Identification and Classification of Olive Oils by High-Resolution ^{13}C Nuclear Magnetic Resonance

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Unsaponifiable matter from 19 olive and olive pomace oils were studied by high-resolution ^{13}C nuclear magnetic resonance spectroscopy. Their spectra showed characteristic peaks that corresponded to molecular substructures rather than the individual constituents present in the unsaponifiable matter. The presence of squalene and other hydrocarbons, sterols and triterpenic alcohols, in addition to other groups of minor compounds, were observed. Based on the analysis of these spectra, it was possible to distinguish among different grades of olive oils by using stepwise discriminant analysis. This direct method of analysis is suggested to be used in artificial neural networks to define oil identity and quality.

KEY WORDS: Artificial neural networks, high-resolution ^{13}C NMR, oil quality, olive oil, olive pomace oil, pattern recognition, stepwise discriminant analysis, unsaponifiable matter.

The identification and classification of olive oils is complicated due to the nature of olive oil. Under ideal conditions, an "extra virgin" olive oil is obtained by purely mechanical extractive means from sound, ripe fruits of the olive tree, with a free fatty acid content below 1%. However, olives themselves are an expensive fruit and must be harvested in timely fashion and with some care to avoid bruising the fruit. Consequently, growers and processors are understandably loathe to allow the least morsel of oil to escape the battery of pressing and extraction processes that have evolved to capture all of the oil. These procedures generate a range of olive oils in addition to the above described "extra virgin." Thus, other olive oils with good flavor but greater acidity may be graded as "fine" or "semifine," and lower grades, including those that have been subjected to refining, are called "lampante" or "pure." In addition, after the initial pressings, the olive fruits are often extracted with organic solvents, typically hexane, to afford "crude olive pomace oil." This grade is generally in need of refining to achieve palatability. Crude olive pomace oil after refining is reduced to 0.5% acidity and may be blended with virgin grades to yield "olive pomace oil". This plethora of possible grades of olive oil, in addition to others seldom encountered in commerce, have been described earlier by Gracián (1) and other authors.

In addition to the several grades of olive oil, commercial olive oil may also be adulterated with other seed oils because of the higher price of olive oil in comparison with other vegetable oils (2). This possibility greatly increases the number of olive oil grades, and numerous studies have been carried out to develop methods that allow to distinguish among them. Many of these methods are based on classical oil analysis, including determination of sterol and triterpenic alcohol compositions or palmitic acid content in the 2-position of the glycerides (3), and, more recently, triglyceride (4) and hydrocarbon (5) analyses have also been considered. However, they are time-consuming, and development of a quick method with widespread usage is desirable. In this respect, the use of on-line liquid chromatography–gas chromatography (LC–GC) for the analysis of the minor components (6), the introduction of artificial neural networks (7), and the use of direct methods of analysis, such as Curie-point pyrolysis mass spectrometry with multivariate data analysis and artificial neural networks, have been suggested (8).

This study was carried out to develop a new method for the identification and classification of olive oils by high-resolution ^{13}C nuclear magnetic resonance (^{13}C NMR). High-resolution NMR, and specially ^{13}C NMR, is used increasingly as a technique to provide insight into the nature of the mixtures present in natural oils and fats and in other lipids (9). Thus, several authors have shown that the chemical shifts for signals associated with the acyl carbon atoms (C1) depend on whether the chain is α- or β-linked to glycerol and on the nature of the unsaturation in the chain, if any (10,11). All these studies have been focused on the triglycerides of oils and have brought some new light on the triacyl composition of oils. However, no studies have been reported on the unsaponifiable fraction of the oil. Quantitative analysis of certain classes of minor components is one of the chief methods for determining an oil's authenticity or for distinguishing between expressed and solvent-extracted oils (12). An NMR study of this unsaponifiable fraction may help in determining grades of olive oils.

MATERIALS AND METHODS

Virgin olive oils were obtained from our Institute's experimental oil mill (samples 1–4), a commercial oil mill (samples 5 and 6), the Institute's (Instituto de la Grasa y sus Derivados, Sevilla, Spain) Department of Analysis (sample 7) and the Institute's pilot plant (samples 8–11). Pure olive oils were purchased in local shops (samples 12 and 13). Refined olive oil (sample 14) was prepared from low-quality virgin sample 11 in the pilot plant by means of a laboratory-scale apparatus. Crude olive pomace oils were obtained in the laboratory by hexane extraction of olive fruits after initial pressing, or from an industrial manufacturer. Refined olive pomace oil (sample 15) was obtained from the Department of Analysis, and other refined olive pomace oils (samples 16–19) were prepared in the pilot plant with a laboratory-scale apparatus.

