1	COH	9.74
	2	CH_2	2.39		2	CH_2	2.39
	3	CH_2	1.61		3	CH_2	1.60
	4, 5	$(CH_2)_2$	1.30		4–13	$(CH_2)_{10}$	1.23
	6	CH_3	0.88		14	CH_3	0.86
Heptanal	1	COH	9.74	Hexenal	1	COH	9.46
	2	CH_2	2.40		2	CH	6.07
	3	CH_2	1.60		3	CH	6.81
	4–6	$(CH_2)_3$	1.30		4	CH_2	2.27
	7	CH_3	0.86		5	CH_2	1.50
					6	CH_3	0.92
Octanal	1	COH	9.74	3-Methyl-	1	COH	9.73
	2	CH_2	2.39	butanal	2	CH_2	2.28
	3	CH_2	1.61		3	CH	2.18
	4–7	$(CH_2)_4$	1.30		4, 5	$(CH_3)_2$	0.96
	8	CH_3	0.86				
Nonanal	1	COH	9.73	2-Methyl-	1	COH	9.62
	2	CH_2	2.39	propanal	2	CH	2.41
	3	CH_2	1.60		3, 4	$(CH_3)_2$	1.11
	4–8	$(CH_2)_5$	1.25				
	9	CH_3	0.85				
Decanal	1	COH	9.74	Benzalde-	1	COH	10.01
	2	CH_2	2.39	hyde	ortho	CH	7.87
	3	CH_2	1.59		metha	CH	7.52
	4–9	$(CH_2)_6$	1.25		para	CH	7.62
	10	CH_3	0.85				

[a]Nuclear magnetic resonance spectra of standard aldehydes have been obtained in the same mixed solvent ($CDCl_3$ + dimethylsulfoxide) used for the oil. Chemical shifts are given in ppm from trimethylsilyl, but were actually measured from the residual signal of chloroform, assumed at 7.26 ppm.

