Application of Stable Isotope Ratio Analysis to the Characterization of the Geographical Origin of Olive Oils

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To gain information about the geographical origin of oil samples, measurements of $\delta^{13}C$ and $\delta^{18}O$ of the whole oil and some of its fractions have been performed on samples coming from fruits of *Olea europaea* L. produced in Greece, Morocco, Spain, Italy, Tunisia, and Turkey. The results obtained by applying statistical procedures have given pieces of evidence that oil samples have shown the trend to cluster according to the different climatic areas of growing environment of fruits. Some confusion has been observed for samples coming from neighboring countries having similar climates.

Keywords: *Carbon and oxygen stable isotopes; olive oil; geographical origin*

INTRODUCTION

Increasing interest has been shown by consumers in knowing the geographical and/or varietal origin of foodstuffs they buy, because such knowledge is regarded as an additional warranty of their quality, authenticity, and typicality.

Two regulations of the European Community, No. 2081/91 and 2082/91 (Denomination of Protect Origin), have considered the expectations of consumers and especially that of producers regarding a better protection of virgin olive oils which, coming from areas particularly suitable for olive growing, have peculiar characteristics from an organoleptic or compositional point of view.

An essential part of these regulations regards the presence of oil components which, because of their chemical nature or their abundance, can be considered specific or peculiar to the product accepted for the denomination. Such components must be ascertainable in an univocal way by means of instrumental and sensory methodologies. However, the same regulations do not provide any parameters or analytical methods that enable the verification of some peculiarities boasted by some olive oils that use this designation.

Chemometric methodologies, applied to the olive oil composition in several studies, sometimes successfully proved that the geographical and varietal origin of samples can be related to some chemical compounds such as fatty acids, sterols, aliphatic and triterpenic alcohols, phenolic substances, tocopherols, phytol, triacylglycerols, and volatile components (Forina and Tiscornia, 1982; Leardi and Paganuzzi, 1987; Armanino et al., 1989; Modi et al., 1992; Alessandri et al., 1992a,b; Aparicio et al., 1994; Aparicio and Alonso, 1994; Balestrieri et al., 1995; Tarandjiiska and Marekov, 1998). However, these methods show the serious restriction requiring the determination of a great number of classes of compounds that, in their turn, are heavily dependent on many factors related to raw material (cultivar, climate, ripening degree, and quality of fruits) and to technological parameters (conditions of harvest and storage of olives, transport, extraction of the oil, and its preservation) (Camera et al., 1976; Camera and Angerosa, 1978; Lotti et al., 1982; Angerosa and Marsilio, 1983; Montedoro et al., 1989; Fiorino and Nizzi Grifi, 1991; Servili et al., 1993; Angerosa and Di Giacinto, 1995; Angerosa et al., 1996a,b, 1997a,b). Therefore, the identification of objective analytical parameters capable of providing useful elements for the characterization of the oil typicality, that is, of its geographical origin, is a very complex problem.

Stable isotope ratio analysis is increasingly spreading as a tool to verify the authenticity of foodstuffs and also their origin, like wine, for instance (Martin et al., 1988; Versini et al., 1995; Rossmann et al., 1996). Recently, relationships between the geographical origin of oils and the $\delta^{13}C$ values of individual fatty acids have been proved for maize oils (Woodbury et al., 1998).

Due to its chemical composition, olive oil can be characterized by ^{13}C, ^{18}O, and D of organic matter. The $^{13}C/^{12}C$ of whole olive oils and of the aliphatic alcohols and sterols has been determined (Bianchi et al., 1993; Angerosa et al., 1997a,b). Recently, the SNIF-NMR of deuterium (Lai et al., 1995; Quemerais et al., 1995; Buddrus et al., 1995) and the $^{18}O/^{16}O$ of the whole oil by pyrolysis-IRMS (Bréas et al., 1998) have been proposed for the characterization of olive oils.

In our preliminary paper we showed that the combined $^{18}O/^{16}O$ and $^{13}C/^{12}C$ isotopic profiles could provide information about the geographical origin of samples. These preliminary results encouraged us to extend the measurement to a greater number of olive oil samples coming from different countries to verify the real possibility of their use for the characterization of geographical origin of oils.