Chemical refining of samples 14 and 16–19 was carried out in a laboratory-scale apparatus as described before (13). Firstly, samples were degummed with phosphoric acid, and free fatty acids were neutralized with sodium hydroxide and filtered out. Secondly, neutralized oils were bleached with bleaching earth (Trisyl) for 10 min at 90°C. Finally, oils were deodorized under vacuum (1 mm) at 250°C for 3 h.

Oil unsaponifiable matter was obtained according to the International Union of Pure and Applied Chemistry reference method (14), but we started with 2.5 g of oil treated with 25 mL 1M potassium hydroxide solution.

^{13}C NMR spectrometry was performed on a Bruker AC 300P (Bruker Instruments, Inc., Karlsruhe, Germany)

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operating at 75.4 MHz. Oil unsaponifiable matter obtained from 2.5 g of oil was dissolved in 800 μL $CDCl_3$ and introduced into a 5-mm NMR tube. Each oil was saponified four times to obtain four replicates. The ^{13}C NMR spectra were obtained by using a proton decoupling technique analogously to Kvalheim *et al.* (15). The free induction decay (FID) of each sample was acquired at room temperature (20°C) with a 1.966-s acquisition time, a sweep width of 16667 Hz and 64K acquisition points to yield a digital resolution of 0.509 Hz/pt. A total of 14000 scans were collected for each sample with a 45° excitation pulse and a 2-s relaxation decay. FIDs were transformed by using absolute intensity, and chemical shifts were related to the signal for tetramethylsilane (δ 0 ppm). The solvent $CDCl_3$ was used as internal standard for height intensity; in the conditions described, the central peak of the solvent showed a height value of 5. This value was used to correct small changes in field homogeneity. Eighty-five peaks at the same chemical shift positions were selected, and peak heights were recorded for use in the data analysis of the intensity patterns. Because each sample of unsaponifiable matter was obtained four times for each individual oil, the values used in the data analysis were the average of each peak intensity for each oil. The recorded intensities for each oil were collected in a matrix, with each row containing all 85 peaks of one spectrum. No further preprocessing of the data was performed.

Statistical data analysis was performed with the SPSS PC+ V4.0 statistical package. Multivariate data analysis included stepwise discriminant analysis (SDA), to select the variables most useful in differentiating the different types of oils, and cluster analysis, to discover natural grouping of the samples.

RESULTS AND DISCUSSION

^{13}C NMR spectra of oil unsaponifiable matters. Figure 1 shows typical spectra of unsaponifiable matter from "virgin" (Fig. 1A), "pure" (Fig. 1B) and "refined" (Fig. 1C) olive oils as well as "refined olive pomace oils" (Fig. 1D). Virgin olive oils showed spectra with the highest number of peaks, in accordance with the higher unsaponifiable content expected in an unrefined oil. On the other hand, refined oils (Figs. 1C and 1D) showed the presence of some new peaks that appeared during refining. They distinguished unrefined from refined oils.

Assignment of peaks to components of unsaponifiable matter is complex because of the high number of compounds present, and because peaks in the spectra may be the consequence of different compounds with carbon atoms in identical structural environments and, therefore, with the same chemical shifts. Because the major constituents in oils are made up of rather few molecular fragments, obtained spectra showed well-resolved parts, even from portions of spectra that contained a large number of constituents. However, this limitation imposed on the interpretation of spectra, considering molecular substructures rather than individual constituents, turns out to be an advantage in the present context (see below).

The spectra of "virgin" olive oils showed, at low fields (δ 145–120 ppm), the presence of several peaks that were lost during refining, and they were also reduced in height when refined oils were blended with virgin oils to obtain "pure" olive oils. These peaks at δ 135.1, 134.9, 131.3, 124.4, 124.3a (when two signals appeared within 0.1 ppm, they were designated with a and b after the chemical shift; the letter a indicated a lower field than the letter b), and

