Classification of Vegetable Oils by High-Resolution ^{13}C NMR Spectroscopy Using Chromatographically Obtained Oil Fractions

Rosario Zamora, Gemma Gómez, and Francisco J. Hidalgo*

Instituto de la Grasa, CSIC, 41012 Sevilla, Spain

ABSTRACT: ^{13}C NMR spectra of oil fractions obtained chromatographically from 109 vegetable oils were obtained and analyzed to evaluate the potential use of those fractions in the classification of vegetable oils and to compare the results with the NMR analysis of complete oils. The oils included the following: virgin olive oils from different cultivars and regions of Europe and north Africa; "lampante" olive, refined olive, refined olive pomace, hazelnut, rapeseed, high-oleic sunflower, corn, grapeseed, soybean, and sunflower oils; and mixtures of virgin olive oils from different geographical origins. Oils were divided into two sets of samples. The training set (98 samples) was employed to select the variables that resulted in significant discrimination among the different oil classes. By using stepwise discriminant analysis, more than 98% of correct validated assignments were obtained; these results were confirmed when applied to the test set (11 blind samples). Results suggest that the use of oil fractions considerably increases the discriminating power of NMR in the analysis of vegetable oils.

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KEY WORDS: High-resolution ^{13}C-NMR; oil analysis; oil characterization; oil classification; pattern recognition; stepwise discriminant analysis; vegetable oils; virgin olive oil.

High-resolution NMR spectroscopy is used increasingly as a technique to provide insight into the nature of the mixtures present in natural oils, fats, and other lipids (1–3). It has many advantages over other spectroscopic techniques, especially because NMR has the potential to obtain and quantify signals corresponding to individual atoms. However, it also has limitations, most of them related to the low sensitivity of this technique compared to other spectroscopic techniques.

Vegetable oils contain more than 95% TAG. Therefore, NMR analysis that involves these molecules may be easily applied to evaluate, for example, FA composition (4) or positional distribution of FA (5). However, determination of other minor components can require longer acquisition times. In other instances, these cannot be determined at all because their signals are hidden by those of the predominant TAG. This happens, for example, in the determination of DAG in virgin olive oils (6), where they do not exceed 2–3%, or in the determination of *trans* FA (3), which are usually under the normal detection limits of ^{1}H or ^{13}C NMR.

In an attempt to overcome these difficulties, we researched the chromatographic isolation of a fraction that contained about 15% unmodified TAG and 85% polar compounds (7). The polar lipid fraction included polymeric TAG, oxidized TAG, DAG, MAG, and FFA, in addition to other minor polar components of the oils. The presence of these compounds in an enriched fraction should provide information about the thermal, oxidative, and hydrolytic alterations of the oils. In addition, this fraction contains many compounds of interest for determining oil genuineness.

The present investigation was undertaken to evaluate the potential use of those enriched polar lipid fractions in the characterization of vegetable oils by high-resolution ^{13}C NMR spectroscopy.

EXPERIMENTAL PROCEDURES

Materials. Two sets of samples were employed in this study: the training set and the test set. To ensure generalizability, the two sets were wholly different and were designed, with respect to virgin olive oils, to encompass a variety of representative cultivars. The training set was composed of 98 oil samples. These included 41 virgin olive oils from different cultivars and regions of Europe and north Africa (specifically Spain, Italy, Greece, and Tunisia); 10 (1:1) mixtures of virgin olive oils using Tunisian or Grecian oils and Spanish oils; 7 "lampante" olive oils, 5 refined olive oils, 6 refined olive pomace oils, 3 hazelnut oils, 3 rapeseed oils, 4 high-oleic sunflower oils, 4 corn oils, 4 grapeseed oils, 5 soybean oils, and 6 sunflower oils. The test set was composed of 11 oils. The identity of these oils was unknown at the time of analysis. 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, and 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 described previously by Dobarganes *et al.* (8). This procedure included degumming

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

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.