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Table 1. $\delta^{13}C$ and $\delta^{18}O$ Values of the Whole Olive Oils and of Sterols and Aliphatic Alcohols of Samples from Different Countries

oil sample	country	$\delta^{13}C$ whole oil	$\delta^{18}O$ whole oil	$\delta^{13}C$ sterols	$\delta^{13}C$ aliphatic alcohols
1	Greece	−28.4	24.3	−26.5	−27.9
2	Greece	−28.3	24.6		
3	Greece	−28.3	22.6		
4	Greece	−28.4	23.1		
5	Greece	−27.8	24.4	−25.9	−27.6
6	Greece	−27.6	24.9	−25.5	−27.4
7	Greece	−28.0	23.1	−26.4	−28.3
8	Greece	−29.1	22.4		
9	Greece	−28.9	22.9		
10	Greece	−28.2	22.3		
11	Morocco	−28.8	28.5		
12	Morocco	−28.9	30.7		
13	Morocco	−28.8	29.7	−28.0	−28.9
14	Morocco	−28.9	27.9	−28.5	−29.2
15	Morocco	−28.9	28.4	−29.1	−29.4
16	Morocco	−29.0	27.1	−29.7	−30.2
17	Morocco	−28.9	29.8		
18	Morocco	−28.4	26.9		
19	Morocco	−29.2	28.0	−29.1	−29.7
20	Morocco	−28.1	28.0	−28.1	−29.8
21	Spain	−29.0	25.9	−28.0	−29.4
22	Spain	−29.1	25.6	−26.2	−30.3
23	Spain	−29.2	24.9	−26.7	−29.9
24	Spain	−28.8	26.0	−28.5	−28.5
25	Spain	−28.6	25.5		
26	Spain	−28.5	24.3		
27	Tunisia	−28.2	26.2		
28	Tunisia	−28.6	26.0		
29	Tunisia	−28.2	27.1		
30	Tunisia	−28.3	25.7	−28.3	−28.3
31	Tunisia	−28.3	25.4	−27.9	−28.8
32	Tunisia	−28.3	25.3		
33	Tunisia	−29.6	26.0	−27.3	−28.2
34	Tunisia	−29.1	27.9		
35	Tunisia	−29.0	26.7	−27.0	−28.5
36	Tunisia	−28.4	26.5	−27.8	−28.7
37	Tunisia	−28.5	25.6	−28.2	−28.4
38	Turkey	−27.7	24.9	−25.6	−27.5
39	Turkey	−29.1	24.6		
40	Turkey	−27.9	25.2	−25.8	−27.7
41	Turkey	−27.9	24.0	−26.6	−27.8
42	Turkey	−28.5	24.6	−26.4	−28.2

In addition, the measurement of the $^{13}C/^{12}C$ ratio of the sterolic and aliphatic alcoholic fractions has been carried out for improving this characterization.

MATERIALS AND METHODS

Materials. Forty-two samples of oils from fruits of *Olea europaea* L. produced in different countries of the Mediterranean basin (Greece, Morocco, Spain, Tunisia, and Turkey), all collected in 1994 regardless of olive varieties and their ripening degree, were investigated. In addition, forty-one olive oils extracted from fruits harvested during the same harvesting year in different regions of Italy were analyzed. The geographical origins of these last samples were known in a more precise way and are shown in Table 2.

All solvents for organic residual analysis were purchased from J. T. Baker (Deventer, Holland), KOH and 2′,7′-dichlorofluorescein, both analytical grade reagents, were from Carlo Erba (Milano, Italy), and silica gel G 60 for thin-layer chromatography was from E. Merck (Schuchardt, Germany). Cholesterol and 1-eicosanol were purchased from Aldrich (Steinheim, Germany).

Pure samples of sterols and aliphatic alcohols were isolated according to EC methods (EC Regulation 2568/91). The oil sample (5 g) was saponified by an ethanolic potassium hydroxide solution (6 g of KOH in 50 mL of 95° ethanol), and then the unsaponifiable matter was extracted by diethyl ether and fractionated by preparative thin-layer chromatography.