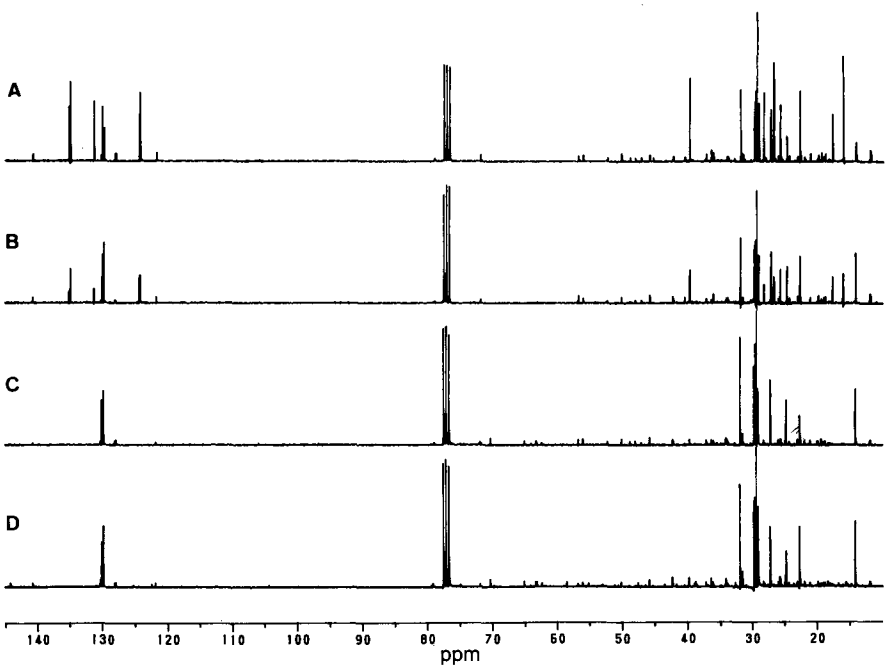

FIG. 1. 75.4 MHz ^{13}C nuclear magnetic resonance spectra of unsaponifiable matters obtained from: A, virgin olive oil; B, pure olive oil; C, refined olive oil; and D, refined olive pomace oil.

124.3b ppm (as well as other peaks at δ 39.8, 39.7, 28.3, 26.8, 26.7, 25.7, 17.7, 16.1 and 16.0 ppm, for the saturated carbons) mainly corresponded to the double bond carbons of squalene. Squalene is the major hydrocarbon in virgin olive oils and may represent as much as 50% of unsaponifiable matter in these oils (16).

Another set of important peaks is related to the presence of sterols, which constitute 20–30% of unsaponifiable matter (3). Sterol fractions in olive oils are composed mainly of β-sitosterol (65–90%) and Δ^5-avenasterol (5–30%) (16). Signals from sterolic carbons are clearly distinguishable at δ 140.7 and 121.8 ppm for carbons C5 and C6, respectively. Other signals (for β-sitosterol) appeared at δ 71.9, 56.8, 56.1, 50.1, 45.8, 42.3, 40.3, 39.8, 37.3, 36.5, 36.2, 34.0, 31.9a, 31.9b, 31.6, 29.1, 28.3, 26.0, 24.3, 23.1, 21.1, 19.8, 19.4, 19.0, 18.8, 12.0 and 11.9 ppm.

Triterpenic alcohols are also major compounds in unsaponifiable matter from olive oils [20–26% of unsaponifiable content (3)]. All these compounds have analogous structures, and some parts of these structures are analogous to sterols. Therefore, they contribute to the intensity of some peaks listed above. Triterpenic alcohols might also be partly responsible for the intensity of peaks at δ 130.0 and 129.8 ppm. Nevertheless, these last two peaks at δ 130.0 and 129.8 ppm are more probably related with the presence of long-chain monounsaturated alcohols (for example, oleyl alcohol showed the unsaturated carbons at δ 129.9 and 129.8 ppm). Although olive oils contain no appreciable amounts of aliphatic alcohols, their esters are present (12) and alcohols are liberated during saponification. This would also explain the high number of aliphatic carbons that appear at δ 20–30 ppm.

Other groups of compounds were also detected, although their assignations are much more tentative because of their low concentration. However, all together they contribute to the final intensity of each peak in each oil and give an intensity pattern that is characteristic for each oil.

Pattern recognition of unsaponifiable matter. Stepwise discriminant analysis was applied to the data matrix by using Wilk's λ as a criterion for the variable selection. Thirteen variables (peaks at δ 40.5, 39.0, 36.9, 32.8, 31.9a, 29.7, 29.6, 29.5a, 29.5b, 29.4, 26.7, 24.9 and 16.1 ppm) resulted in significant discrimination between virgin, refined and pure groups (Table 1), and they were used to calculate the coefficients of canonical variables. The plot of the 19 samples on the plane defined by the two canonical variables is shown in Figure 2. A 100% correct assignment was made with this parametric method.

Many of the most discriminating variables (highest *F* values), used for classifying olive and olive pomace oils among virgin, refined and pure groups, resulted also in significantly discriminating among virgin olive, refined olive, pure olive and refined olive pomace groups (Table 2), and they were used to calculate the coefficients of canonical variables. The plot of the 19 samples on the plane defined by the first two canonical variables is shown in Figure 3. A 100% correct assignment was also obtained with this discriminant analysis.