Oil fractionation. Oil fractionation was carried out as described (7). Briefly, triplicate samples of the oils were fractionated by column chromatography using 19 g of silica gel (particle size 0.063–0.200 mm) as absorbent. The column was equilibrated with the initial elution solvent of a mixture of hexane and diethyl ether (87:13). The oil (6 g) was dissolved in 10 mL of the same solvent and introduced onto the column. Most nonpolar compounds were eluted with 100 mL of the elution solvent and discarded. The polar lipid fraction, containing polar compounds as well as small amounts of TAG and other nonpolar compounds, was eluted with 100 mL of acetone.

NMR spectroscopy. ^{13}C NMR spectroscopy was performed on a Bruker AC 300P (Bruker Instruments, Inc., Karlsruhe, Germany) operating at 75.4 MHz. The whole oil fractions obtained by column chromatography, as described in Reference 7, were dried, dissolved in 700 μL of $CDCl_3$ (containing 0.03% vol/vol tetramethylsilane), and introduced into a 5-mm NMR tube. The ^{13}C NMR spectra were obtained by using a one-pulse sequence (9), analogous to that described by Kvalheim *et al.* (10). The free induction decay of each sample was acquired at room temperature (20°C) with a 1.966-s acquisition time, a sweep width of 16667 Hz, and 64 K acquisition points to yield a digital resolution of 0.509 Hz/pt. A total of 3500 scans was collected for each sample with a 45° excitation pulse and a 2-s relaxation decay (the total acquisition time for each sample was 4 h). Free induction decays were transformed by using absolute intensity, and chemical shifts were related to the signal for tetramethylsilane (δ0 ppm). The solvent $CDCl_3$ was used as the internal standard for height intensity and to correct for small changes in field homogeneity. One hundred thirty-five peaks at the same chemical shifts/positions were selected, and peak heights were recorded for use in the data analysis of intensity patterns. The recorded intensities for each oil were collected in a matrix, with each row containing all 135 peaks of one spectrum. The values used in the data analysis were the average of the three replicates obtained for each oil. No further preprocessing of the data was performed.

Data analysis. Statistical data analysis was performed with the SPSS for Windows (version 9.0.1) statistical package (SPSS Inc., Chicago, IL). The data matrix, prepared as described above for the training set, was submitted to stepwise discriminant analysis (SDA) to select the variables most useful in differentiating the different types of oils. These variables were then employed to study the test set.

RESULTS

^{13}C NMR spectra of oil fractions. Figure 1 shows the typical spectra of oil fractions obtained from four representative types of oils: a virgin olive oil (Fig. 1A), a refined olive pomace oil (Fig. 1B), a high-oleic sunflower oil (Fig. 1C), and a soybean oil (Fig. 1D). ^{13}C NMR spectra of oil fractions were similar to those obtained for complete oils (9) but exhibited more than double the number of signals. This is a consequence of the composition of the oil fractions (7). They were composed mostly of TAG and DAG, which were responsible for the highest observed signals. However, the spectra showed many other signals that are usually absent from the spectra of complete oils.

