Use of High-Resolution ^{13}C Nuclear Magnetic Resonance Spectroscopy for the Screening of Virgin Olive Oils

Rosario Zamora, Virginia Alba, and Francisco J. Hidalgo*

Instituto de la Grasa, CSIC, 41012 Sevilla, Spain

ABSTRACT: ^{13}C Nuclear magnetic resonance (NMR) spectra of 104 oil samples were obtained and analyzed in order to study the use of this technique for routine screening of virgin olive oils. The oils studied included the following: virgin olive oils from different cultivars and regions of Europe and north Africa, and refined olive, "lampante" olive, refined olive pomace, high-oleic sunflower, hazelnut, sunflower, corn, soybean, rapeseed, grapeseed, and peanut oils, as well as mixtures of virgin olive oils from different geographical origins and mixtures of 5–50% hazelnut oil in virgin olive oil. The analysis of the spectra allowed us to distinguish among virgin olive oils, oils with a high content of oleic acid, and oils with a high content of linoleic acid, by using stepwise discriminant analysis. This parametric method gave 97.1% correct validated classifications for the oils. In addition, it classified correctly all the hazelnut oil samples and the mixtures of hazelnut oil in virgin olive oil assayed. All of these results suggested that ^{13}C NMR may be used satisfactorily for discriminating some specific groups of oils, but to obtain 100% correct classifications for the different oils and mixtures, more information than that obtained from the direct spectra of the oils is needed.

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Olive oil has gained popularity in recent years not only because of its superior flavor but also because of reports of potential health benefits, including a specific reduction in plasma non-high-density lipoprotein cholesterol levels and a protective effect in some types of cancer (1,2). As a consequence of this popularity, its relatively small production area, and the amount of labor needed for its production, olive oil commands a high price on the market, and its admixture with cheaper oils has been frequently practiced.

Unlike most other seed oils, virgin olive oil is a "natural fruit juice" that has been obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions that do not lead to alterations in the oil. International regulations have established analytical criteria to define olive oil genuineness (detection of adulterations with seed oils or other solvent-extracted oils) and quality grade (extra virgin, virgin, "lampante," "refined," "pure," etc.) (3,4). However, there are too many different assays to be applied for a broad routine control, and most of these methods require the isolation and analysis of minor compounds from the unsaponifiable matter by means of procedures that are laborious and time-consuming. Therefore, many studies have been carried out to develop new analytical techniques that, with very little or no manipulation of the sample, can afford results similar to or approximately the same as those obtained by the classic, more accurate procedures. Specifically, spectroscopic techniques have emerged as potential tools in recent years, although the complexity and intrinsic variability of biological samples such as olive oil and its potential adulterants require the application of multivariate calibration or pattern-recognition techniques to aid interpretation of the data obtained using these instrumental methods. In this context, the use of pyrolysis mass spectrometry (5), mid- and near-infrared spectroscopy (6), and Raman spectroscopy (7,8) has been suggested. However, at present, none of these methods seems to have found universal acceptance.

One spectroscopic technique with a high potential in this field is high-resolution nuclear magnetic resonance (NMR) spectroscopy. Both ^{1}H and ^{13}C NMR have already been employed in the analysis of olive oils. For example, NMR has been applied to the determination of mono- and diglycerides, to the analysis of the positional distribution of fatty acids in triacylglycerols, and to the characterization of virgin olive oils (9–14). Also, previous research from this laboratory used high-resolution ^{13}C NMR spectra of the unsaponifiable material obtained from the oils for the identification and classification of olive oils (15). However, most of these procedures either were applied to a limited number of samples or were much too time-consuming to be used routinely.

As a continuation of these studies, the present investigation was undertaken to analyze the potential use of high-resolution ^{13}C NMR spectroscopy for the screening of virgin olive oils by testing over 100 samples, and including in the procedure mixtures of special difficulty for the present methods of analysis, specifically mixtures of different virgin olive oils from various origins or mixtures of virgin olive oils with hazelnut oils.

