JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

A Metabolomic Approach to the Evaluation of the Origin of Extra Virgin Olive Oil: A Convenient Statistical Treatment of Mass Spectrometric Analytical Data

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The selection of suitable markers from the secondary metabolism of lipoxygenase, in experimental olive oils produced from drupes harvested in different areas of the Italian Calabria region and of Tunisia, allows an easy discrimination between each cluster of samples. The origin of the foodstuff can be ascertained even when the distances between the production zones are very close to each other as in Calabria. Olive oils produced from irrigated and nonirrigated farms in Tunisia were also clearly distinguishable. The markers were detected by chemical ionization mass spectrometry with an ion trap gas chromatography−mass spectrometry apparatus. The quantitative data of Calabrian olive oil samples were subjected to linear discriminant analysis, whereas the Tunisian data were treated by means of other two statistical tools, i.e., the Kruskal−Wallis test and the Wald−Wolfowitz test.

KEYWORDS: Olive oil; metabolomics; lipoxygenase pathway; traceability; GC-CI−ITD; SPME analysis; statistical analysis

INTRODUCTION

Olive oil represents the most consumed edible fat in the food intake of the Mediterranean basin. Its increasing use in other developed and developing countries, where other sources of vegetable and animal fats are traditionally present in the local diet, has to be ascribed to the healing effects (*1*−*3*) associated with a regular intake of this peculiar foodstuff, in the framework of the so-called "Mediterranean diet". In contrast to other vegetables oils, extra virgin olive oil, produced by modern milling technologies, can be directly consumed without any further manipulation.

Protected Designation of Origin, Protected Geographical Indication, and others are official European Union classifications, which should guarantee both the quality and the origin of the foodstuff (*4*). Unfortunately, only some works have been conducted on the identification of markers for the origin of monovarietal oils by analytical methods (*5*−*7*). Therefore, on the basis of objective scientific clues, the solution to this important problem is still far too away.

A possible approach could be establishing correlation of the profiling for the products of secondary metabolism of primary metabolites, such as olive lipids, volatile components of which are responsible in part for the aroma of the oil (*8*), with geographic area of production. Among the many factors affecting the profiling of the volatile components, a peculiar role is, in fact, played by the cultivar, the atmospheric, pedologic, and fostering conditions, the ripening degree, the olive and oil storing procedure, and the technology of oil extraction from drupes (*9*−*17*).

In a recent work, we have correlated some typical parameters, such as cultivar and ripening stage, of southern Italian olive oils with the distribution of five biomarkers, present in the volatile fraction, which were selected from the number of species originating from the lipoxygenase (LOX) cascade (*17*), the principal components of this aerobic secondary metabolism being mainly represented by aliphatic C_6 species (**Figure 1**) (*9*, *18*−*20*).

We now explore a statistical method based on the metabolomics of monovarietal olive oils to recognize their origin. In the case of Tunisian oils, the effect of irrigation will be also evaluated.

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10.1021/jf062929u CCC: $37.00 © 2007 American Chemical Society
Published on Web 01/20/2007