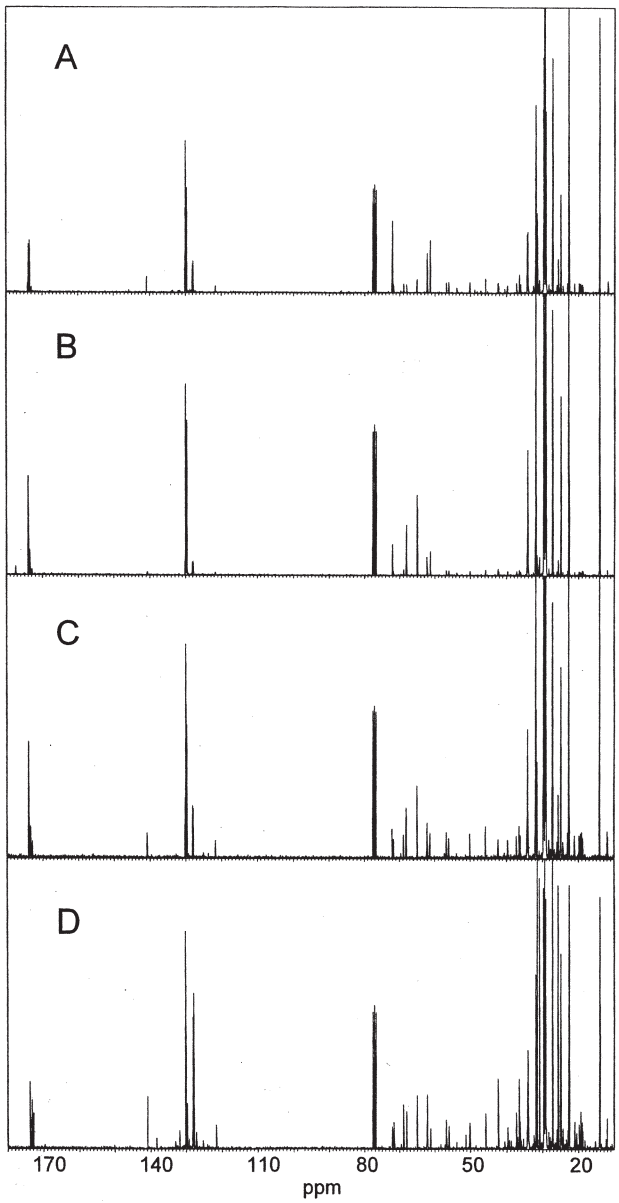

FIG. 1. ^{13}C NMR spectra at 75.4 MHz of oil fractions obtained chromatographically from (A) a virgin olive oil, (B) an olive pomace oil, (C) a high-oleic sunflower oil, and (D) a soybean oil.

The resonances obtained could be grouped into different regions. The carbonyl carbons appeared between 177.8 and 172.8 ppm, and corresponded to the different carbonyl carbons present in the fractions, i.e., TAG, DAG, and MAG, and FFA as main components. The unsaturated carbons ranged

TABLE 1
^{13}C NMR Signals Used in Stepwise Discriminant Analysis (SDA)