EXPERIMENTAL PROCEDURES

Materials. Oil samples (104) were analyzed in this study. These included: 25 virgin olive oils from different cultivars and regions of Europe and north Africa (specifically Spain, Italy, Greece, and Tunisia), 12 (1:1) mixtures of virgin olive

*To whom correspondence should be addressed at Instituto de la Grasa, Avenida Padre García Tejero, 4, 41012-Sevilla, Spain.
E-mail: fhidalgo@cica.es

oils between Tunisian or Grecian oils and Spanish oils, 4 refined olive oils, 5 "lampante" olive oils, 8 refined olive pomace oils, 2 high-oleic sunflower oils, 6 hazelnut oils, 5 sunflower oils, 4 corn oils, 3 soybean oils, 2 rapeseed oils, 3 grapeseed oils, 1 peanut oil, and 24 mixtures of 5–50% hazelnut oil in virgin olive oil. Most of the samples were obtained from our Institute's experimental oil mill (Instituto de la Grasa, Sevilla, Spain), the Institute's Department of Analysis, the Institute's pilot plant, or Koipe S.A. (Andujar, Jaén, Spain). In addition, some of the refined oils were prepared and refined in our laboratory using a laboratory-scale apparatus as described previously by Dobarganes *et al*. (16). This procedure included degumming with phosphoric acid, neutralization with sodium hydroxide, bleaching with bleaching earth (Trisyl) for 10 min at 90°C, and deodorization under vacuum (1 mm) at 250°C for 3 h.

Samples were classified into three groups. The first included only virgin olive oils (37 samples including the 25 virgin olive oils and the 12 mixtures of virgin olive oils). The second group (51 samples) corresponded to the high-oleic oils. It comprised refined olive, "lampante" olive, refined olive pomace, high-oleic sunflower, and hazelnut oils as well as the mixtures of 5–50% hazelnut oil in virgin olive oil. This group also included the rapeseed oils. According to the well-known fact that no chain differentiation on the basis of chain length can be achieved by NMR, the erucic acid had a behavior similar to the oleic acid. Finally, the last group (15 samples) included sunflower, corn, soybean, grapeseed, and peanut oils.

NMR spectroscopy. ^{13}C NMR spectroscopy was performed on a Bruker AC 300P (Bruker Instruments, Inc., Karlsruhe, Germany) operating at 75.4 MHz. Triplicate samples of the oils (250 mg) were dissolved in 750 μL of $CDCl_3$ and introduced into a 5-mm NMR tube. The ^{13}C NMR spectra were obtained by using a one-pulse sequence with broad-band proton decoupling analogously to Kvalheim *et al*. (17). 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 16,667 Hz, and 64 K acquisition points to yield a digital resolution of 0.509 Hz/point. A total of 800 scans was collected for each sample with a 45° excitation pulse and a 2-s relaxation decay. FID 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 and to correct small changes in field homogeneity. Sixty-one peaks at the same chemical shifts positions were selected, and peak heights were recorded for use in the data analysis of the intensity patterns. The recorded intensities for each oil were collected in a matrix, with each row containing all 61 peaks of one spectrum. No further preprocessing of the data was performed.

Data analysis. Statistical data analysis was performed with the SPSS for Windows (v. 9.0.1; Chicago, IL) statistical package. Four matrixes (M1–M4) were used in the analyses. M1 was obtained by using triplicates of the oils and three acquisitions for each sample. Each intensity in this matrix was therefore the mean of nine measurements. M2 was obtained by using triplicates of the oils, and the intensities used were the mean of these three measurements. M3 was obtained by acquiring three times one sample for each oil, and the intensities were the mean of the three measurements obtained. Finally, M4 was obtained by using one sample and one acquisition for each oil. These matrixes were submitted to stepwise discriminant analysis (SDA) to select the variables most useful in differentiating the different types of oils.

RESULTS

^{13}C NMR spectra of vegetable oils. ^{13}C NMR spectra of vegetable oils have been broadly reported in the literature and can be easily assigned according to the chemical shifts previously reported (11,13,18). ^{13}C NMR resonances of the oils could be grouped into four well-defined spectral regions: carbonyl carbons ranging from 173.3 to 172.8 ppm; unsaturated carbons ranging from 132.1 to 126.8 ppm; glycerol carbons ranging from 69.1 to 61.6 ppm; and aliphatic carbons ranging from 34.5 to 13.9 ppm. All assayed oils differed in quantitative chain composition rather than in qualitative acid profile, therefore exhibiting analogous signals, although with varying intensities which were characteristic for each oil.