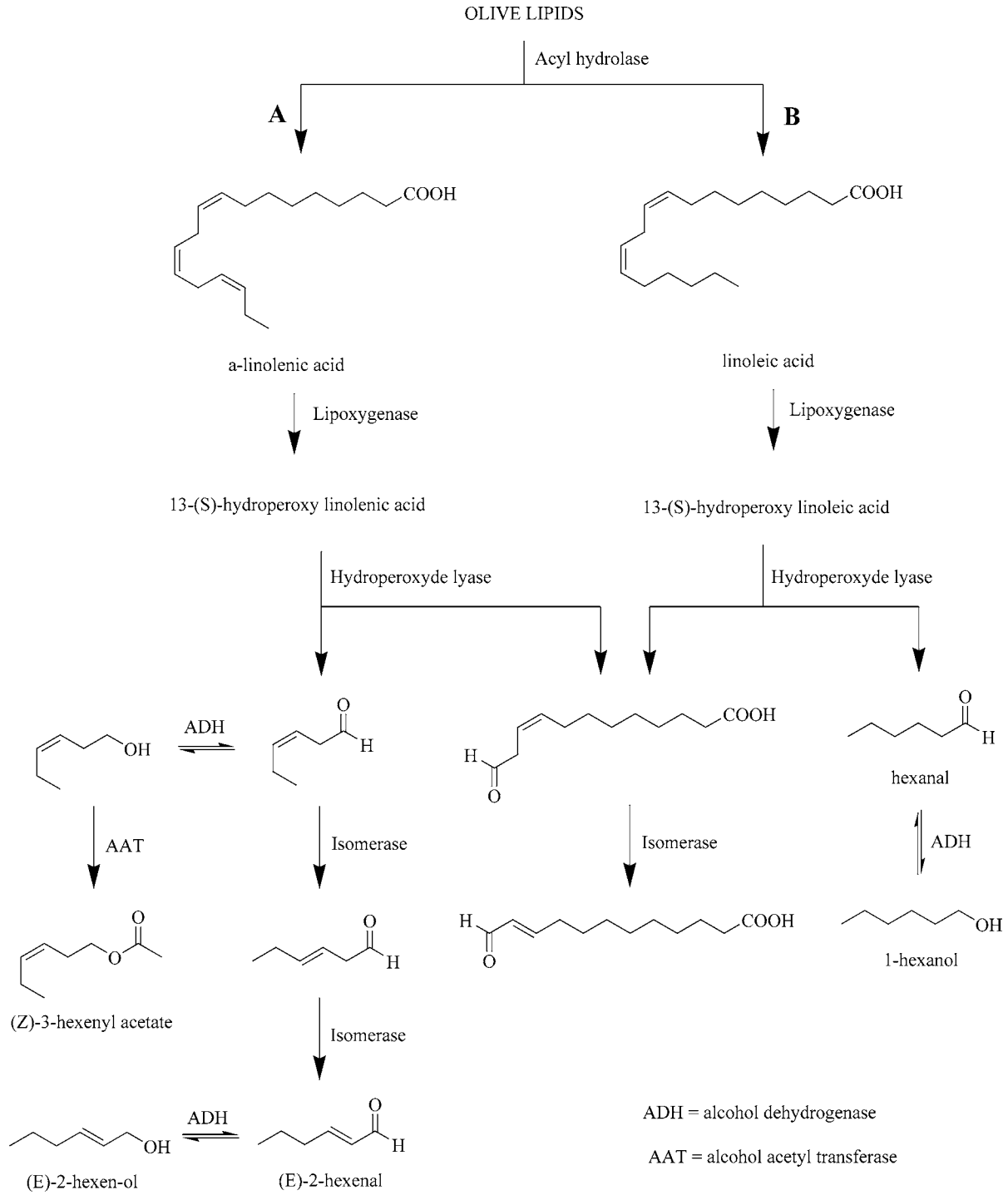

Figure 1. Lipoxygenase pathway.

MATERIALS AND METHODS

The survey was carried out on four samples from each Calabrian areas, i.e., Catanzaro, Corigliano, Rende, and Nocera Terinese, and on 12, 10, and 11 samples from Enfidha, Sidi, Bouzid, and Taous (Tunisian areas) (**Figure 2**), respectively, with similar Jaen indexes. For each sample, 10 kg of olive was picked from three to five trees and then milled in a laboratory scale hammer mill. After 20 min of malaxation, the oil was separated by centrifugation.

Experimental Procedure. The most suitable solid-phase microextraction (SPME) conditions for quantitative assay of the five selected compounds, hexanal (**1**), (*E*)-2-hexenal (**2**), (*E*)-2-hexen-1-ol (**3**), 1-hexanol (**4**), and (*Z*)-3-hexen-1-yl acetate (**5**), were described in a previous study (*17*). Briefly, SPME was performed with a 65 μm Carbowax/divinylbenzene (DVB) fiber (Supelco, Bellefonte, PA). Two grams of sample was placed in each septum-closed vial, and the extraction was performed in the headspace volume (~8 mL) at 40 °C for 20 min. The adsorbed analytes were thermally desorbed by introducing the fiber into the injector set at 250 °C for 3 min.

The quantitative assay was performed in chemical ionization (CI) mode, using isobutane as the reagent gas and ethyl isobutanoate (**6**) as the internal standard (IS) at the concentration ranges 0.2−2 and 5−100 mg/kg, using 1 and 40 mg/kg of IS, respectively.

Preparation of Samples. A mother solution was prepared by dissolving 0.020 g (1.72×10^{-4} mol) of IS in 2.000 g of each sample. Concentrations of 1 and 40 mg/kg, for the two calibration curves, respectively, were achieved through serial dilutions.