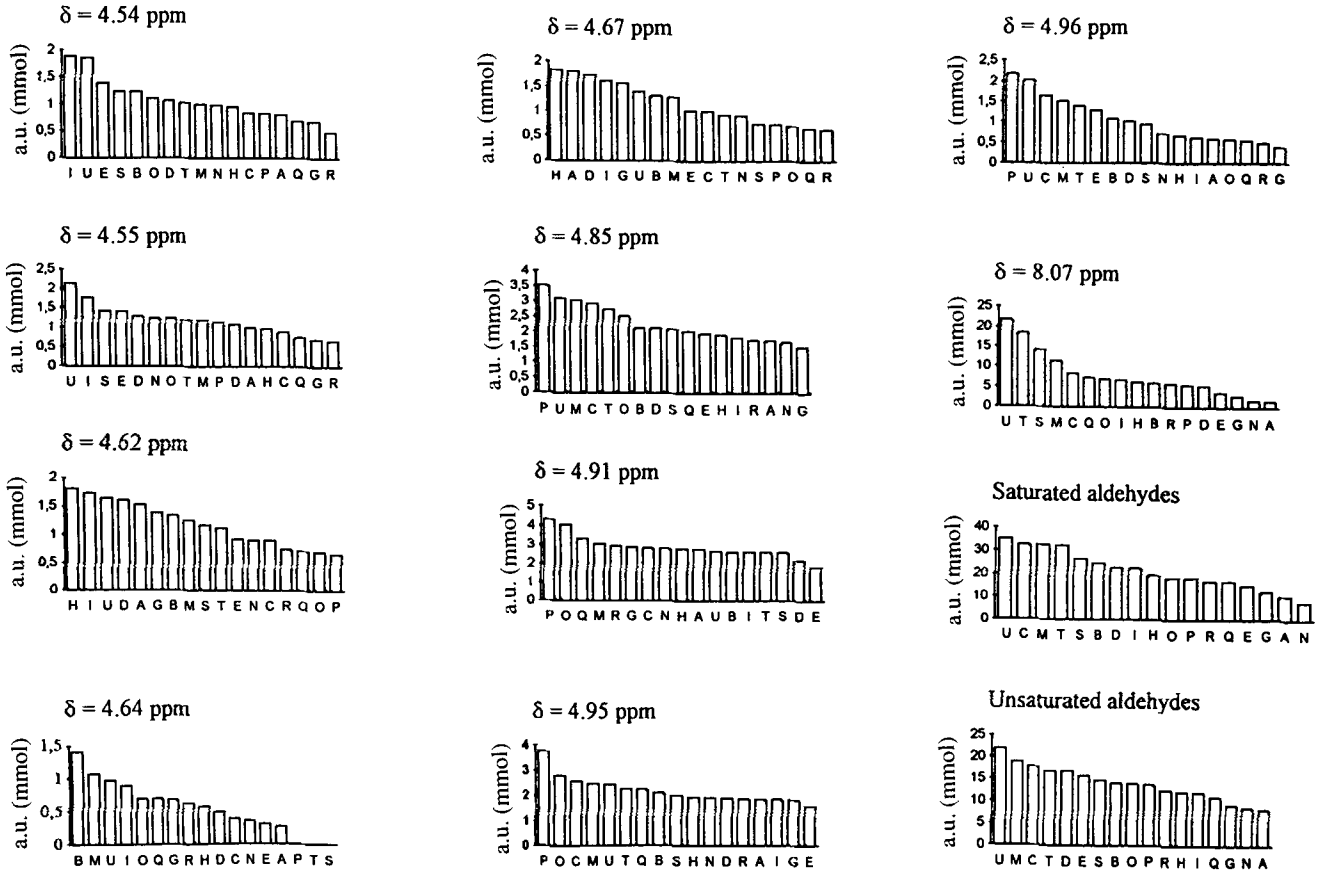

FIG. 10. Histograms relative to the intensities of resonances due to volatile compounds in different virgin olive oils.

(normalized to 10,000, to give an index that is proportional to the molar ratio between each volatile compound and total fatty chains). The signal's intensity, proportional to the molar concentration of each compound, has been evaluated in the spectra of different olive oil samples obtained from olives of different variety and degree of ripening (Table 3). A statistical analysis was then performed to assess the relation between minor components and olive ripeness and variety. The aims of the statistical analysis were twofold: A) to select the most important frequency bands, i.e., those that have the highest discrimination power to distinguish between different oils; and B) to classify oils with respect to the selected frequency bands. To satisfy the first aim, a principal component analysis was performed and nonredundant variables (frequency bands) were selected according to the method described by Mardia *et al.* (22). The number n of selected variables is chosen by the following iterative procedure: (i) let n be the number of measured variables minus one; (ii) choose n variables according to (22); (iii) classify the oils with the methods described in (B), applied to the n variables chosen in step (ii); (iv) if the classification is the same as that given by retaining all variables, then put $n = n - 1$ and go to (ii); otherwise, put $n = n + 1$ and stop. Given n variables, the oils were classified by using a hierarchical method of cluster analysis (22). Several hierarchical methods have been tried, coupled with different choices of the distances of the distance function. The results were stable with respect to both the method and the distance function used. Therefore, the single linkage method, based on the Euclidean distance, was routinely used as shown in Figure 11. The procedure described above has been implemented in the S language (23) and makes use of macros (principal components analysis, hierarchical clustering) belonging to the S-Plus system (12). Data obtained by means of high-field ^1H NMR allowed the discrimination of oils coming from different olive varieties grown in the same environment and picked at the same ripening stage.

As shown in Table 4, samples O–P and R–Q give quite similar values for some signals. They belong to the same olive oil variety harvested within two weeks. Signals between 4.62 and 4.96 ppm seem to be related to the olive variety. Other signals, such as 4.54 and 4.55 ppm, showed a variation that could be due to degree of ripening. The ratio between *trans*-2 hexenal and hexanal showed significant variations for the same variety (Table 3). Considering different ripening stages in the cultivars studied (i.e., "Frantoio"), we observed that the distribution of volatile compounds is also related to the ripening stage of olive fruits. Thus, our observations are in agreement with previous findings regarding the composition of volatiles (19) and phenolic derivatives (24,25). In particular, oils from the "FS-17" clone, characterized by an early oil ac-