Positions and intensities of the signals were a consequence of the fatty acid profile of the oils and the position of these acyl chains in the triacylglycerols. This could be observed in different parts of the spectra. Thus, for example, Figure 1 shows the portion of the ^{13}C NMR spectra that corresponded to the unsaturated carbons of several oils. It includes: a soybean oil, as an example of a vegetable oil with a high content of linoleic acid; a high-oleic sunflower oil, as a model of high-oleic oils; and two olive oils, one from Greece, with a fatty acid composition common for olive oils, and another from Tunisia, with a content in saturated and linoleic acids higher than is common for most olive oils produced in European countries. Thus, the Tunisian oils studied had about 20% linoleic acid, in contrast with the typical 10% obtained for most of the virgin olive oils analyzed. Assignments of the signals are included in the figure. A simple view of the spectra indicated the approximate relative composition of fatty acids in the oils. In fact, a good correlation between, for example, the intensity of olefinic carbon atom signals and the fatty acid composition determined by gas chromatography has been observed (19). In addition, it was possible to determine the proportion of unsaturated fatty acids at the α or β position (9), which previously could only be determined by the method of pancreatic lipase. The difference between substituents in both positions may be appreciated, for example, in Figure 1 by comparing the spectra of high-oleic sunflower or soybean oils (with a higher content of linoleyl residues at the α position) with those of Tunisian olive oil (which has a higher content of linoleyl residues at the β position). Therefore, a single ^{13}C NMR spectrum provided more information than what is obtained with a simple analysis of fatty acids by gas chromatography.

Use of ^{13}C NMR for the screening of vegetable oils. The information contained in the spectra was employed to study