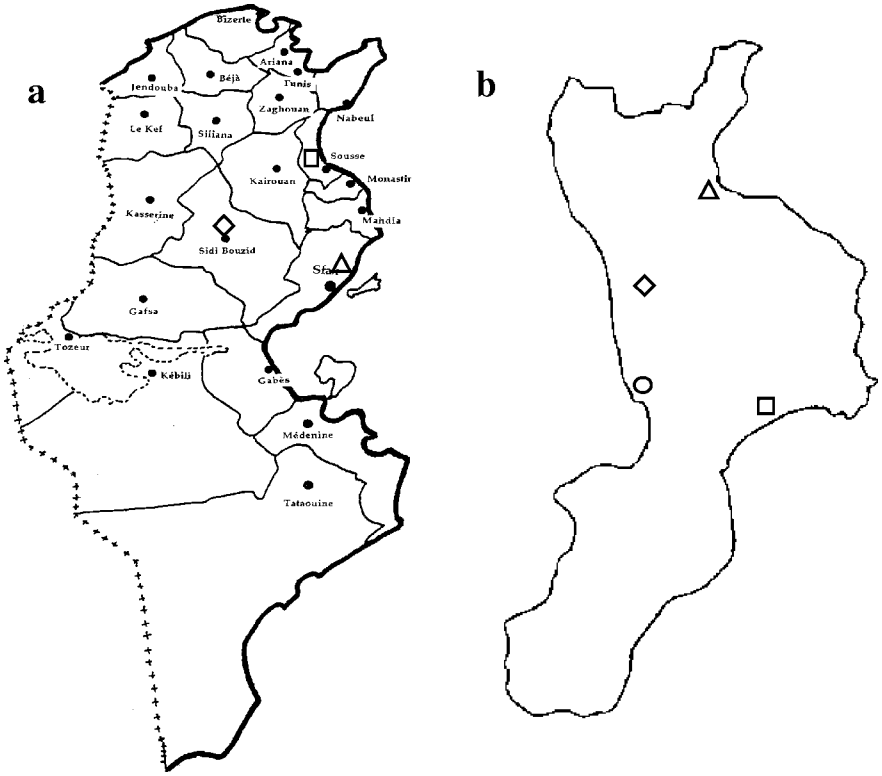

Figure 2. (**a**) Tunisia map [Agro-Combinat Enfidha (Sousse), □; Domaine Ettaous (Taous, Sfax), △; Agro-Combinat Touila (Sidi Bouzid), ◇]. (**b**) Calabria map (Nocera Terinese, ○; Catanzaro, □; Rende, ◇; and Corigliano, △).

Instrumentation. Sample analyses were performed using a Varian (Walnut Creek, CA) Saturn 2000 GC-MS ion trap (ITD) system in positive CI modes, with isobutane as the reagent gas, coupled to a Varian 3400 gas chromatograph equipped with a Varian 8200 autoinjector. The ion trap temperature was set at 210 °C with an ionization time of 2 ms, a reaction time at 50 ms, and a scan rate at 1000 ms. The transfer line temperature was set at 230 °C. The column was a 30 m Chrompack CP-Sil 8 CB low-bleed/MS (0.25 mm i.d., 0.25 μm film thickness). The gas chromatography (GC) oven temperature was initially held at 40 °C for 3 min, then increased at 1 °C/min to 70 °C, increased again at 20 °C/min to 250 °C, and held for 8 min. The carrier gas was helium at 1 mL/min. Analyses were performed in splitless mode. For SPME analyses, a narrow-bore Supelco 0.8 mm i.d. GC inlet liner was used.

The isobutane pressure was adjusted to produce a ratio of *m*/*z* 43−57 of approximately 1/1.2. The selective ejection chemical ionization (SECI) scan mode parameters were as follows: CI storage level, *m*/*z* 19; ejection amplitude, *m*/*z* 15; and background mass, *m*/*z* 65.

Statistical Analysis. The quantitative data of Calabrian olive oil samples were subjected to linear discriminant analysis (LDA) to classify samples with a priori hypothesis, which is the number of groups (areas of production), and to find the variables with the highest discriminant power. On the contrary, the Tunisian data were subjected to another two statistical tools: the Kruskal−Wallis test and the Wald−Wolfowitz test. These are the analogous nonparametric methods of one-way between-groups of variance (analysis of variance, ANOVA) and *t*-test, respectively. Nonparametric techniques are also called "distribution-free methods", since they are not dependent on a given distribution (such as in the case of ANOVA) but generally work for a broad range of different distributions. Statistical treatment of data was performed by Statistica 7.1 (StatSoft 2005 edition).

RESULTS AND DISCUSSION

The profiling of the components of volatile fraction from olive oil is recognized as a fundamental parameter for quality assessment and as a unique tool for authenticity control. Different methods for the identification of olive oil aroma components have been devised, most of which are based on dynamic headspace and SPME experimental approaches (*21*).

In particular, our group has proposed that the concentrations of the biomarkers **1**−**5**, i.e., five secondary metabolites out of the number of species present in the volatile fraction of olive oil, could provide clues for cultivar and ripening phase discrimination of different oils (*17*).

It is expected that the biosynthetic pathways of LOX cascade could be affected by the different expression of the enzyme pool involved, whose availability could be controlled by pedoclimatic parameters. It could be likely, therefore, that the same markers are useful in the identification of origin. Accordingly, we are now exploring the correlation, by statistical methods, of the origin of the oil with the concentrations of these biomarkers monitored by GC-CI-ITD mass spectrometry.

The volatile compounds absorbed on carbowax/DVB fibers and desorbed, by an autosampler device, into the mass spectrometer previously described were tested in both electron ionization (EI) and CI modes. A typical reconstructed chromatogram obtained in this condition is shown in **Figure 3** where the ion current profile due to the five markers and to the IS is indicated.