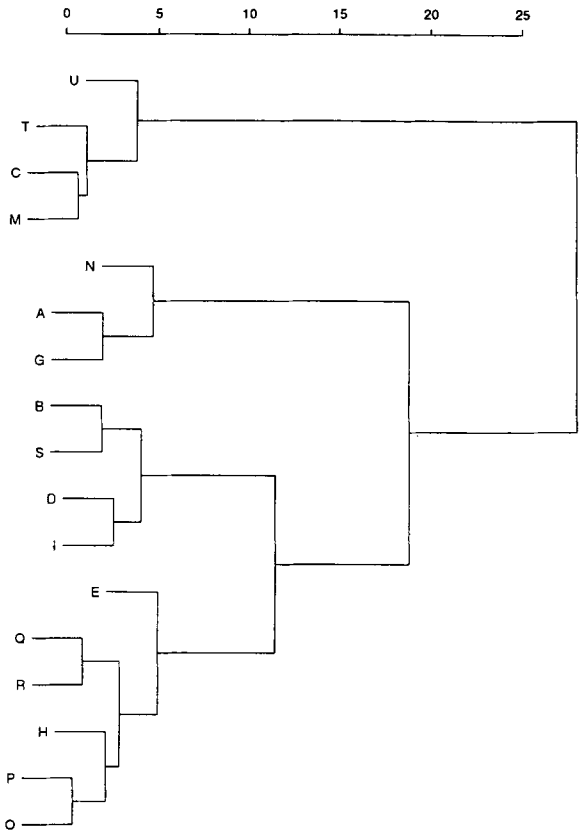

FIG. 11. Clustering of virgin olive oils based on peak intensity of volatile compounds resonances in the 600.13-MHz ^{1}H nuclear magnetic resonance spectrum.

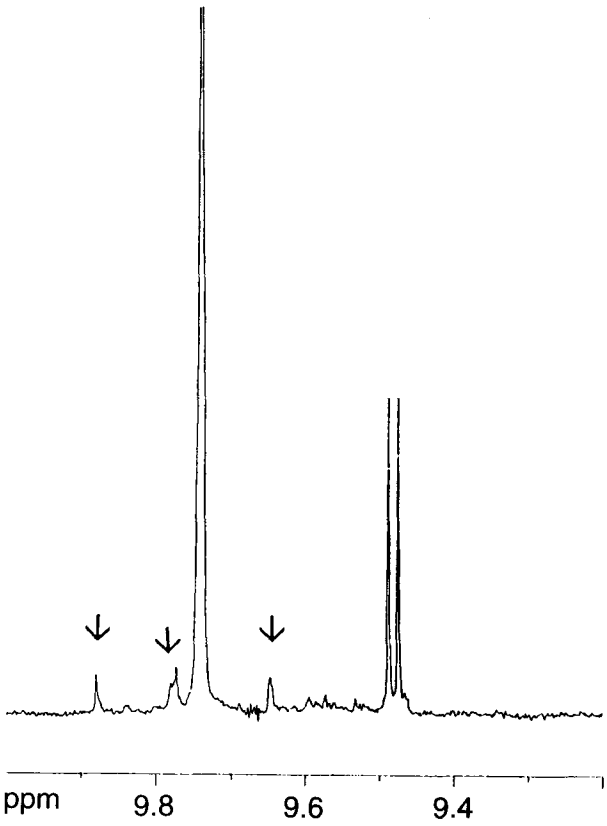

FIG. 12. 600.13-MHz ^{1}H nuclear magnetic resonance spectral region of aldehydic protons for a sample presenting a rancid smell. Small signals marked with an arrow are absent in nonrancid samples.

cumulation process (26), and "Coratina," "Ascolana Semitenera," and "Toscanina" cultivars, characterized by their strong flavor, besides a high level of hexenal show a general high intensity of resonances related to the presence of other volatile compounds in the range 4.5–5 ppm and 8.07 ppm. The profile of aldehydic compounds in virgin olive oils can also be followed during storage to evaluate the shelf life of oils. The presence of other peaks in the aldehyde profile is obvious when the oil presents a rancid smell (Fig. 12). These newly formed compounds can probably be related to lipid autooxidation as well as the modification of the ratio between unsaturated and saturated aldehydes.

Water content. From the proton NMR spectrum obtained in pure chloroform-*d* solution, other information can be obtained; in fact, the water content can be evaluated directly from the broad signal at 1.59 ppm (Fig. 13). The measure of