Table 2. $\delta^{13}C$ and $\delta^{18}O$ Values of the Whole Olive Oils and of Sterols and Aliphatic Alcohols of Samples from Italy

oil sample	region	city	$\delta^{13}C$ whole oil	$\delta^{18}O$ whole oil	$\delta^{13}C$ sterols	$\delta^{13}C$ aliphatic alcohols
43	Abruzzo	Fossacesia (CH)	−27.8	20.0		
44	Abruzzo	Pianella (PE)	−28.1	22.5		
45	Abruzzo	Pianella (PE)	−28.2	23.9		
46	Calabria	Rende (CS)	−30.3	20.6		
47	Calabria	Rende (CS)	−29.3	21.9		
48	Emilia Romagna	Brisighella (RA)	−28.7	20.3		
49	Lazio	Paliano (FR)	−30.3	18.1		
50	Lazio	Canino (VT)	−30.0	21.0		
51	Liguria	Taggia (IM)	−28.8	20.7		
52	Liguria	Arnasco (SV)	−29.1	19.6		
53	Lombardia	Iseo (BS)	−30.3	22.0		
54	Lombardia	Sulzano (BS)	−30.7	18.6		
55	Marche	S. Benedetto del T. (AP)	−31.3	18.1		
56	Marche	Cartoceto (PS)	−29.1	20.0		
57	Molise	Termoli (CB)	−29.9	21.0		
58	Puglia	Andria (BA)	−27.7	23.9		
59	Puglia	Molfetta (BA)	−29.6	20.6		
60	Puglia	Monopoli (BR)	−27.4	21.3		
61	Sardegna	Alghero (SS)	−27.0	23.1		
62	Sardegna	Sorso (SS)	−28.8	22.7		
63	Sicilia	Lentini (CT)	−28.6	23.7		
64	Sicilia	Santa Ninfa (TP)	−28.3	22.2		
65	Toscana	Gaiole in Chianti (SI)	−30.3	19.3		
66	Toscana	Massarosa (LU)	−29.8	19.3		
67	Toscana	Trequanda (SI)	−28.6	21.1		
68	Toscana	Montepulciano (SI)	−30.6	19.1		
69	Toscana	Trequanda (SI)	−30.2	19.3		
70	Toscana	Pienza (SI)	−29.5	20.3		
71	Toscana	Cerbaia (FI)	−29.5	19.1		
72	Toscana	Ponte a Chiani (AR)	−30.2	19.8		
73	Umbria	Passignano (PG)	−29.5	20.5		
74	Umbria	Spoleto (PG)	−29.5	20.1	−27.1	−28.8
75	Umbria	Spoleto (PG)	−30.6	19.3	−26.5	−28.6
76	Umbria	Spoleto (PG)	−29.7	20.4	−26.6	−28.7
77	Lazio	Monterotondo (RM)	−29.8	19.6	−28.1	−29.5
78	Lazio	Monterotondo (RM)	−30.5	20.7	−30.5	−31.7
79	Lazio	Canneto Sabino (RI)	−30.6	20.5	−28.6	−28.2
80	Lazio	Canneto Sabino (RI)	−31.3	20.9	−29.8	−30.7
81	Lazio	Canneto Sabino (RI)	−29.4	21.9	−26.7	28.5
82	Lazio	Vetralla (VT)	−30.7	20.9	−28.4	−29.3
83	Lazio	Vetralla (VT)	−31.2	21.2	−29.8	−30.6

Sterol and aliphatic alcohol bands, identified by comparison with reference cholesterol and 1-eicosanol standards, were recovered from silica gel by the usual workup.

Isotopic Determination. Stable isotope ratios were determined with a Finnigan MAT (Bremen, Germany) Delta S mass spectrometer, interfaced on-line with a Carlo Erba (ThermoQuest, Milan, Italy) CHN 1108 elemental analyzer for sample combustion.

The analytical procedures adopted for measuring the $^{13}C/^{12}C$ ratios are described in Angerosa et al. (1997b), whereas the analytical procedures adopted for measuring the $^{18}O/^{16}O$ ratios are described in Bréas et al. (1998).

The measurements are expressed as per thousand (‰) versus an international standard, according to the equation

$$\delta X‰ = (R_{sample}/R_{standard} - 1) \times 10^3 \quad (1)$$

where X = ^{13}C or ^{18}O and R = $^{13}C/^{12}C$ or $^{18}O/^{16}O$.

The international standard for ^{13}C is Pee Dee Belemnite (PDB), and the international standard for ^{18}O is standard mean ocean water (SMOW).