Among the possible reagent gases, isobutane favors the obtainment of highly specific mass spectra, preventing also, at a great extent, interferences with coeluted analytes. In particular, a more satisfactory integration was obtained in CI mode for (*E*)-2-hexenal, even though its retention time, 9.30, is close to that of (*Z*)-3-hexen-1-ol, 9.41 min. In CI mode, in fact, the ion at *m*/*z* 99 chosen for assay of (*E*)-2-hexenal because of its relative intensity is absent in the spectrum of (*Z*)-3-hexen-1-ol spectra obtained in the same ionization conditions (**Figure 4A**). On the contrary, in EI mode (**Figure 4B**), any abundant ion selected for the assay of the aldehyde **2** may suffer from

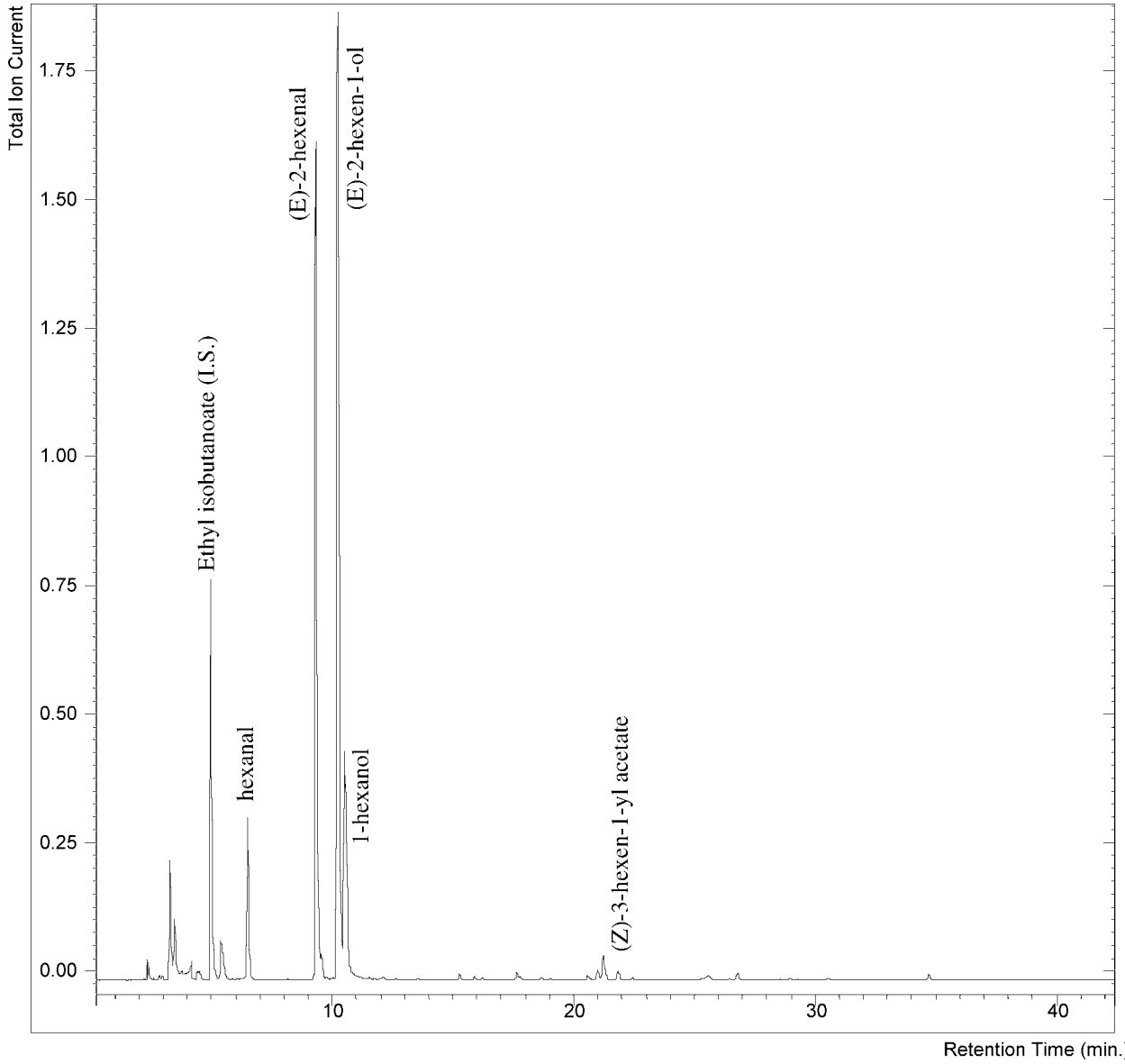

Figure 3. Typical total ion chromatogram in CI mode of a Tunisian oil (E6) obtained from trees grown in the irrigated farmlands.

interferences with similar, or identical, ionic species present in the EI spectrum of (*Z*)-3-hexen-1-ol.

The CI (isobutane) mass spectra of (**a**) the internal standard used for quantitative measurements and (**b**−**f**) those of the five biomarkers previously described are presented in **Figure 5**. It can be easily verified that the total ion current of each spectrum is associated mainly to a single cluster of ions. Moreover, the selection of identical ionic species, i.e., those at *m*/*z* 83 for **b**, **c**, and **f**, refers to peaks eluted at different times and does not cause any overlapping problem (**Figure 5**).

The extensive use of chemiometric tools for data handling and retrieving was explored in the identification of the geographical origin of olive oils originating from different Calabrian and Tunisian areas (**Figure 2**). For the Tunisian oils, the effect of irrigating farmlands was also considered to account for severe drought periods that may be encountered in this country.

