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Detection of Extra Virgin Olive Oil Adulteration with Lampante Olive Oil and Refined Olive Oil Using Nuclear Magnetic Resonance Spectroscopy and Multivariate Statistical Analysis

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High-field ^{31}P NMR (202.2 MHz) spectroscopy was applied to the analysis of 59 samples from three grades of olive oils, 34 extra virgin olive oils from various regions of Greece, and from different olive varieties, namely, 13 samples of refined olive oils and 12 samples of lampante olive oils. Classification of the three grades of olive oils was achieved by two multivariate statistical methods applied to five variables, the latter being determined upon analysis of the respective ^{31}P NMR spectra and selected on the basis of one-way ANOVA. The hierarchical clustering statistical procedure was able to classify in a satisfactory manner the three olive oil groups. Subsequent application of discriminant analysis to the five selected variables of oils allowed the grouping of 59 samples according to their quality with no error. Different artificial mixtures of extra virgin olive oil−refined olive oil and extra virgin olive oil−lampante olive oil were prepared and analyzed by ^{31}P NMR spectroscopy. Subsequent discriminant analysis of the data allowed detection of extra virgin olive oil adulteration as low as 5% w/w for refined and lampante olive oils. Further application of the classification/prediction model allowed the estimation of the percent concentration of refined olive oil in six commercial blended olive oils composed of refined and virgin olive oils purchased from supermarkets.

KEYWORDS: ^{31}P NMR spectroscopy; extra virgin olive oil; refined olive oil; lampante olive oil; hierarchical cluster analysis; discriminant analysis; adulteration of olive oil

INTRODUCTION

Olive oil is a fine product with high nutritional value and significant health benefits (*1*−*3*). It is known for its superior organoleptic characteristics (aroma and taste) and its remarkable antioxidant properties. The cultivation of olive trees, the harvesting of the olive fruits, and the extraction of olive oil are hard and time-consuming tasks, which add to its relatively high commercial price. Therefore, attempts to adulterate this commodity with less expensive materials, such as seed oils and/or olive oils of lower quality (refined olive oil), are by no means rare. Needless to say, this practice deteriorates its quality and nutritional value and causes major economic losses.

European Mediterranean countries, which are major suppliers of olive oil in the world market, adopted common legislations to protect olive oil growers and consumers from olive oil fraud. The current European Union legislation (*4*) classifies olive oil into various categories reflecting its quality. These include various grades of virgin olive oils, in which extra virgin olive oil (EVOO) is considered to be the oil of the highest quality. Its free acidity expressed as oleic acid must be <0.8%. Lampante olive oil (LOO) is virgin olive oil with high acidity (>2.0%) and poor organoleptic properties. Refined olive oil (ROO) is an oil of lower grade obtained usually from virgin olive oil mechanically extracted from damaged olive fruits or from olives stored in unsuitable conditions and using refining methods that do not lead to alterations of the initial glyceridic structure. Its free acidity does not exceed 0.3%.

Addition of ROO to EVOO is expected to deteriorate the antioxidant properties and organoleptic characteristics of EVOO (*1*−*3*). The presence of foreign oils in EVOO is usually reflected on measured physical and chemical parameters, such as $K_{232-270}$, peroxide value, and ratio of *trans*-2-hexenal to hexanal, as well as on organoleptic assessments (*4*). Adulteration of EVOO with LOO is not a common practice and is detected quite easily by conventional methods. However, LOO distinction from EVOO is important because LOO cannot be consumed without refining (*6*), and the present methodology is suitable for this purpose.

Authentication and detection of olive oil adulteration is based on chromatographic techniques including high-resolution gas chromatography and high-performance liquid chromatography (see, for instance, ref *5*), which is the current basis of olive oil adulteration standards. Chromatography is the most accurate analytical technique with low detection limits, although it is time-consuming because it requires several steps to complete quantification. This methodology is useful for routine work for

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an assessment of the olive oil quality according to EU regulations (ECN 42, spectroscopic constants $K_{232-270}$, triglycerides, peroxide value, etc.).

In the past decade, there has been an increasing interest in the classification of edible oils including olive oil as an alternative means to examine authentication and to detect possible adulteration of extra virgin olive oils with seed oils and/or olive oils of lower grade. Classification of various grades of olive oil has been carried out in several instances by using a variety of analytical techniques and chemometric procedures. Among these are classical methods based on gas chromatography and high-performance liquid chromatography (*7*, *8*), mass spectrometry (*9*), isotope mass ratio analysis (*10*), metal oxide sensors (*6*), vibrational spectroscopy (*11*−*16*), and nuclear magnetic resonance (NMR) spectroscopy (*17*−*20*).