Table 3. Mean Values, Standard Deviations (SD), Minima, and Maxima of $\delta^{13}C$ and $\delta^{18}O$ Values and Mean Confidence Intervals (CI) for All Considered Countries

	Greece		Italy		Morocco		Spain		Tunisia		Turkey	
	$\delta^{13}C$	$\delta^{18}O$	$\delta^{13}C$	$\delta^{18}O$	$\delta^{13}C$	$\delta^{18}O$	$\delta^{13}C$	$\delta^{18}O$	$\delta^{13}C$	$\delta^{18}O$	$\delta^{13}C$	$\delta^{18}O$
N	10	10	41	41	10	10	6	6	11	11	5	5
mean	−28.3	23.5	−29.5	20.7	−28.8	28.5	−28.9	25.4	−28.6	26.2	−28.2	24.6
SD	0.45	0.99	1.08	1.47	0.31	1.21	0.28	0.65	0.45	0.79	0.58	0.44
min	−29.1	22.3	−31.3	18.1	−29.2	27.1	−29.2	24.3	−29.6	25.3	−29.1	24.0
max	−27.6	24.9	−27.0	23.9	−28.4	30.7	−28.5	26.0	−28.2	27.9	−27.7	25.2
CI	±0.3	±0.7	±0.3	±0.5	±0.2	±0.9	±0.3	±0.7	±0.3	±0.6	±0.8	±0.6

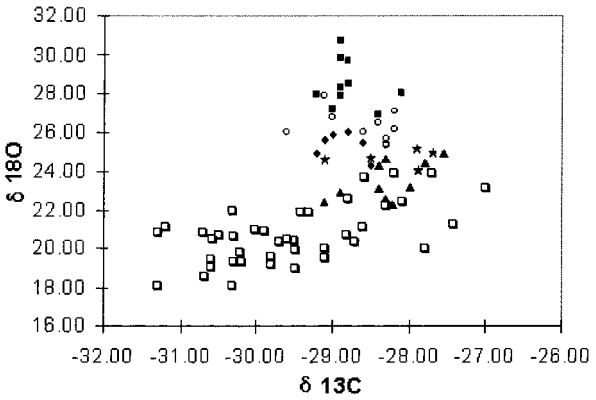

Figure 1. $\delta^{13}C$ values versus $\delta^{18}O$ values of all examined samples: (◆) Spain; (■) Morocco; (▲) Greece; (○) Tunisia; (★) Turkey; (□) Italy.

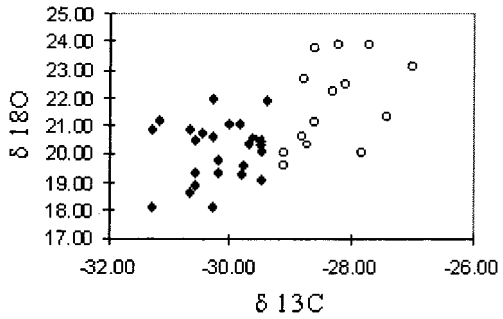

Figure 2. $\delta^{13}C$ values versus $\delta^{18}O$ values of oils from (◆) inland and (○) seaside Italian samples.

RESULTS AND DISCUSSION

$\delta^{13}C$ and $\delta^{18}O$ values found for samples produced in different countries of the Mediterranean basin are listed in Tables 1 (Greece, Morocco, Spain, Tunisia, and Turkey) and 2 (Italy), respectively.

In Table 3 mean values, standard deviations, confidence intervals, and minima and maxima of ^{13}C and ^{18}O isotopic values for all of the countries are reported.

Most of the samples coming from the considered countries show $\delta^{13}C$ values ranging between −29.5 and −27.5, proving the strict discrimination of the Calvin biosynthetic process in the olive fruit that previous research had already shown (Bianchi et al., 1993). Instead, $\delta^{18}O$ values show a greater variability ranging between 18.1 and 30.7.

By plotting $\delta^{13}C$ values obtained for all of the examined oils versus their corresponding $\delta^{18}O$ data (Figure 1), samples seem to arrange according to the climate of the country of the oil production and put in evidence a clear trend to cluster in relation to their geographical origin.

Italian oils show a greater variability of $\delta^{13}C$ values than the other countries, probably due to a very high number of different Italian cultivars, the particular geographical arrangement, and the special orography of Italy.

$\delta^{18}O$ values of samples coming from Morocco, Tunisia, and Spain point out a greater enrichment in ^{18}O than those produced in the other considered countries. In particular, the Italian oils show $\delta^{18}O$ values ranging between 18.1 and 23.9, with an average value (20.7) lower than those of the other considered countries (Table 3). Moreover, for the Italian oils the same two variables evidence a differentiation among the oils produced in spots of the middle and southern regions located near the sea and those obtained from fruits grown in inland areas (Figure 2).