No.	δ	Assignment[a]	No.	δ	Assignment	No.	δ	Assignment
1	177.75	CA (FFA1)	46	57.30	UK	91	29.60	ME
2	174.32	CA (1MAG1α)	47	57.25	UK	92	29.53	ME
3	173.94	CA (1,3DAG1α)	48	56.73	ST14	93	29.49	ME
4	173.90	CA (1,3DAG1α)	49	56.59	ST17	94	29.37	ME
5	173.80	CA (1,2DAG1α)	50	55.99	UK	95	29.33	ME
6	173.76	CA (1,2DAG1α)	51	53.73	UK	96	29.29	ME
7	173.46	CA (1,2DAG1β)	52	52.25	UK	97	29.18	ME
8	173.44	CA (1,2DAG1β)	53	51.21	UK	98	29.09	ME
9	173.35	CA (TAG1α)	54	50.09	ST9	99	29.03	ST
10	173.31	CA (TAG1α)	55	48.77	UK	100	28.99	UK
11	173.26	CA (TAG1α)	56	48.03	UK	101	28.25	ST
12	172.90	CA (TAG1β)	57	47.08	UK	102	27.79	UK
13	172.85	CA (TAG1β)	58	45.76	ST	103	27.33	UK
14	140.74	OL (ST5)	59	45.25	UK	104	27.20	AL (O11)
15	132.73	OL	60	42.78	UK	105	27.18	AL (L8; L14)
16	132.17	OL	61	42.28	ST13	106	27.16	AL (O8)
17	130.17	OL (L13)	62	42.14	ST4	107	26.60	UK
18	129.97	OL (O10; L9)	63	40.52	UK	108	26.44	UK
19	129.81	OL	64	39.74	ST12	109	26.01	UK
20	129.68	OL (O9)	65	39.32	UK	110	25.96	ST
21	128.82	OL	66	39.00	UK	111	25.61	AL (L11)
22	128.21	OL	67	38.76	UK	112	25.48	UK
23	128.18	OL	68	37.42	UK	113	24.90	O3; S3; L3β
24	128.03	OL (L10)	69	37.24	ST1	114	24.86	L3α
25	127.87	OL (L12)	70	36.77	UK	115	24.28	ST
26	127.69	OL	71	36.47	ST10	116	23.01	ST
27	127.06	OL	72	36.13	STchain	117	22.69	Oω2; Sω2
28	125.31	OL	73	35.84	UK	118	22.58	Lω2
29	121.63	OL (ST6)	74	35.66	UK	119	22.23	UK
30	86.79	UK	75	35.31	UK	120	21.97	UK
31	86.56	UK	76	34.25	UK	121	21.83	UK
32	85.50	UK	77	34.16	O2β; L2β; S2β	122	21.06	ST
33	78.89	UK	78	34.06	S2α	123	20.51	UK
34	72.04	GL (1,2DAG2)	79	34.00	O2α; L2α; ST	124	19.93	UK
35	71.69	ST3, HD	80	31.93	Sω3	125	19.81	ST
36	70.17	GL (1MAG2)	81	31.91	Oω3; ST	126	19.38	ST
37	69.96	UK	82	31.86	ST	127	19.30	UK
38	68.85	GL (TAG2)	83	31.79	ST	128	18.99	ST
39	68.06	GL (1,3DAG2)	84	31.52	Lω3	129	18.75	ST
40	64.99	GL (1,3DAG1/3;1MAG3)	85	31.48	UK	130	18.20	UK
41	63.31	GL (1MAG1; FH1)	86	30.90	UK	131	14.26	UK
42	62.17	GL (1,2DAG3; 2MAG1/3)	87	29.76	ME	132	14.12	Oω1; Sω1
43	62.09	GL (TAG1/3)	88	29.70	ME	133	14.08	Lω1
44	61.24	GL (1,2DAG1)	89	29.67	ME	134	11.94	ST
45	57.93	UK	90	29.63	ME	135	11.83	ST

[a]Assignments are abbreviated with carbon type followed by compound and carbon number, if known. AL, allylic, CA, carbonyl; DAG, diacylglycerol; FFA, free fatty acid; FH, fatty alcohol; GL, glycerol; HD, hydroxy derivative; L, linoleic; ME, methylene envelope; MAG, monoacylglycerol; O, oleic; OL, olefinic; S, saturated; ST, sterol; TAG, triacylglycerol; UK, unknown.

from 141.0 to 121.0 ppm, and the largest signals corresponded to unsaturated carbon atoms in the FA and to the olefinic carbons of sterols. Glycerol carbons appeared between 72.1 and 61.0 ppm, although this region was overlapped with signals corresponding to carbon atoms bonded to hydroxyl groups in sterols and fatty alcohols. In addition, and different from the spectra of complete oils, this region was not well separated from that of aliphatic carbons, which appeared from 58.0 and 11.8 ppm.

Assignment of peaks to components in the polar lipid fractions is complex because of the number of compounds present. However, knowledge of the composition of the main components present in the fractions (7) allowed us to assign a substantial number of the signals obtained. Table 1 gives the 135 signals selected to be used in SDA analysis and their corresponding assignments.

The main components of the polar lipid fractions are TAG and DAG (7). ^{13}C NMR spectra of both types of components

TABLE 2
Summary of SDA of Intensity Data from ^{13}C NMR Chemical Shifts[a]