Calabrian Oils. The relative ratios of the five markers obtained by the method previously described (**Table 1**) show that substantial differences can be observed among the oils produced in the selected different regions.

Samples C-**1** to C-**4** show, on the average, a higher concentration of alcohols with respect to aldehydes, in particular hexanal, which might be due to an overexpression or overactivity of the dehydrogenase (ADH) enzymes. This behavior is even more pronounced in the oils produced in Corigliano (C-**9** to C-**12**); in this case, in fact, the concentration of the two alcohols is more than doubled as compared to the concentration of the aldehydes. Moreover, the much higher absolute content of the four analytes than in the Nocera Terinese oils could be either due to higher activity of LOX enzymes or to a higher concentration of linolenic and linoleic acids in the drupes harvested in Corigliano area. The detection of hexenyl acetate, even if as a minor component, in the C-**9** to C-**12** samples, only, could be related to the relative concentration of the alcoholic components in the aroma of the drupes harvested in the Corigliano area.

Similar observations can be drawn for the oils produced from drupes of the other Calabrian areas. Different activity or different expression of the LOX enzymes as a function of the different pedoclimatic parameters might affect the observed behavior.

A statistical evaluation of the data could provide better tools for interpreting the meaning of the results, providing clues to identifying the origin of production. The objective could be

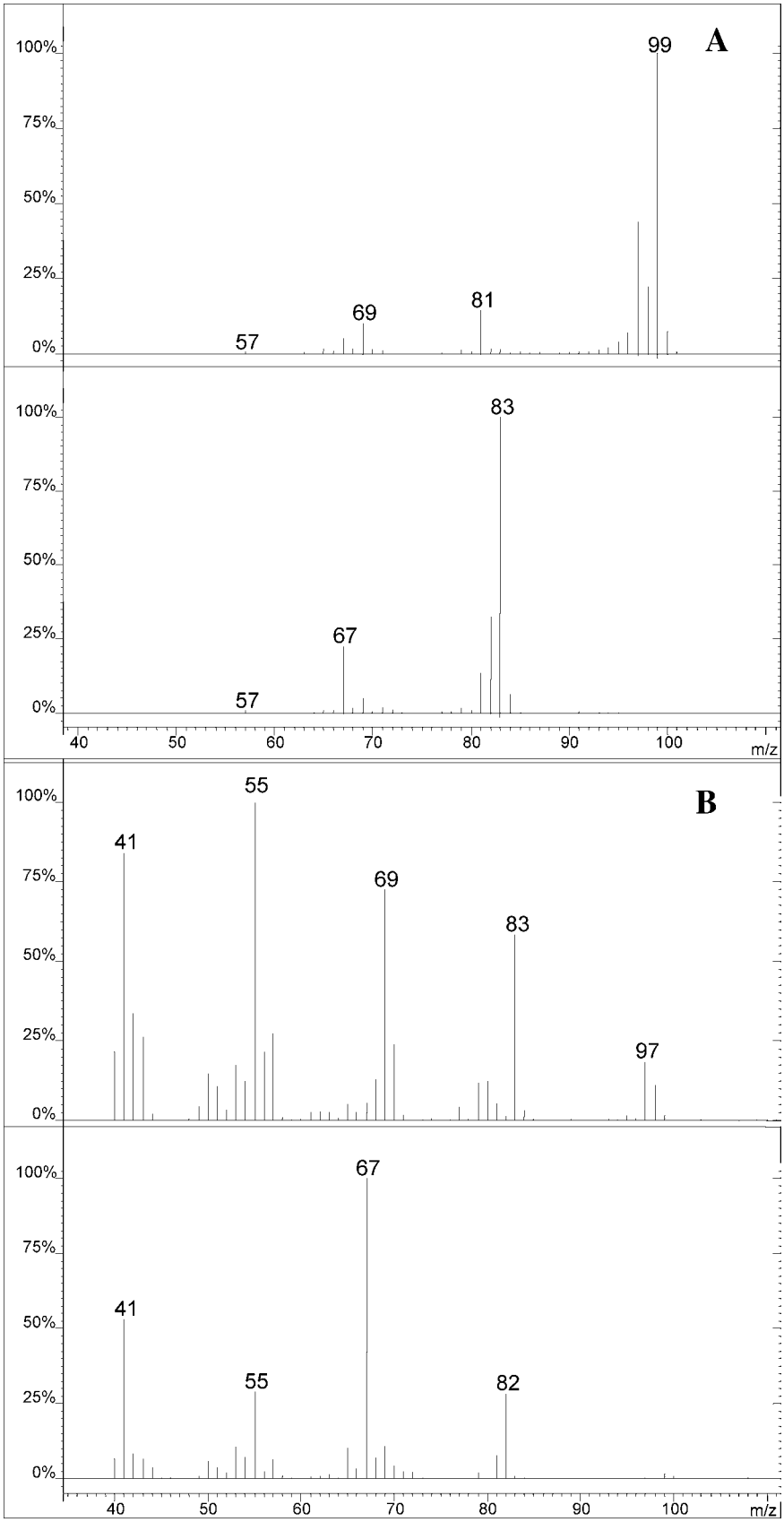

Figure 4. Spectra of (*E*)-2-hexenal and (*Z*)-3-hexen-1-ol in CI (**A**) and EI (**B**) modes.

attended by considering the data in **Table 1** as a data matrix that can be subjected to a statistical multivariate analysis.