To improve the geographical differentiation of these oils, we also considered the isotopic values of sterols and aliphatic alcohols since a previous research (Bianchi et al., 1993) pointed out that their isotopic values were significantly different from those of the whole oils.

Thirty-five oils from the different countries have been submitted to isolation and measurement of the $^{13}C/^{12}C$ ratio of their sterolic and aliphatic alcohol fractions. The isotopic values are summarized in Tables 1 and 2.

Data concerning the $\delta^{18}O$ of sterolic and alcoholic fractions have not been taken into account because of their poor reproducibility, probably due to the low oxygen content in these fractions. A study is in progress to improve the methodology.

To group samples similar in their isotopic data and to evidence a possible geographical differentiation, a hierarchical cluster analysis (HCA), using the statistical package SPSS (SPSS Inc., Chicago, IL), has been performed using the four isotopic standardized variables.

Figure 3 represents the dendrogram, that is, the graphic drawing, of the similarity of the oil samples. The more similar oils group at the first steps of the clustering process. As shown in Figure 3, the most similar samples are grouped at the first step into four main clusters identified by oils from Greece and Turkey, from Tunisia and Spain, all oils from Morocco, and some Italian ones, respectively. As the clustering process proceeds, there appears a sharp differentiation of oil samples into three groups formed by (i) Greek and Turkish samples, (ii) oils coming from Morocco, Tunisia, and Spain, and (iii) Italian oils. The next clustering step separates Italian oils from the all remaining samples.

Principal component analysis (PCA), using the statistical package SPSS (SPSS Inc., Chicago, IL), has been applied using the isotopic values measured on the whole oil and on sterolic and aliphatic fractions reported in Tables 1 and 2. All variables were standardized by *z*-scores, dividing data of each variable by its standard deviation, because there is a great difference between $\delta^{13}C$ and $\delta^{18}O$ values, so that the initial importance of all variables weighted equally. The two principal components (PC) have been calculated, and they altogether

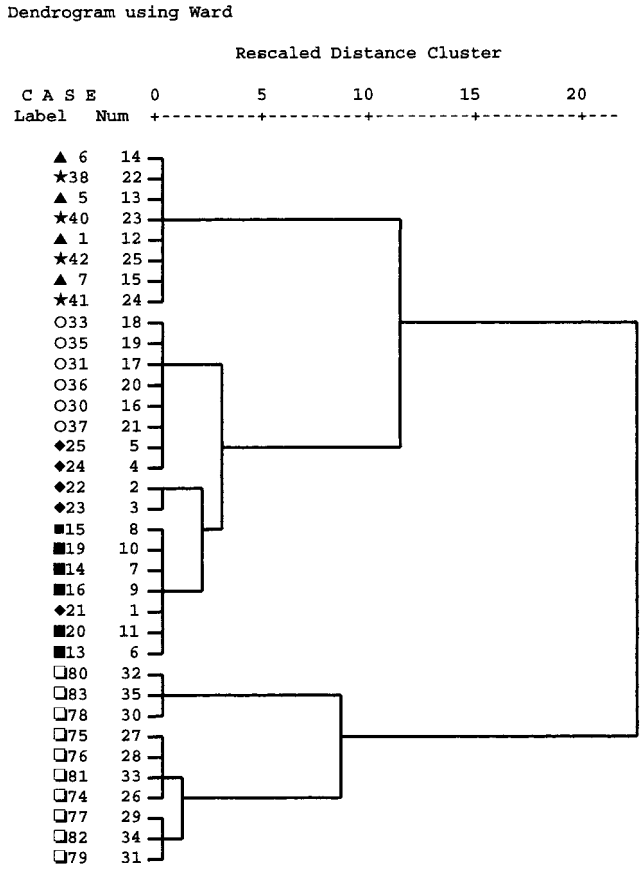

Figure 3. Dendrogram showing the clustering of the virgin olive oil samples using the four isotopic variables: (□) Italy; (■) Morocco; (○) Tunisia; (◆) Spain; (★) Turkey; (▲) Greece.