In a recent publication (*21*) we proposed a methodology based on NMR spectroscopy in combination with multivariate statistical analysis to classify 13 types of edible oils and to detect virgin olive oil adulteration with corn, soybean, sunflower, and hazelnut oils. In particular, ^{31}P NMR spectroscopy was used to determine in a single experiment 1,2-diacylglycerols (1,2-DGs), 1,3-diacylglycerols (1,3-DGs), total diacylglycerols (TDGs), the ratio *D* of 1,2-DGs over TDGs, total free sterols, and free acidity. This method (*21*) is based on the derivatization of the labile hydrogens of hydroxyl and carboxyl groups with 2-chloro-4,4,5,5-tetramethyl dioxaphospholane (**1**) and the use of the ^{31}P chemical shifts of the phosphitylated compounds to identify the labile centers. The phosphorus reagent reacts rapidly and quantitatively under mild conditions with the hydroxyl and carboxyl groups (*21*). The same procedure will be used in the present study to discriminate among three different grades of olive oil, namely, EVOO, ROO, and LOO, and to detect EVOO adulteration with these oils.

MATERIALS AND METHODS

Samples and Reagents. Thirty-four EVOO harvested between 2001and 2003 were provided by local cooperatives. Twenty-one olive oils were sampled in Crete (Peza, Kolymbari, Heraklion, Sitia), 3 oils in Messinia, and 1 in Ilia. They were extracted from the olive variety Koroneiki, whereas the oils from Lakonia (6 oils), Lesvos (1 oil), and Pilion (2 oils) were extracted from the varieties Athinolia, Kolovi, and a local variety, respectively. The samples were extracted by centrifugation within 48 h after harvesting and stored immediately in brown screw-capped bottles at −20 °C prior to spectrum acquisition and were coded as extra virgin olive oils according to the official limits (*4*). A total of 25 samples of olive oil of lower quality were kindly supplied by the olive oil company Minerva (Athens, Greece): 12 samples of LOO and 13 samples of ROO. Refined olive oils were deodorized in the final step of the refining process. Finally, six commercial samples of blended olive oil composed of refined olive oil and virgin olive oil (BOO) were purchased from local supermarkets.

The ^{31}P NMR method was applied to detect olive oil adulteration. Fresh EVOO samples were mixed with LOO and ROO samples. Two set of mixtures of 1, 3, 5, 10, 15, 20, 35, 50, 65, and 80% (w/w) and 1, 3, 5, 10, 15, 20, 35, and 50% for ROO adulterant in EVOO and one set of 1, 3, 5, 10, 15, and 20% (w/w) of LOO in EVOO were prepared. EVOO samples of different geographical and botanical origins and different ROO samples were used for the preparation of the various mixtures. Finally, three EVOO samples of different geographical and botanical origins (1 from Pilion, 1 from Sitia, and 1 from Lesvos), not included in the 34 EVOO samples used for the present analysis, were adulterated with different ROO samples at concentrations of 60, 65, 70, 75, and 80% (w/w). These mixtures were analyzed by ^{31}P NMR spectroscopy and used as blank tests to estimate the composition of BOO samples. All olive oil samples and their mixtures were analyzed twice.

Pinacol, triethylamine, phosphorus trichloride, protonated solvents (reagent or analytical grade), and deuterated solvents used in the present study were purchased from Sigma-Aldrich (Athens, Greece). The derivatizing phosphorus reagent 2-chloro-4,4,5,5-tetramethyldioxaphospholane (**1**) was synthesized from pinacol and phosphorus trichloride following the method described in the literature (*23*). However, to increase the yield of the reaction, we utilized hexane solvent instead of benzene and pyridine instead of triethylamine as suggested in the original method. This modification resulted in ∼45% yield of the product against 19% obtained with the original method.

Sample Preparation for ^{31}P NMR Spectral Analysis. A stock solution was prepared by dissolving 0.6 mg of chromium acetylacetonate, $Cr(acac)_3$ (0.165 μM), and 13.5 mg of cyclohexanol (13.47 mM) in 10 mL of a mixture of pyridine and $CDCl_3$ solvents (1.6:1.0 volume ratio) and protected from moisture with 5A molecular sieves. Cyclohexanol was used as an internal standard for quantification purposes. Olive oil (100−150 mg) was placed in a 5 mm NMR tube. The required volumes of the stock solution (0.4 mL) and the reagent **1** (15 μL) were added. The reaction mixture was left to react for ∼15 min at room temperature. Upon completion of the reaction, the solution was used to obtain the ^{31}P NMR spectra.