The statistical approach selected for Calabrian oils is the LDA, which could allow the classification of unknown samples after having verified the possible differences among samples of known origin. Data treatment has been applied to the concentrations of the five markers, using the four groups, corresponding to the four selected areas, as a priori input.

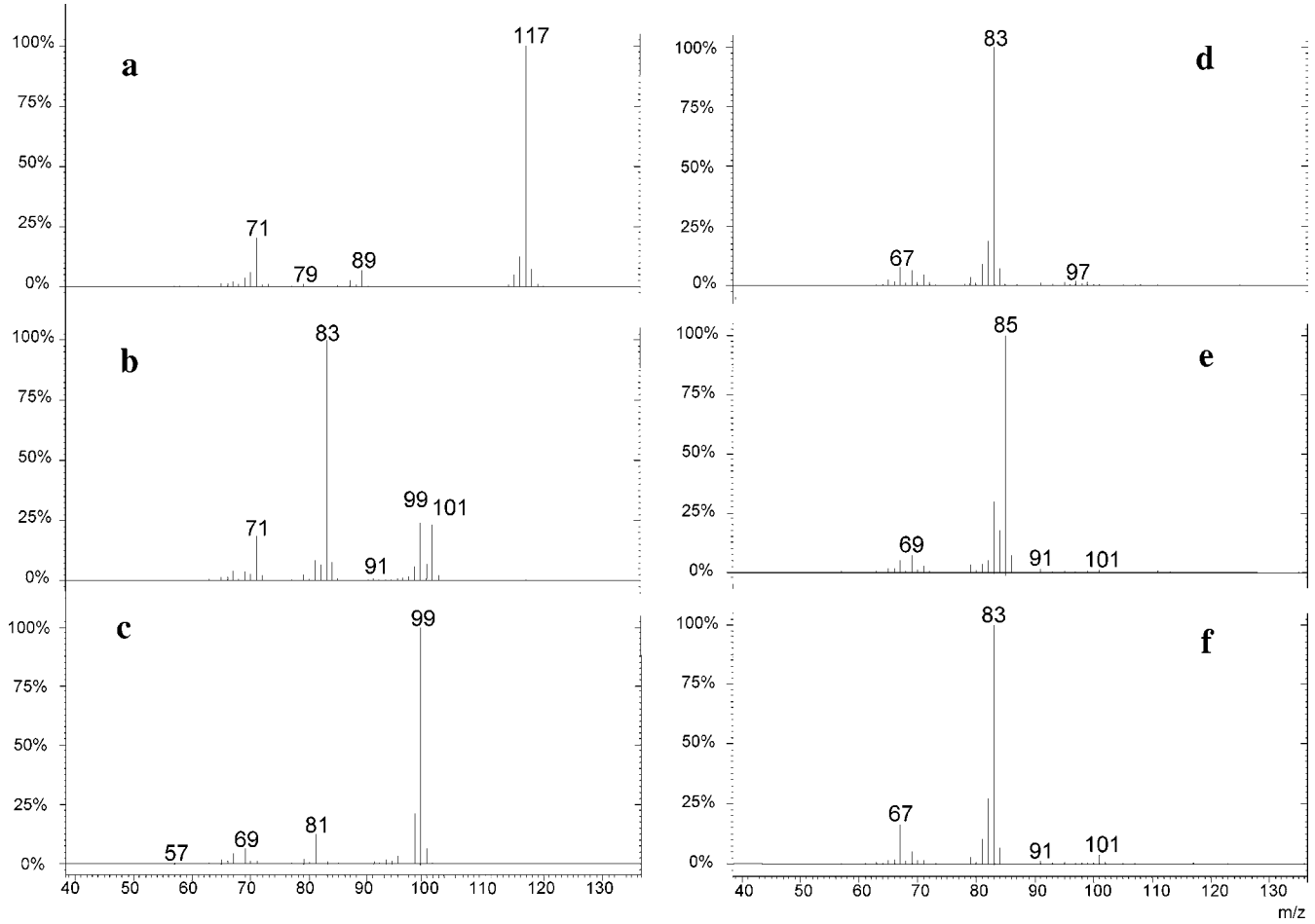

Figure 5. CI spectra of (**a**) ethyl isobutanoate (IS), (**b**) hexanal, (**c**) (*E*)-2-hexenal, (**d**) (*E*)-2-hexen-1-ol, (**e**) 1-hexanol, and (**f**) (*Z*)-3-hexen-1-yl acetate.