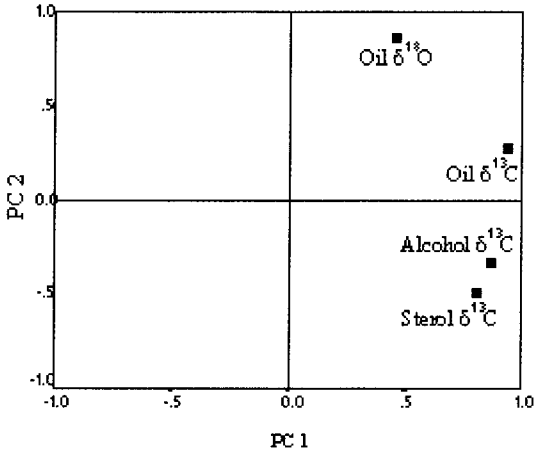

Figure 4. Distribution of loadings onto the two first PCs.

explain 91% of the whole variability. Figure 4 displays the distribution of loadings of each variable onto the two-dimensional plot of the two first principal components. The whole oil $\delta^{13}C$ is the high loading on PC1, whereas PC2 is characterized by the whole oil $\delta^{18}O$ variable. Figure 5 shows the arrangement of samples onto the two-dimensional plot of the two principal components. On PC2 it is possible to evidence a trend in discriminating the oils in relation to their geographical origin, whereas the first principal component allows only the discrimination of some Italian oils. Samples from Morocco and Italy are completely differentiated on PC2, whereas samples from Spain and Tunisia and Greece and Turkey, respectively, overlap.

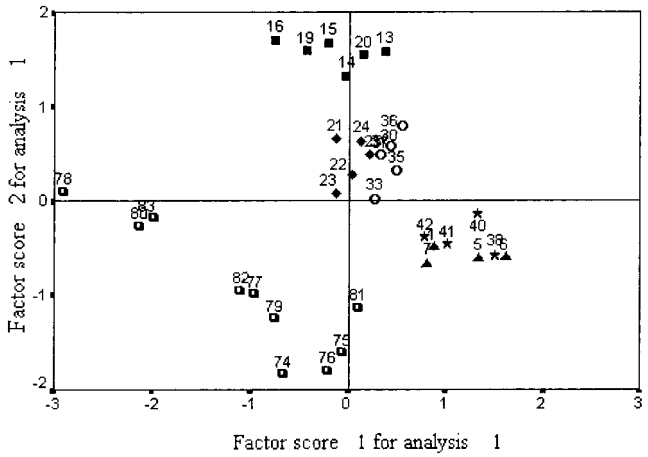

Figure 5. Arrangement of samples onto the two-dimensional plot of the two PCs: (★) Turkey; (▲) Greece; (◆) Spain; (○) Tunisia; (■) Morocco; (□) Italy.

The results obtained up to now point out that by considering the $\delta^{18}O$ values of the whole oil and the $\delta^{13}C$ values of the whole oil and the sterolic and alcoholic fractions, we do not obtain a clear differentiation of the producing countries, as they are defined from a political point of view. However, isotopic measurements give us information about the climatic growing conditions of fruits used for the olive oil production.

$\delta^{18}O$ values of samples coming from countries characterized by higher temperature and dry climate, for example, Morocco, Tunisia, and Spain, point out a greater enrichment in ^{18}O than is generally found for oils produced in the other considered countries. The observed dependence of $\delta^{18}O$ values on geoclimatic parameters should not be surprising because it is known that an enrichment in heavy isotopes of oxygen and hydrogen occurs in plant water due to evapotranspiration, the importance of which is greatly dependent on the climatic characteristics of the different geographical areas, as observed, for instance, in European wines (Bréas et al., 1994).

Oils from Greece and Turkey, the production areas fo which are characterized by a more temperate climate due to the nearness to the sea, show similar $\delta^{13}C$ and $\delta^{18}O$ data, with lower isotopic values—in particular ^{18}O values—than those observed for samples from Morocco, Tunisia, and Spain.

CONCLUSIONS

The results, which should be in any case confirmed in subsequent harvesting years, show that isotopic data measured on the oil and some of its fractions could be useful for the geographical characterization of olive oils of the Mediterranean basin.

An improvement of the differentiation of samples could probably be obtained by the introduction of the $\delta^{18}O$ values of sterols and aliphatic alcohols among the variables. We would like to continue in this direction, and work is in progress to improve the technique of the measurement of $^{18}O/^{16}O$ ratio values of the mentioned fractions of olive oil. Other isotopic parameters (e.g., deuterium content) could be considered.

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