^{13}P NMR Experiments. ^{13}P NMR experiments were conducted on a Bruker AMX500 spectrometer operating at 202.2 MHz for the phosphorus-31 nucleus at 30 ± 1 °C. The spectra were recorded by employing the inverse gated decoupling technique in order to suppress NOE. Typical spectral parameters for quantitative studies were as follows: 90° pulse width = 12.5 μs, sweep width = 10 kHz, relaxation delay = 30 s, memory size = 16K (zero-filled to 32K). Line broadening of 1 Hz was applied, and drift correction was performed prior to Fourier transform. Polynomial fourth-order baseline correction was performed before integration. For each spectrum 32 transients were accumulated. All ^{31}P chemical shifts are relative to the product of the reaction of **1** with water (moisture contained in all samples), which gives a sharp signal in pyridine/$CDCl_3$ at δ 132.20. It should be noted that the presence of the paramagnetic metal center of $Cr(acac)_3$ in the samples lowers the relaxation times of the phosphorus nuclei, shortening thus the duration of the measurements significantly. The relaxation delay in the 90° pulse sequence is based on the ^{31}P spin−lattice relaxation time of 4.9 s measured for the internal standard. Lower values were measured for other model compounds (3.5 s for stearic acid and 2.3 s for 1,2-diolein). Therefore, the relaxation delay (5 times the longest relaxation time) is suitable for quantitative analysis. The applicability of this method to quantitative analysis, as well as its reproducibility and repeatability, has been tested thoroughly in previous studies (*20*, *21*).

Statistical Analysis. One-way ANOVA was used to find the variables with the highest discriminatory power. The results of this analysis are reported as the Fisher *F* ratios and *p* level. The *F* ratio with the degrees of freedom tests whether the between and within variances are significantly different. The *p* level represents a decreasing index of reliability of a result and gives the probability of error involved in accepting a result as valid. The results reported below show that the selected variables were significantly different for the three grades of olive oil. The unsupervised statistical method of hierarchical cluster analysis (HCA) was applied to explore similarity (or dissimilarity) among the various olive oil samples, whereas the supervised method discriminant analysis (DA) classified olive oils according to different grades and detected adulteration. Discriminant function analysis is used to determine which variables (e.g., compositional parameters of olive oil samples) discriminate between two or more naturally occurring groups (e.g., olive oil grades). The DA method applied to a matrix of 5 independent variables and 59 olive oil samples provided good results (see below) while being very straightforward in calculation and interpretation. Visualization of the oil classification is achieved by plotting the individual scores for two principal discriminant functions, whereas for the detection of EVOO adulteration the discriminant functions obtained from the DA model and the Mahalanobis distances were used. The statistical package Statistica for Windows 5.1B (StatSoft Inc.) was used.

RESULTS AND DISCUSSION

Data Analysis. Results are summarized in **Tables 1**−**3**. **Table 1** contains the percentage content of five chemical components

Table 1. Compositional Parameters, Means, and Standard Deviations of Extra Virgin Olive Oils from Various Regions of Greece and of Commercial Blended Olive Oils Determined by ^{31}P NMR Spectroscopy

sample	area	1,2-DGs	1,3-DGs	TDGs	D	sterols	acidity
EVOO[a]							
1	Sitia	2.15	0.17	2.32	0.93	0.140	0.33
2	Sitia	1.58	0.09	1.67	0.95	0.148	0.21
3	Sitia	1.88	0.15	2.03	0.93	0.093	0.24
4	Sitia	2.05	0.17	2.22	0.92	0.115	0.35
5	Sitia	2.04	0.13	2.17	0.94	0.126	0.33
6	Sitia	1.74	0.15	1.89	0.92	0.089	0.20
7	Sitia	1.86	0.12	1.98	0.94	0.121	0.25
8	Heraklion	2.49	0.16	2.65	0.94	0.155	0.40
9	Heraklion	1.57	0.15	1.72	0.92	0.091	0.23
10	Heraklion	2.08	0.16	2.24	0.93	0.143	0.24
11	Heraklion	1.70	0.13	1.83	0.93	0.128	0.21
12	Heraklion	1.90	0.14	2.04	0.93	0.149	0.24
13	Heraklion	1.58	0.12	1.70	0.93	0.118	0.38
14	Kolymbari	1.76	0.18	1.94	0.91	0.093	0.39
15	Kolymbari	2.30	0.23	2.53	0.91	0.141	0.30
16	Kolymbari	1.69	0.14	1.83	0.92	0.106	0.26
17	Peza	1.72	0.14	1.86	0.93	0.112	0.32
18	Peza	2.22	0.19	2.41	0.92	0.167	0.41
19	Peza	1.39	0.11	1.50	0.93	0.103	0.18
20	Peza	1.49	0.11	1.60	0.93	0.124	0.18
21	Peza	1.90	0.12	2.02	0.94	0.160	0.22
22	Lakonia	1.94	0.20	2.14	0.91	0.098	0.21
23	Lakonia	2.05	0.13	2.18	0.94	0.071	0.24
24	Lakonia	1.79	0.12	1.91	0.94	0.081	0.16
25	Lakonia	2.08	0.17	2.25	0.93	0.113	0.20
26	Lakonia	2.06	0.16	2.22	0.93	0.077	0.22
27	Lakonia	2.15	0.14	2.29	0.94	0.101	0.21
28	Messinia	1.74	0.12	1.86	0.94	0.094	0.22
29	Messinia	1.77	0.18	1.95	0.91	0.080	0.20
30	Messinia	1.56	0.18	1.74	0.90	0.099	0.24
31	Pilion	2.29	0.17	2.46	0.93	0.099	0.63
32	Pilion	2.10	0.15	2.25	0.93	0.076	0.46
33	Lesvos	1.77	0.16	1.93	0.92	0.104	0.42
34	Ilia	2.37	0.08	2.45	0.97	0.154	0.20
av		1.90	0.15	2.05	0.93	0.114	0.28
SD		0.27	0.03	0.28	0.01	0.027	0.10
35[b]	Pilion	2.02	0.14	2.16	0.94	0.08	0.46
36[b]	Lesvos	1.97	0.23	2.20	0.90	0.10	0.77
37[b]	Sitia	2.15	0.17	2.32	0.93	0.14	0.32
BOO[c]							
1		1.71	1.72	3.43	0.50	0.07	0.53
2		1.41	3.28	4.69	0.30	0.07	0.68
3		1.20	1.88	3.08	0.39	0.06	0.56
4		1.63	3.08	4.71	0.35	0.05	0.22
5		0.92	0.84	1.76	0.52	0.07	0.19
6		1.43	2.24	3.67	0.39	0.06	0.30