Table 1. Calabrian Olive Oils; Markers Concentration (ppm) in the Volatile Fraction of the Oils Produced in Four Different Selected Areas

sample	origin	hexanal	(*E*)-2-hexenal	(*E*)-2-hexen-1-ol	1-hexanol	(*Z*)-3-hexen-1-yl acetate
C1	Nocera T.	0.080	0.256	0.296	0.412	ND
C2	Nocera T.	0.072	0.218	0.275	0.324	ND
C3	Nocera T.	0.090	0.374	0.331	0.308	ND
C4	Nocera T.	0.062	0.305	0.214	0.454	ND
C5	Catanzaro	0.745	0.379	0.175	0.315	ND
C6	Catanzaro	0.72	0.392	0.083	0.174	ND
C7	Catanzaro	0.657	0.298	0.112	0.250	ND
C8	Catanzaro	0.569	0.302	0.287	0.241	ND
C9	Corigliano	0.758	1.105	1.863	1.802	0.001
C10	Corigliano	0.625	0.892	1.750	1.825	0.015
C11	Corigliano	0.703	0.748	1.678	1.786	0.009
C12	Corigliano	0.108	0.670	1.480	1.813	0.028
C13	Rende	0.498	2.203	0.264	0.299	ND
C14	Rende	0.712	1.785	0.122	0.240	ND
C15	Rende	0.767	1.938	0.096	0.240	ND
C16	Rende	1.065	1.161	0.214	0.799	ND

In the bidimensional plot of the first two roots, the clear separation of the four clusters representing the four areas of production is clearly displayed (**Figure 6**). From a statistical point of view, this differentiation is also very significant since the very low Wilks λ value (0.0000146) shows that the model is highly discriminating, whereas the high value (83.23) of the F (15.22) parameter indicates a significant difference among the means of the groups. Finally, the information from data treatment is characterized by a high degree of reliability since the p level is extremely low (<0.00001).

To verify the stability of the model, the method was checked with unknown samples. In particular, one set of four samples, containing one sample for each production zone, was randomly removed in three independent runs and the model was recalculated. In all cases, the samples were correctly classified, thus showing the stability of the system.

The LDA (**Table 2**) shows that a major contribution to the differentiation is provided by the aldehydes as compared to alcohol and ester components even if all of the components contribute in a significant manner to group separation and identification.

Tunisian Oils. Experimental Tunisian oils provided by the Institut de l'Olivier of Sfax (Tunisia) were produced from drupes of the same Chemlali cultivar, harvested in the three different

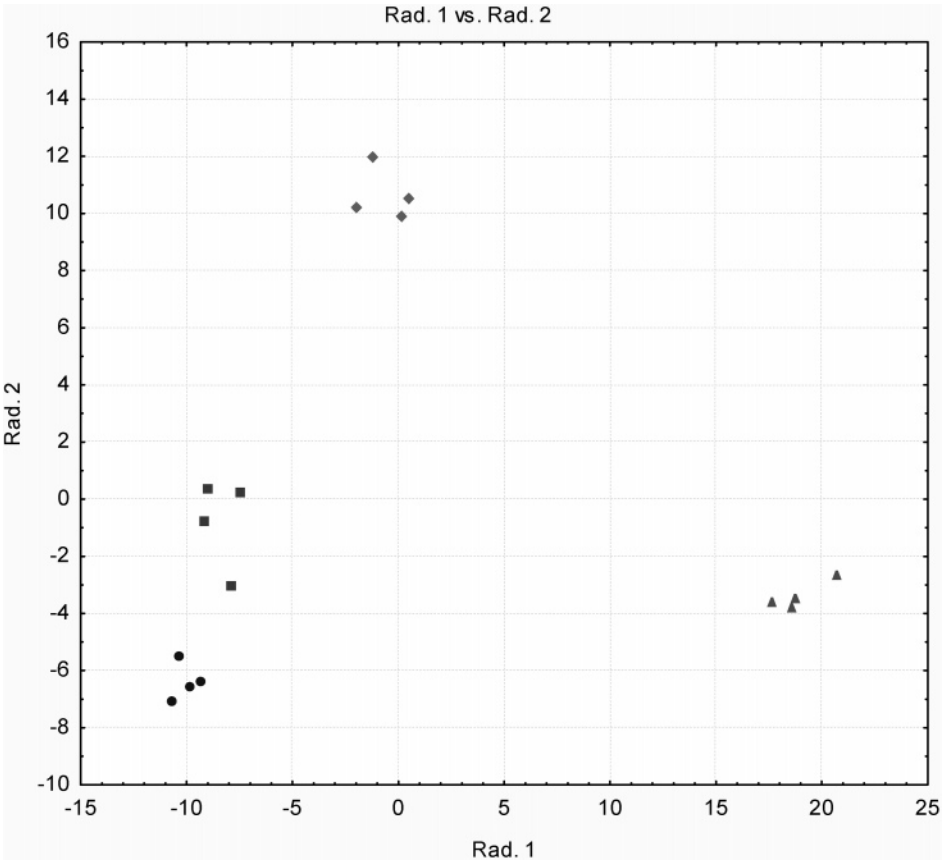

Figure 6. LDA plot of 16 olive oil samples based on quantitative values of the five selected compounds (Nocera Terinese, ●; Catanzaro, ■; Rende, ♦; and Corigliano, ▲).