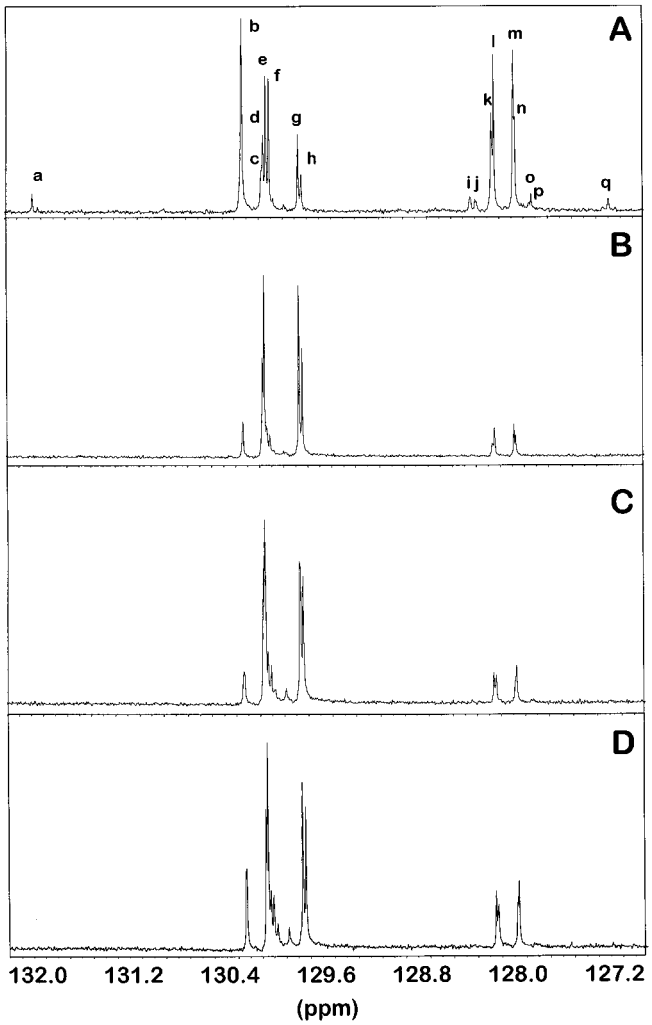

FIG. 1. Olefinic carbon signals obtained by nuclear magnetic resonance at 75.4 MHz of (A) a soybean oil, (B) a high-oleic sunflower oil, (C) a virgin olive oil from Greece, and (D) a virgin olive oil from Tunisia. Assignments of peaks are: a, 16 Lnα + β; b, 9 Lnα + β + 13 Lα + β; c, 10 Oβ; d, 10 Oα; e, 9 Lα; f, 9 Lβ; g, 9 Oα; h, 9 Oβ; i, 13 Lnα + β; j, 12 Lnα + β; k, 10 Lβ; l, 10 Lα; m, 12 Lα; n, 12 Lβ; o, 10 Lnβ; p, 10 Lnα; and q, 15 Lnα + β. Abbreviations: 1(3)- and 2-positions of glycerol are designated by the Greek symbols α and β, respectively. Labeling of acyl chains: O, oleyl; L, linoleyl; Ln, linolenyl chain.

the potential for using ^{13}C NMR spectroscopy to screen vegetable oils. The spectra of the oils were taken from one to nine times in the spectrometer for about 1 h, and four matrixes were elaborated with the results obtained, which contained the intensities of the 61 peaks selected. The matrixes M1–M4 were defined in the Experimental Procedures section. The signals considered are collected in Table 1 with their corresponding assignments.

SDA was applied to the data matrixes M1–M4 by using Wilk's λ as a criterion for the variable selection. For M1, 17 variables (peaks at δ 129.90, 129.69, 129.67, 62.09, 34.19, 31.83, 29.75, 29.72, 29.53, 29.49, 29.14, 27.21, 27.19, 25.65, 24.86, 22.62, and 16.04 ppm) resulted in significant discrimination between virgin olive, high-oleic, and high-linoleic

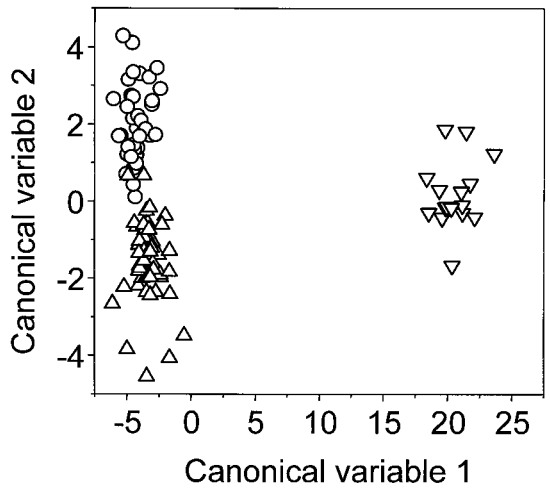

FIG. 2. Plot of the 104 oil samples on the plane defined by the two canonical variables obtained with the selected variables from stepwise discriminant analysis among virgin olive (○), high-oleic (△), and high-linoleic (▽) oils.

groups, and they were used to calculate the coefficients of canonical variables. Table 2 is a summary of the SDA analysis. The plot of the 104 samples on the plane defined by the two canonical variables obtained for matrix M1 is shown in Figure 2. A 97.1% correct assignment was made with this parametric method, and the validation of the method also produced 97.1% correct assignment.

Similar results, although with slightly worse assignments, were also obtained for matrixes M2–M4. M2 gave a 98.1% correct assignment, and the validation of the method produced 95.2% correct assignment. The use of only one sample per oil decreased the correct assignments. Thus, M3 gave a 92.3% correct assignment, and the validation of the method produced 86.5% correct assignment. Finally, M4 gave a 92.3% correct assignment, and the validation of the method produced 88.5% correct assignment.

By using SDA and the matrix M1, all the samples of hazelnut oil and the mixtures of 5–50% hazelnut oil in virgin olive oil were classified correctly. Figure 3 shows the plot of the 67 samples (25 virgin olive oils, 12 mixtures of virgin olive oils, 6 hazelnut oils, and 24 mixtures of hazelnut oils in virgin olive oils) on the plane defined by the two canonical variables obtained for the 104 oil samples and the matrix M1.