Variables selected for these two discriminant analyses are related to the different families of compounds that constitute the unsaponifiable matter. Therefore, analogous results to the above might be expected by using classical oil analysis. However, this new method requires much less manipulation of samples and could be applied easily to the development of artificial neural networks with a higher number of samples.

Figure 4 shows the results of the application of cluster analysis (method of average linkage within groups) to virgin and pure olive oils (samples 1–13). It shows similarities among the different samples, which may allow detection of further features. Different groups were obtained, suggesting that intensity patterns from ^{13}C NMR might also be useful in determining different grades of virgin olive oils. Additional studies should be carried out to correlate these results with compositional and organoleptic characteristics of the several grades of commercial olive oils.

TABLE 1

Stepwise Discriminant Analysis (SDA) of Intensity Data from ^{13}C Nuclear Magnetic Resonance Chemical Shifts of Virgin, Refined and Pure Oils

Peaks used for SDA (δ in ppm)	*F*	Coefficients for canonical variables	
		1	2
24.9	495.0	30.13800	2.20878
29.7	49.3	−0.04455	18.09853
36.9	35.5	−15.67138	2.96696
32.8	34.1	−14.64620	0.60857
29.5a	27.4	−5.75676	−6.94593
31.9a	23.8	−13.66307	−4.78169
29.5b	16.2	29.50243	−1.44072
29.6	15.2	16.32485	−2.67587
26.7	12.5	3.22570	11.51002
40.5	3.8	−0.01218	1.76901
16.1	3.6	−2.60226	−5.41346
39.0	2.6	5.84926	−0.83836
29.4	2.3	2.78694	−8.21476
Eigen values		4398.8716	69.2479
Percentage of variance		98.45	1.55
Canonical correlations		0.9999	0.9929

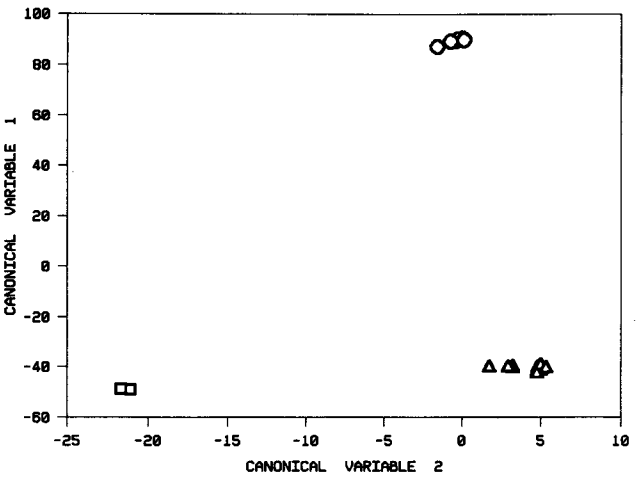

FIG. 2. Plot of olive and olive pomace oils on the plane defined by the two canonical variables obtained with the selected variables from stepwise discriminant analysis among virgin (△), refined (○) and pure (□) groups.

TABLE 2

Stepwise Discriminant Analysis (SDA) Among Three Grades of Olive Oil (virgin, refined and pure) and One of Olive Pomace Oil (refined) Using Intensity Data from ^{13}C Nuclear Magnetic Resonance Spectra

Peaks used for SDA (δ in ppm)	*F*	Coefficients for canonical variables		
		1	2	3
29.5b	46.7	25.56717	6.31349	−2.04941
36.9	31.3	−15.63146	0.42597	0.54181
32.8	21.8	−10.62306	−4.69652	−0.22392
31.9a	18.2	−12.26964	−5.65562	−2.33928
24.9	14.0	10.66114	−26.61330	7.03711
70.3	10.5	9.54125	25.01093	−5.50990
29.7	6.4	4.08033	2.46715	5.63605
29.5a	6.4	−3.60595	−0.34116	−2.37523
31.9b	4.5	2.92380	2.75057	−0.21583
39.8	3.5	−0.20464	−0.69529	2.24997
127.9	2.7	1.61873	0.20363	0.68854
40.5	2.1	−0.02357	1.13997	1.09521
Eigen values		2081.2057	37.2856	15.0192
Percentage of variance		97.47	1.80	0.73
Canonical correlations		0.9998	0.9869	0.9683