[a] EVOO, extra virgin olive oils. [b] EVOO samples 35–37 were used for the preparation of blank tests (see text). [c] BOO, blended olive oils composed of refined olive oils and virgin olive oils.

Table 2. Compositional Parameters, Means, and Standard Deviations of Lampante and Refined Olive Oils Determined by ^{31}P NMR Spectroscopy

sample	1,2-DGs	1,3-DGs	TDGs	D	sterols	acidity
LOO[a]						
1	1.35	2.85	4.20	0.32	0.104	2.81
2	1.85	3.08	4.93	0.38	0.099	6.45
3	1.67	4.14	5.81	0.29	0.080	7.33
4	2.08	2.55	4.63	0.45	0.107	5.10
5	2.10	3.19	5.29	0.40	0.107	8.73
6	1.29	1.92	3.21	0.40	0.168	2.96
7	2.23	5.38	7.61	0.29	0.090	7.55
8	2.38	4.64	7.02	0.34	0.08	10.17
9	1.77	4.31	6.08	0.29	0.07	7.78
10	1.00	2.23	3.23	0.31	0.100	1.88
11	0.73	1.84	2.56	0.28	0.08	2.29
12	2.41	5.80	8.21	0.29	0.07	10.86
av	1.74	3.49	5.18	0.34	0.096	6.16
SD	0.55	1.34	1.79	0.05	0.027	3.11
ROO[b]						
1	1.47	2.95	4.42	0.33	0.066	0.03
2	1.68	3.16	4.84	0.35	0.047	0.00
3	1.55	3.33	4.88	0.32	0.099	0.07
4	1.61	2.83	4.44	0.36	0.063	0.04
5	1.85	3.52	5.37	0.35	0.048	0.02
6	2.14	3.71	5.85	0.37	0.108	0.02
7	2.10	3.71	5.81	0.36	0.086	0.00
8	1.39	2.63	4.02	0.35	0.096	0.00
9	1.60	3.89	5.49	0.29	0.077	0.03
10	1.53	3.50	5.03	0.31	0.067	0.06
11	0.97	2.31	3.28	0.30	0.00	0.015
12	1.49	3.58	5.07	0.29	0.066	0.03
13	0.99	2.23	3.22	0.31	0.066	0.04
av	1.57	3.18	4.75	0.33	0.068	0.03
SD	0.35	0.55	0.85	0.03	0.028	0.02

[a] LOO, lampante virgin olive oils. [a] ROO, refined virgin olive oils.

and the ratio *D* (1,2-DGs/TDG) for the various samples of EVOO and BOO, whereas **Tables 2** and **3** depict the same parameters for LOO, ROO, and their mixtures with EVOO, respectively. Spectral assignments and methods of quantification of the NMR data have been reported in detail in previous publications (*20*, *21*). The mean values in **Tables 1** and **2** show that there are discernible differences in 1,3-DGs, TDGs, the ratio *D* (1,2-DGs/TDGs), and free acidity. These parameters appear to differentiate the olive oil samples. In particular, EVOO are characterized by high values of the ratio *D* and low values of TDGs and free acidity relative to the same parameters of the other oils. It is known that the natural content of TDGs in EVOO does not exceed 1−3% depending on the olive fruit ripeness and olive fruit variety (*20*, *24*−*26*), whereas the level of TDGs (mainly 1,3-DGs) is higher (4−5%) in ROO (*27*). Moreover, in EVOO samples, 1,3-DGs are much lower than the corresponding values in the ROO samples. This is due to the fact that the isomerization of 1,2-DGs to 1,3-DGs that usually occurs during prolonged olive oil storage is very rapid upon olive oil refinement (*24*, *28*). The LOO samples have characteristics similar to those of ROO except free acidity. The discrimination of EVOO with respect to the other oils can be seen graphically in **Figure 1**, where the ratio *D* is plotted against the difference of TDGs minus free acidity (TDG−FFA, where FFA stands for free fatty acids). It is seen clearly that the EVOO samples are clustered in the upper part of the graph, whereas LOO and ROO are dispersed in the lower part of the graph. What is most interesting in this graph is the observation that adulterated EVOO samples (solid symbols) with LOO and ROO lie between the group of EVOO and the respective group of the lower quality olive oils, depending on the amount of the latter oils in the mixtures. This finding may suggest an alternative method to detect EVOO adulteration with LOO and ROO, although this observation needs further exploration on an experimental and theoretical basis. At any rate, it appears that the parameter TDG−FFA is able to discriminate EVOO from the other oils.