DISCUSSION

High-resolution ^{13}C NMR is considered among the most powerful techniques yet described for analysis of vegetable oils. For example, as a consequence of the information contained in the spectra, NMR is a useful technique to distinguish among different cultivars, grades, and geographical origins of olive oils (13,20). The above results, as well as previous results in the literature, have shown that one NMR spectrum contained most of the information that previously could be obtained only by two different official chromatographic

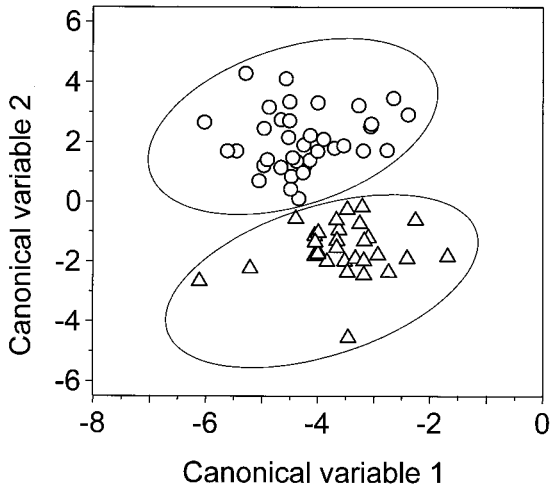

FIG. 3. Plot of the 67 oil samples of virgin olive oils (○) and oils containing hazelnut in a proportion of 5–100% (△) on the plane defined by the two canonical variables obtained with the selected variables from stepwise discriminant analysis.

methods. However, to develop a general application of this technique for quality control of virgin olive oils, it is necessary to know if this information is enough to classify these oils correctly when olive oils from different origins and mixtures of them are considered.

The results obtained in this study showed that ^{13}C NMR was a powerful technique for screening virgin olive oils. However, when it was applied to a broad number of samples, some incorrect classifications were observed. Thus, the percentage of correct classifications increased with the number of replicates used in the analysis, but it was almost independent of the number of times that the spectrum of the sample was acquired in the spectrometer. The above results showed that, by using three replicates and a total time of 3 h, it was possible to obtain more than 95% of correct validated assignments. According to Figure 2, it was not difficult to distinguish between virgin olive oils and vegetable oils containing a high content of linoleic acid, but the difference between virgin olive oils and some high-oleic oils was much less clear. In using the matrix

TABLE 1
^{13}C Nuclear Magnetic Resonance Chemical Shifts Used in SDA Analysis

Signal	Chemical shift (ppm)	Assignment[a]	Signal	Chemical shift (ppm)	Assignment[a]
1	130.16	13 Lα + β/9 Lnα + β	32	29.53	5 Pα
2	129.99	10 Oβ	33	29.49	Unknown
3	129.98	10 Oα	34	29.42	13 Pα + β/15 Stα + β
4	129.96	9 Lα	35	29.39	15 Lα + β
5	129.93	9 Lβ	36	29.38	13 Oα + β
6	129.90	Unknown	37	29.37	15 Oα + β
7	129.81	Unknown	38	29.32	6 Pα + β/6 Stα + β
8	129.69	9 Oα	39	29.24	5 Oβ/5 Lβ/5 Lnβ
9	129.67	9 Oβ	40	29.22	5 Oα/5 Lα/5 Lnα
10	128.08	10 Lβ	41	29.16	6 Oβ/6 Lβ/6 Lnβ/4 Sα
11	128.07	10 Lα	42	29.14	6 Oα/6 Lα/6 Lnα/4 Sβ
12	127.90	12 Lα	43	29.12	4 Oα/4 Lα/4 Lnα
13	127.89	12 Lβ	44	29.08	4 Oβ/4 Lβ/4 Lnβ
14	68.89	β-Glycerol	45	29.03	Unknown
15	62.09	α-Glycerol	46	27.24	11 Oα + β
16	34.19	2 Oβ/2 Lβ/2 Lnβ/2 Pβ/2 Stβ	47	27.22	14 Lα + β/8 Lnα + β
17	34.04	2 Pα/2 Stα	48	27.21	8 Lα + β
18	34.02	2 Oα/2 Lα/2 Lnα	49	27.19	8 Oα + β
19	31.98	14 Pα + β/16 Stα + β	50	25.65	11 Lα + β/11 Lnα + β/14 Lnα + β
20	31.95	16 Oα + β	51	24.90	3 Oβ/3 Pβ/3 Stβ
21	31.83	Unknown	52	24.89	3 Oα/3 Lβ/3 Lnβ/3 Pα/3 Stα
22	31.56	16 L α+ β	53	24.86	3 Lα/3 Lnα
23	29.80	12 Oα + β	54	22.74	15 Pα + β/17 Stα + β
24	29.75	7 Oβ/11 Stα + β/12 Stα + β 13 Stα + β/14 Stα + β	55	22.73	17 Oα + β
25	29.74	7 Oα/11 Pα + β/12 Pα + β/10 Stα + β	56	22.71	Unknown
26	29.72	8 Sα + β/9 Sα + β/10 Pα + β/7 Stβ	57	22.62	17 Lα + β/17 Lnα + β
27	29.67	7 Pβ/7 Stα	58	16.04	Unknown
28	29.66	7 Lβ/7 Pα	59	16.00	Unknown
29	29.64	7 Lα/7 Lnα + β	60	14.14	18 Oα + β/16 Pα + β/18 Stα + β
30	29.58	14 Oα + β/5 Stβ	61	14.11	18 Lα + β
31	29.55	5 Stα/5 Pβ			