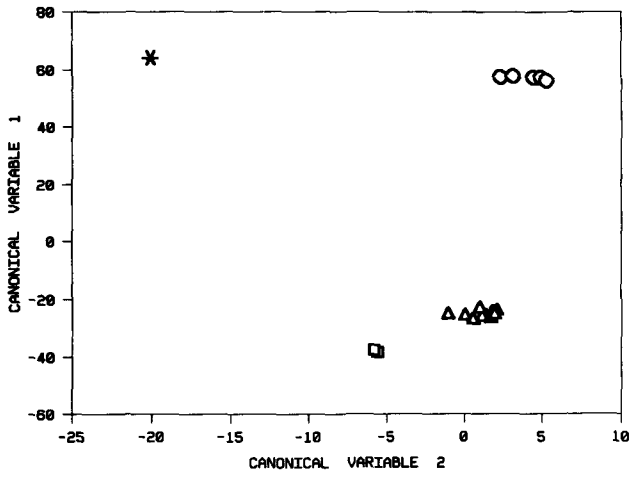

FIG. 3. Plot of olive oils on the plane defined by the two first canonical variables obtained with the selected variables from stepwise discriminant analysis among virgin (△), refined (*) and pure (□) olive, and refined (○) olive pomace groups.

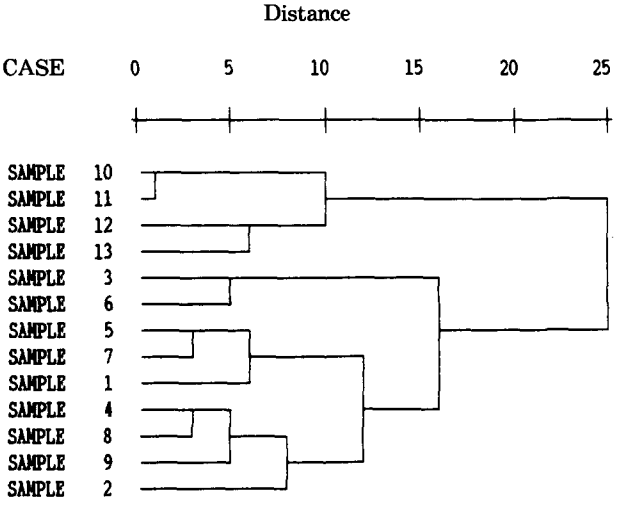

FIG. 4. Clustering of virgin and pure olive oils based on peak height patterns obtained from ^{13}C nuclear magnetic resonance spectroscopy.

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REFERENCES

1. Gracián Tous, J., in *Analysis and Characterization of Oils, Fats and Fats Products*, Vol. 2, edited by H.A. Boekenoogen, John Wiley & Sons (Interscience), New York, 1968.
2. Kiritsakis, A.K., and P. Marakakis, in *Essential Oils and Waxes*, edited by H.F. Linskens, and J.F. Jackson, Springer, Heidelberg, 1991.
3. Kiritsakis, A.K. (ed.), *Olive Oil*, American Oil Chemists' Society, Champaign, 1990.
4. Flor, R.V., L.T. Hecking and D. Martin, *J. Am. Oil Chem. Soc. 70*:199 (1993).
5. Lanzón, A., A. Cert and T. Albi, *Grasas Aceites 40*:385 (1989).
6. Artho, A., K. Grob and C. Mariani, *Fat Sci. Technol. 95*:176 (1993).
7. Aparicio, R., T. Albi, A. Lanzón and M.A. Navas, *Grasas Aceites 38*:9 (1987).
8. Goodacre, R., D.B. Kell and G. Bianchi, *Nature 359*:594 (1992).
9. Gunstone, F.D., *J. Am. Oil Chem. Soc. 70*:361 (1993).
10. Ng, S., *Lipids 20*:778 (1985).
11. Wollenberg, K.F., *J. Am. Oil Chem. Soc. 67*:487 (1990).
12. Fedeli, E., *Prog. Chem. Fats Other Lipids 15*:57 (1977).
13. Dobarganes, M.C., M.C. Pérez-Camino, G. Márquez-Ruiz and M.V. Ruiz-Méndez, in *Edible Fats and Oil Processing: Basic Principles and Modern Practices*, edited by D.R. Erickson, American Oil Chemists' Society, Champaign, 1990.
14. International Union of Pure and Applied Chemistry, *Standard Methods for the Analysis of Oils, Fats and Derivatives*, Blackwell Scientific Publications, Oxford, 1992, Method 2.401.
15. Kvalheim, O.M., D.W. Aksnes, T. Brekke, M.O. Eide, E. Sletten and N. Telnaes, *Anal. Chem. 57*:2858 (1985).
16. Tiscornia, E., M. Forina and F. Evangelisti, *Riv. Ital. Sostanze Grasse 59*:519 (1982).

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