When free acidity was plotted against 1,3-DGs for all oil samples, a good linear correlation ($r = 0.95$) was obtained, indicating that the concentration of free fatty acids increases with increasing concentration of 1,3-DGs. This pattern, which has been observed previously (*26*), can be explained as follows: hydrolysis of triglycerides results in 1,2- (and/or 2,3-) DGs and 1,3-DGs in a concentration ratio of 2:1 and free fatty acids, followed by isomerization of 1,2-DGs to 1,3-DGs. The isomerization reaction proceeds more rapidly than hydrolysis (*28*), and the consumption of 1,2-DGs shifts the equilibrium of

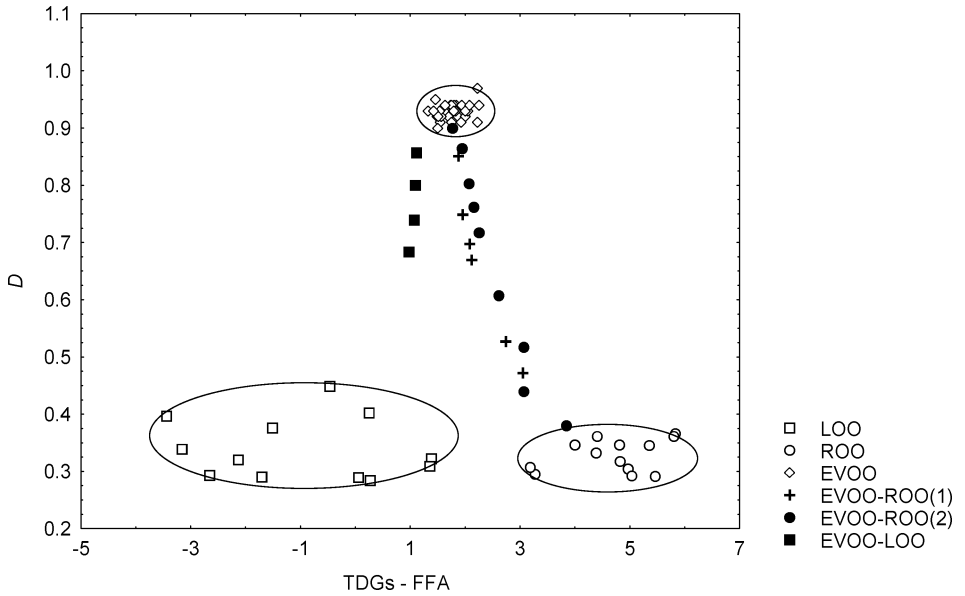

Figure 1. Plot of the *D* ratio against the difference TDGs–FFA for 59 samples of extra virgin olive oils (EVOO), refined olive oils (ROO), and lampante olive oils (LOO) (open symbols) and for the EVOO mixtures with the lower quality olive oils (solid symbols). Ellipses were drawn as an aid to the eye.

Table 3. Compositional Parameters of Mixtures of Extra Virgin Olive Oils with Refined and Lampante Olive Oils Determined by ^{31}P NMR Spectroscopy

mixture	concn (%, w/w)	1,2-DGs	1,3-DGs	TDGs	D	sterols	acidity
EVOO–ROO (1)[a]	1	1.85	0.16	2.01	0.92	0.100	0.21
	3	1.81	0.23	2.04	0.89	0.100	0.18
	5	1.76	0.31	2.07	0.85	0.095	0.16
	10	1.61	0.54	2.15	0.75	0.090	0.15
	15	1.57	0.68	2.25	0.70	0.095	0.15
	20	1.54	0.75	2.29	0.67	0.090	0.14
	35	1.52	1.36	2.88	0.53	0.078	0.11
	50	1.49	1.67	3.16	0.47	0.075	0.10
EVOO–ROO (2)[b]	1	1.79	0.17	1.96	0.91	0.057	0.15
	3	1.72	0.19	1.91	0.90	0.078	0.14
	5	1.79	0.28	2.07	0.86	0.072	0.13
	10	1.77	0.43	2.20	0.80	0.082	0.13
	15	1.73	0.54	2.27	0.76	0.081	0.12
	20	1.69	0.67	2.36	0.72	0.073	0.11
	35	1.65	1.07	2.72	0.61	0.075	0.10
	50	1.62	1.51	3.13	0.52	0.083	0.07
	65	1.37	1.75	3.12	0.44	0.060	0.04
	80	1.49	2.41	3.90	0.38	0.070	0.06
EVOO-LOO[c]	1	1.47	0.15	1.62	0.91	0.118	0.37
	3	1.41	0.20	1.61	0.88	0.145	0.42
	5	1.40	0.23	1.63	0.86	0.109	0.52
	10	1.35	0.34	1.69	0.80	0.111	0.60
	15	1.33	0.47	1.80	0.74	0.090	0.73
	20	1.29	0.60	1.89	0.68	0.121	0.90
EVOO–ROO(3)[d]							
1	60	1.79	1.2.07	3.86	0.46	0.12	0.17
2	65	1.76	2.22	3.98	0.44	0.114	0.16
3	70	1.75	2.55	4.30	0.40	0.074	0.15
4	75	1.70	2.90	4.60	0.37	0.080	0.20
5	80	1.60	3.15	4.75	0.34	0.108	0.13