[a]1(3)- and 2-Positions of glycerol are designated by the Greek symbols α and β, respectively. Labeling of acyl chains: S, saturated; P, palmitic; St, stearic; O, oleyl; L, linoleyl; Ln, linolenyl chain. SDA, stepwise discriminant analysis.

TABLE 2
Summary of SDA of Intensity Data from ^{13}C Nuclear Magnetic Resonance Chemical Shifts of Virgin Olive, High-Oleic, and High-Linoleic Oils[a]

	Canonical function 1			Canonical function 2			Correct	
Matrix	Eigen value	Variance (%)	Canonical correlation	Eigen value	Variance (%)	Canonical correlation	assignment (%)	Validation (%)
M1	79.270	96.6	0.994	2.780	3.4	0.858	97.1	97.1
M2	79.613	97.3	0.994	2.238	2.7	0.831	98.1	95.2
M3	33.228	95.1	0.985	1.705	4.9	0.794	92.3	86.5
M4	38.450	96.8	0.987	1.271	3.2	0.748	92.3	88.5

[a]For abbreviation see Table 1.

M1, the model incorrectly classified one sample of a 1:1 mixture of virgin olive oils (composed of one oil from Greece and one oil from Spain), which was identified as high-oleic oil, and one sample of refined olive oil and another sample of refined olive pomace oil, which were classified as virgin olive oils. These three oils were always incorrectly classified by using any of the four matrixes considered in this study. If a much higher difference between virgin olive oil and high-oleic oil groups is needed in order to obtain 100% correct assignments, acquisition of additional information should be considered.

Although the procedure did not give 100% correct classification for all the different oils considered, some difficult classifications were resolved satisfactorily. Thus, for example, at present no methods have proved to be reliable for detecting the adulterations of olive oil with hazelnut oil, although it is certainly a widely applied adulteration which so far has not been detectable at the concentrations of interest (around 5–20% of hazelnut oil in olive oil) (21). The ^{13}C NMR procedure applied in the present study produced satisfactory results for distinguishing hazelnut oils. Thus, all hazelnut oils assayed as well as the mixtures of hazelnut oil in olive oil, which ranged from 5 to 50%, were classified correctly.

The above results suggest that, although ^{13}C NMR is satisfactory for discriminating among some specific groups of oils, the general application of this methodology for the screening of virgin olive oils may fail for complicated samples or mixtures. In these cases more information than that obtained from the direct spectra of the oils is needed. This additional information might be obtained from the high-resolution ^{1}H NMR spectra (12) or by fractionation of the oil to increase the content of the unsaponifiables.

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