	Training set								Test set
	Canonical function 1			Canonical function 2					
Number of groups	Eigenvalue	Variance (%)	Canonical correlation	Eigenvalue	Variance (%)	Canonical correlation	Correct assignment (%)	Validation (%)	Correct assignment (%)
2	3.070	100.0	0.868	—	—	—	98.0	98.0	90.9
3	48.801	93.8	0.990	3.200	6.2	0.873	100.0	98.0	100.0
4a	25.011	84.8	0.981	2.638	8.9	0.852	98.0	98.0	100.0
4b	27.738	85.7	0.982	2.740	8.5	0.856	100.0	98.0	90.9
5	53.473	67.2	0.991	15.511	19.5	0.969	100.0	98.0	90.9
7	75.331	70.1	0.993	18.390	17.1	0.974	100.0	96.9	100.0
8	50.918	53.7	0.990	22.173	23.4	0.978	100.0	98.0	81.8
10	142.509	66.1	0.997	38.211	17.7	0.987	100.0	94.9	81.8

[a]The training set was divided into the following groups for SDA analysis. 2: V and others; 3: V, high-oleic (O + P + H + R + X), and high-linoleic (C + U + S + F); 4a: V, (O + P), (H + R + X), and (C + U + S + F); 4b: V, O, (P + H + R + X), and (C + U + S + F); 5: V, O, P, (H + R + X), and (C + U + S + F); 7: V, O, P, H, R, X, and (C + U + S + F); 8: V, O, P, (H + R + X), C, U, S, and F; 10: V, O, P, H, R, X, C, U, S, and F. V, virgin olive oils; O, olive oils; P, olive pomace oils; H, hazelnut oils; R, rapeseed oils; X, high-oleic sunflower oils; C, corn oils; U, grapeseed oils; S, soybean oils; F, sunflower oils. For other abbreviation see Table 1.

have been broadly reported in the literature and can easily be assigned according to the chemical shifts previously reported (5,6,11,12). In addition, some of these assignments were confirmed by correlation studies between the data matrix used for SDA analysis and the content of TAG and DAG determined by high-performance gel filtration chromatography. Thus, for example, DAG were correlated with intensity of peaks 34, 39, 40, 42, and 44 ($r = 0.835$, $P < 0.0001$), indicating that these peaks were mainly due to DAG.

Assignments of other signals were carried out by comparison with the spectra of model compounds, which were found by GC to be present in the isolated fractions. In particular, the spectrum of β-sitosterol was clearly observed in the spectra of all olive oils analyzed.

Use of ^{13}C NMR spectra of oil fractions for the classification of vegetable oils. The information contained in the spectra was employed to classify vegetable oils. Oils included in the training set were classified in different groups by using SDA and Wilks' λ as a criterion for variable selection. The results have been summarized in Table 2. Only the first two canonical functions obtained are shown. The results depended on the number of groups in which the oils were grouped. Because a higher number of groups implied a smaller number of oils in each group, the results were worse for a higher number of groups because some groups were not big enough to be representative of that type of oil. Nevertheless, the training set always produced almost 100% correct classification for the 98 oils, and the validation of the method produced about 98% correct assignments. The variables selected for each SDA analysis are given in Table 3.

As an example of the results obtained, Figure 2 shows the plot of the 98 oils on the plane defined by the two canonical variables obtained with the selected variables from SDA among virgin olive, high-oleic, and high-linoleic oils. In addition, Figure 3 shows the plot of the 98 oils on the plane defined by the first two canonical variables obtained with the selected variables from SDA among the 10 groups of oils assayed.

Application of the functions obtained to the test set produced a number of correct assignments that depended on the number of groups into which the training set was divided. Thus, the 11 samples of the test set were correctly classified when the training set was divided into 3, 4a, or 7 groups; 10 samples were correctly classified when the training set was divided into 2, 4b, or 5, groups, and 9 samples were correctly classified when the training set was divided into 8 or 10 groups (Table 2).