[a] First set of mixtures obtained from EVOO sample 27 (**Table 1**) and ROO sample 2 (**Table 2**). [b] Second set of mixtures obtained from EVOO sample 24 (**Table 1**) and ROO sample 4 (**Table 2**). [c] Mixtures obtained from EVOO sample 13 (**Table 1**) and LOO sample 6 (**Table 2**). [d] Samples for blank tests were prepared as follows: 1 and 2 from EVOO sample 37 (**Table 1**) and ROO 3 (**Table 2**); 3 from EVOO sample 35 (**Table 1**) and ROO 10 (**Table 2**); 4 and 5 from EVOO sample 36 (**Table 1**) and ROO 9 (**Table 2**).

hydrolysis toward the products and, hence, increases the concentration of free fatty acids.

Classification of Olive Oils with Multivariate Statistical Analysis. A study of skewness and kurtosis on each variable demonstrated that all had an almost normal distribution, except perhaps free acidity, which showed positive skewness (2.25) and kurtosis (3.91). Logarithmic transformation was applied to this variable before further analysis was performed. Multivariate outliers among samples were detected by applying principle component analysis (PCA). No outliers were identified in the present data. The variables with the highest discriminating power were selected by employing one-way ANOVA. On the basis of the Fisher *F* ratio, we selected the following variables (in parentheses the *F* values with degrees of freedom 2 and 56): 1,3-DGs, (176.2), TDGs (74.0), *D* (3017.9), sterols (13.4), log of acidity (434.7), TDG-FFA (135.2) with the probability $p < 0.000017$ for sterols and $p < 0.000001$ for the remaining variables. The *F* ratios are higher than the critical values obtained from standard statistical tables at $p = 0.005$, F_{critical} $(2, 56) = 5.80$, or upon comparison with F_{critical} $(2, 11) = 7.20$ at $p = 0.01$, 12 being the number of oil samples minus 1 for the group of olive oils with the least number of samples (LOO with 12 samples). The strict criteria for the chosen probability (usually $p = 0.05$) was justified by the fact that the groups of olive oils contain different numbers of samples. The relatively low Fisher *F* ratio observed for sterols is indicative of its limited discriminatory power, and thus it is excluded from further statistical analysis.

The selected variables were used next to examine the similarity (or dissimilarity) of the oils by means of HCA. This statistical method organizes the clustering of different objects without any a priori knowledge of the of class membership on the basis of a similarity indicator (distance) and an amalgamation algorithm that joins similar objects into clusters. The results of HCA for the present olive oil are presented in the form of a dendrogram in **Figure 2** obtained with Euclidean distances and single linkage. Apart from one lampante oil sample, a clear grouping of the three olive oil grades is achieved.

For further statistical analysis using DA, the type of oil was chosen as a grouping variable or code. Also, this supervised statistical method allows detection of adulteration (see below). **Figure 3** shows the graph of the two canonical functions (roots) obtained, which succeeded in classifying the 59 oil samples into