TABLE 4
Normalized Intensity of Resonances Due to Volatile Compounds

Sample	P	U	C	M	T	E	B	D	S	N	H	I	A	O	Q	R	G
4.96 ppm	2.18	2.02	1.65	1.51	1.40	1.29	1.08	1.01	0.93	0.71	0.67	0.63	0.59	0.57	0.55	0.50	0.36
4.95 ppm	3.77	2.44	2.56	2.47	2.26	1.54	2.09	1.87	1.99	1.89	1.91	1.82	1.83	2.80	2.24	1.84	1.79
4.91 ppm	4.32	2.69	2.84	3.05	2.64	1.82	2.64	2.19	2.62	2.82	2.81	2.64	2.80	4.06	3.34	2.95	2.88
4.85 ppm	3.52	3.09	2.92	3.03	2.72	1.95	2.12	2.12	2.10	1.68	1.91	1.82	1.73	2.51	2.03	1.74	1.52
4.67 ppm	0.74	1.40	1.01	1.29	0.94	1.02	1.32	1.73	0.75	0.92	1.83	1.61	1.80	0.71	0.67	0.64	1.56
4.64 ppm	0.0	0.97	0.39	1.07	0.0	0.31	1.41	0.49	0.0	0.36	0.57	0.89	0.27	0.70	0.70	0.62	0.68
4.62 ppm	0.66	1.64	0.91	1.27	1.13	0.93	1.35	1.61	1.18	0.91	1.81	1.73	1.53	0.71	0.72	0.75	1.39
4.55 ppm	1.14	2.14	0.89	1.18	1.19	1.41	1.29	1.08	1.42	1.23	0.98	1.76	1.00	1.23	0.75	0.66	0.70
4.54 ppm	0.83	1.85	0.84	1.00	1.03	1.38	1.23	1.07	1.23	0.98	0.95	1.88	0.81	1.11	0.68	0.48	0.68
Hexenal																	
9.46 ppm	6.86	10.95	8.92	9.41	8.43	8.83	7.02	8.42	7.34	4.33	6.07	6.01	4.11	6.96	5.51	6.26	4.58
Hexanal																	
9.72 ppm	18.20	34.98	32.58	32.55	32.3	14.96	24.52	22.67	26.37	7.83	19.45	22.58	10.63	18.33	16.71	16.87	12.48
8.07 ppm	5.59	21.75	8.32	11.48	18.43	3.73	6.05	5.39	14.16	1.73	6.23	6.67	1.66	6.90	7.29	6.00	2.88

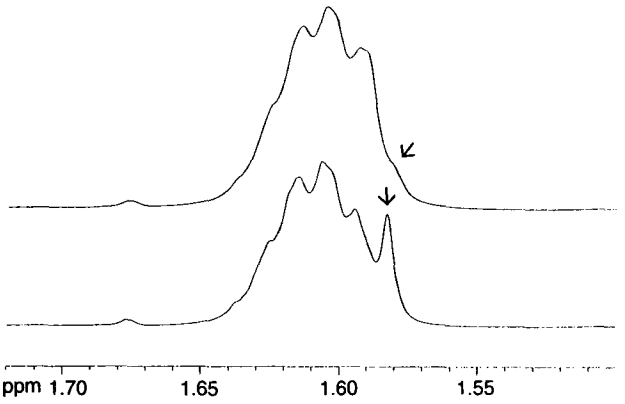

FIG. 13. Expansion of the 600.13-MHz ^{1}H nuclear magnetic resonance resonances used for evaluation of the water content in different virgin olive oils. The signal marked with an arrow is due to the water resonance.

water content can be relevant because a high level of dispersed water can enhance degradation phenomena during storage with the formation of off-flavors, such as those of "wine-vinegar" and "acid" (also arising from the fermentation of olives that are stored for a long time or are overripe), which compromise the organoleptic quality of virgin olive oil. A sample of oil, characterized by a clear vinegar off-flavor, was also analyzed, and acetic acid resonance was clearly singled out.

The 600-MHz proton NMR spectra of virgin olive oils show a large number of structural and compositional data related to oil quality. Diglycerols can be determined, thus allowing the definition of oil freshness and also verification of the quality of the starting material (olive ripeness) and the extraction technology. Fatty acid composition can be obtained accurately by comparing minor signals with those due to ^{13}C satellites of major resonances. In this way, the quantitative measure of other minor components, such as linolenic acid or sterols, can be obtained. Furthermore, for virgin olive oil, analysis of the aldehyde profile seems to be interesting for a rapid and structure-specific evaluation of both herbaceous flavor intensity and oxidative degradation. The possibility of determining other minor components, such as phenols or alcohols, without any extraction procedure or chemical derivatization appears to be promising and opens new application possibilities for the instrumental quality assessment of virgin olive oils. Moreover, potential information for the definition of source derivation of virgin olive oil might arise from the careful identification of alcohols and other volatile components. The comparison of NMR data with other instrumental techniques, such as gas chromatography and olfactometry, as well as the analysis of quantitative NMR data by multivariate statistic methods, is currently in progress.

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