TABLE 3
Variables Selected by SDA of Intensity Data from ^{13}C NMR Chemical Shifts[a]

Number of groups	Selected variables
2	7,8,41,56,70,94,114
3	5,17,19,21,24,29,31,46,49,56,64,65,67,73,96,100,113,115,116
4a	7,9,42,54,57,65,71,94,104,111,114,119,131
4b	4,19,24,49,56,58,65,67,68,96,98,99,113,115,126
5	7,9,16,24,33,44,46,58,60,62,63,65,67,68,69,75,80,83,90,94, 99,100,105,113,115,135
7	16,18,19,22,24,28,44,46,49,53,56,62,65,67,68,69,75,94,96, 113,115,122,129,133
8	16,24,25,33,46,49,53,65,66,67,75,76,80,94,99,100, 102,113,115,119,126
10	22,24,25,47,49,53,60,65,66,67,75,76,80,99,100,102,113, 115,116,119,129

[a]Chemical shifts of selected variables and tentative assignments are given in Table 1. For abbreviation see Table 1.

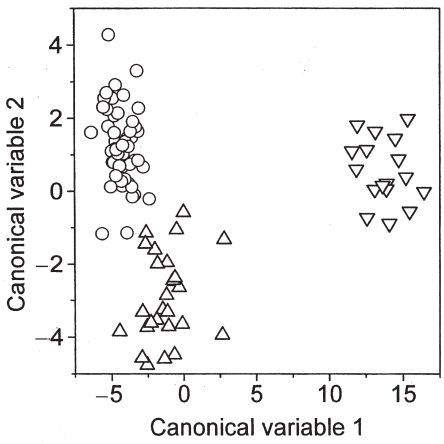

FIG. 2. Plot of the 98 oil samples on a plane defined by the two canonical variables obtained with the selected variables from stepwise discriminant analysis among (○) virgin olive, (△) high-oleic, and (▽) high-linoleic oils.

DISCUSSION

High-resolution ^{13}C NMR is considered among the most powerful techniques yet described for analysis of vegetable oils (5,6,11,13–16). However, recent studies in this laboratory found that the general application of this method to the screening of vegetable oils may fail for complicated samples or mixtures (9). In an attempt to improve the potential of the technique, the present study employed chromatographically enriched polar fractions in place of complete oils. This produced considerably richer spectra (135 signals vs. 61 signals obtained from complete oils) that contained signals corresponding to unmodified TAG, polymeric TAG, oxidized TAG, DAG, MAG, and FFA, in addition to other minor polar components of the oils. These compounds collectively characterize different oils better than the TAG signals analyzed in the spectra of the complete oils.

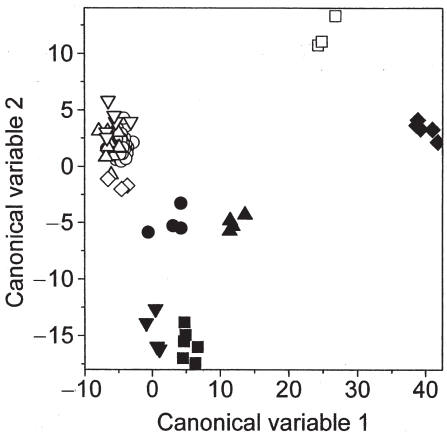

FIG. 3. Plot of the 98 oil samples on a plane defined by the two canonical variables obtained with the selected variables from stepwise discriminant analysis among (○) virgin olive, (△) olive, (▽) olive pomace, (◇) hazelnut, (□) rapeseed, (●) high-oleic sunflower, (▲) corn, (▼) grapeseed, (◆) soybean, and (■) sunflower.

Thus, when 98 vegetable oils were studied by SDA, it was possible to discriminate among the diverse types of oils, obtaining about 98% correct validated assignments. These results, which were also confirmed using a test set of 11 previously unknown samples different from those used in the training set, were better than those obtained by employing complete oils (9), thereby confirming that NMR spectroscopy may obtain much more information from the enriched polar oil fractions than from the original oils using only slightly longer acquisition times. In addition, these results were analogous to those obtained by using the unsaponifiable content of the oils (14), but in a much shorter time period and with a simpler procedure.

These results suggest that the use of oil fractions considerably increases the potential of NMR in the analysis of vegetable oils and may convert this technique into a valid routine alternative to some of the analytical methods currently employed.

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