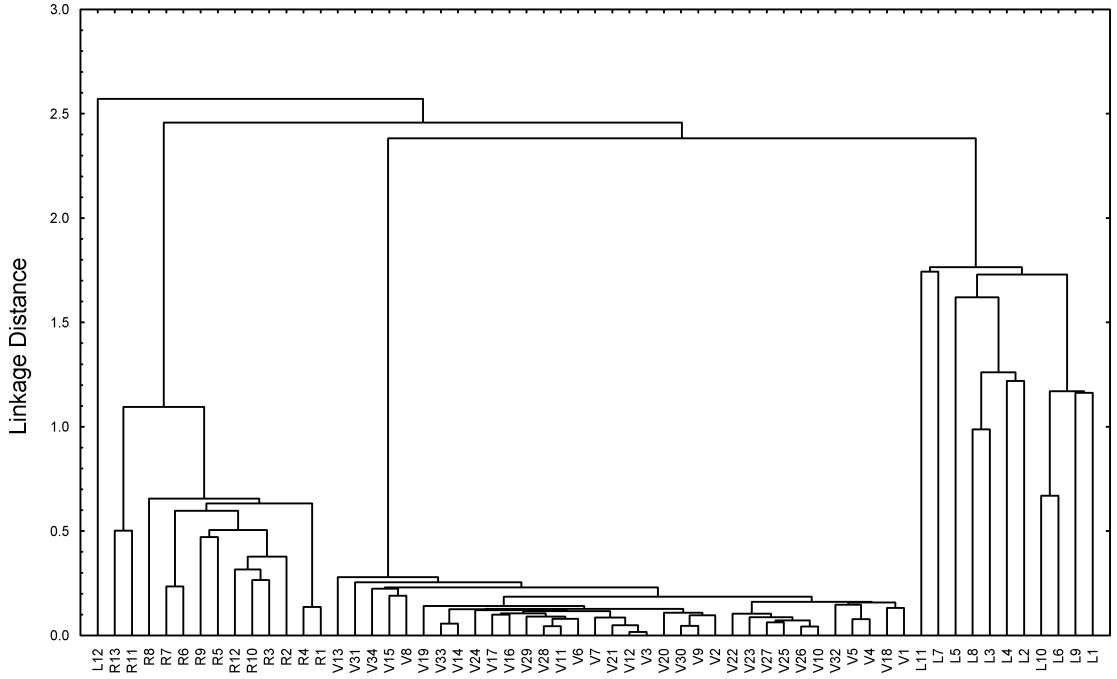

Figure 2. Dendrogram showing the clustering of the 59 samples of olive oils based on the NMR data set. Samples labeled with the same letter originate from the same olive oil grade: extra virgin olive oil (V), refined olive oil (R), lampante olive oil (L).

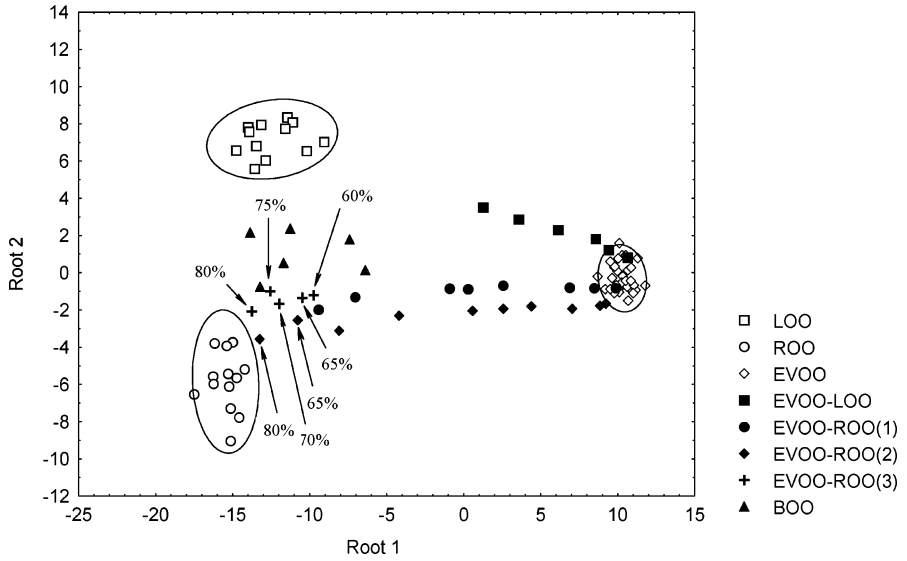

Figure 3. Plot of the discriminant functions roots 1 and 2 for three grades of olive oils, extra virgin olive oils (EVOO), refined olive oils (ROO), and lampante olive oils (LOO) (open symbols) and two sets of mixtures, EVOO–ROO (1) and EVOO–ROO (2), containing 1–50 and 1–80% w/w ROO in EVOO, respectively, and one set of mixtures 1–20% of EVOO with LOO (solid symbols). Crosses denote the blank tests, EVOO–ROO (3), and solid triangles denote the commercial blended olive oils (BOO). Arrows indicate mixtures of EVOO containing ROO at concentrations >50%.

three groups. The ellipses denote the 95% probability that a certain sample belongs to its own group of oils within the region defined by the ellipse. The raw coefficients, the tolerance values, and the Wilk's λ criterion assigned to the selected variables are shown in **Table 4**. Variables 1,3-DGs, TDGs, *D*, and log acidity have the largest raw coefficients of the canonical functions, reflecting their significant contribution to the discrimination between groups. The values of Wilk's λ are very small, indicating that the five selected variables are characterized by an effective discriminatory power. Also, the high tolerance values show no indication of redundancy among the selected variables.

The reliability of the system used to classify the oils has been validated by its ability to correctly classify unknown samples. In this respect, the data set of all oils was split into the training set (around two-thirds of the samples) and the test set (around one-third of the samples) considered as unknowns. The samples of the training and test sets were selected three times at random from 59 oil samples. Each time, different training and test sets were used. Application of the DA classification functions obtained for the training set of oil samples in all three runs produced 100% correct assignments for the test set of EVOO, LOO, and ROO.

Adulteration of Extra Virgin Olive Oils. DA was applied to the analysis of the oil mixtures (adulterated EVOO) analyzed by ^{31}P NMR spectroscopy and considered as unknown samples. Results were calculated using the model obtained by the training set and depicted in **Figure 3**. The adulterated EVOO samples with ROO and LOO (solid symbols) (see also **Table 3** for the origin of mixtures) lie between the group of EVOO and the

Table 4. Discriminant Analysis: Raw Coefficients, Wilk's λ Values, and Tolerance Levels for Each Variable Used for the Classification of Four Grades of Olive Oils

variable	root 1	root 2	Wilk's λ	tolerance level
1,3-DGs	3.727	–0.026	0.000446	0.0474
TDGs	–2.605	–0.511	0.000459	0.0506
D	48.509	–2.765	0.000638	0.4588
log of acidity	1.907	5.638	0.001134	0.5598
TDGs–FFA	–0.335	–0.069	0.000344	0.6429
constants	–29.471	6.70		
eigenvalues	152.923	19.01		

Table 5. Squared Mahalanobis Distances of Adulterated EVOO Samples from Olive Oil Centroid

adulterant	1%	3%	5%	10%	15%	20%	35%	50%	65%	80%
ROO[a]	0.6	3.7	12.3	61.3	102.2	128.0	304.8	394.5		
ROO[b]	3.7	5.2	14.2	38.6	64.24	99.5	217.1	349.9	452.0	576.6
LOO[c]	3.6	5.5	9.7	26.2	57.61	99.4				

[a] First set of mixtures (1–50%) of EVOO with ROO. [b] Second set of mixtures (1–80%) of EVOO with ROO. [c] Set of mixtures (1–20%) of EVOO with LOO.

respective group of ROO and LOO depending on the amount of the latter oils in the mixtures. The first set of mixtures containing the lowest amount of adulterant ROO in EVOO (1%) falls inside the 95% ellipse of EVOO, whereas the second set of mixtures with the same concentration of adulterant lies in the border of the ellipse of EVOO (**Figure 3**). The adulterated EVOO with LOO show higher detection limits, because only the 5% mixture is outside the 95% ellipse of EVOO group,

A more accurate means to detect adulteration is the Mahalanobis distance, which measures the distances between each point and the group centroid. The calculated Mahalanobis distances for mixtures (**Table 5**) are to be compared with the Mahalanobis distance of EVOO samples from the centroid of their own group. Apart from 2 EVOO samples of 34 with Mahalanobis distances 6.7 and 5.3, all other EVOO samples are characterized by distances lower than those calculated for the EVOO with 5% of ROO and EVOO adulterated with 5% LOO. These data clearly show that the minimum amounts of ROO and LOO that can be detected by using the present methodology are 5%.

Although application of DA to adulteration studies does not lead to precise quantitative conclusions, the almost linear array of the adulterated EVOO samples in the plot of **Figure 3** may allow a semiquantitative estimation of the proportion of added foreign oil. First, we prepared mixtures of EVOO with ROO of known composition (60, 65, 70, 75, and 80%) using different EVOO samples not included in the previous statistical treatment (see **Table 3** and footnote). These samples, used as blank tests, were treated as unknowns using the model obtained by the training set of 59 oils. As shown in **Figure 3**, these mixtures indicated by arrows are lying close to the array of the adulterated EVOO with ROO used in the statistical analysis at positions depending on the amount of the added ROO. The Mahalanobis distances for 65% (436.4) and 80% (586.4) adulteration agree closely with those reported for the corresponding values of the mixtures in the array with the same composition (**Table 5**). Next, we consider real oils represented by six blended olive oils (BOO) consisting of refined olive oil and virgin olive oil and purchased from supermarkets in an attempt to estimate the percent concentration of ROO in these commercial products. These samples were treated again as unknowns using the model obtained by the training set of the 59 oil samples (**Figure 3**). The observed deviations of the commercial oils from the arrays is justified by the fact that the EVOO and ROO samples used to prepare these oils are characterized by different compositions of diglycerides and acidity. However, their compositions can be estimated on the basis of the calculated Mahalanobis distances from the centroid of the group of EVOO samples, which are to be compared with the corresponding distances of all the artificial mixtures in **Figure 3**. Two of the six BOO samples (1 and 5) show Mahalanobis distances of 286.03 and 323.45, respectively, which are within the respective distances of the adulterated EVOO samples with 35 and 50% ROO (**Table 5**) and lower than the Mahalanobis distance of 405.5 for the 60% blank sample. The Mahalanobis distances of BOO samples 3 (477.8) and 6 (488.0) are close to the corresponding value (501.1) of the blank mixture with 70% ROO, whereas the final commercial products 2 and 4 contain ~80% ROO as indicated by their Mahalanobis distances of 606.4 and 560.0, respectively, which agrees closely with those of the oils in the array (**Table 5**) and the blank sample (586.4) of the same concentration.

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