Table 2. Linear Discriminating Analysis of the Five Selected Compounds

	Wilks' λ	partial Wilks' λ	*F* remove	*p* level
hexanal	0.000188	0.077425	31.77538	0.000086
(*E*)-2-hexenal	0.000491	0.029639	87.30497	0.000002
(*E*)-2-hexen-1-ol	0.000097	0.150666	15.03254	0.001189
1-hexanol	0.000050	0.292956	6.43596	0.015853
(*Z*)-3-hexen-1-yl acetate	0.000087	0.166399	13.35907	0.001756

regions. The effect of irrigation on the profiling of the volatile components was checked for oil produced from drupes harvested in Enfidha and Sidi Bouzid regions.

Quantitative data (**Table 3**) show that alcohols are nearly absent whereas (*Z*)-3-hexen-1-yl acetate is present at very low amounts. This result is in part contradictory to the data obtained by Marzouk et al. (*22*) that showed that a percentage of alcohols accounted for about 15% of the whole C_6 fractions of Chemlali cultivar. This discordance could be explained by the different production area of analyzed oil samples (locality of Bouargoub in the region of Cap-Bon) or by different ripening degrees of olives.

An attempt of differentiation of geographical areas of production for the Chemlali cultivar was tried by applying the Kruskal−Wallis test to hexanal and (*E*)-2-hexenal variables (**Figures 7** and **8**). **Figure 8** shows that there are not significant differences among concentrations of (*E*)-2-hexenal in olive oil produced in different geographical areas of Tunisia. On the contrary, hexanal seems to be a suitable variable to distinguish oils produced in the Sidi Bouzid region from samples from Enfidha and Taous (**Figure 7**). In fact, the Kruskal−Wallis test

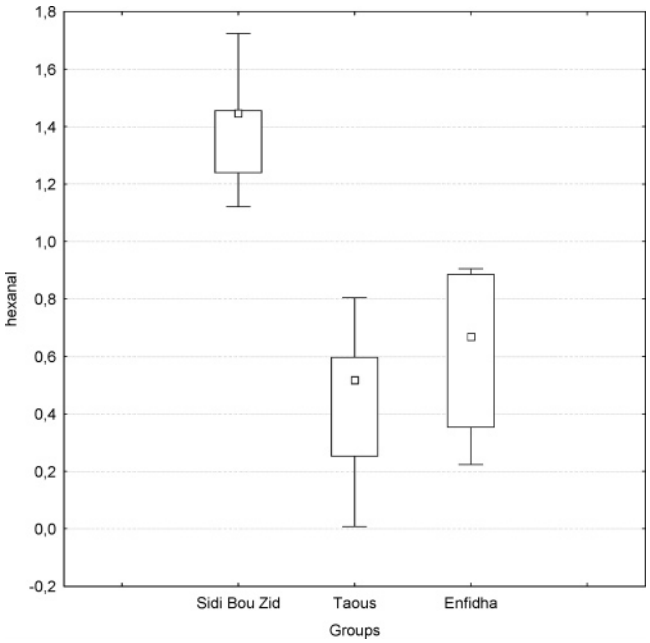

Figure 7. Box and Whisker plot of the quantitative data of hexanal in Tunisian oils (medians, small squares; 25–75%, large bars; and min–max, lines).

applied to the three groups of oils showed a highly significant difference between Sidi Bouzid and Taous ($p = 0.002268$) and a less marked difference between Sidi Bouzid and Enfidha ($p = 0.074797$). Enfidha and Taous did not result different ($p = 1.00000$).

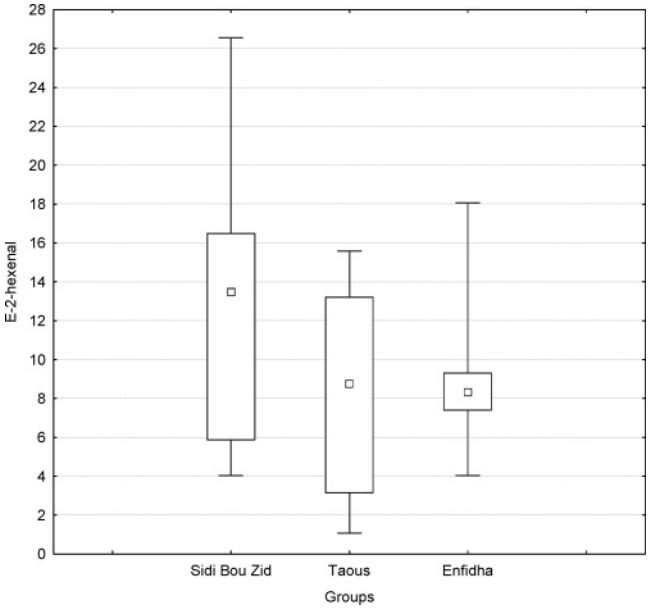

Figure 8. Box and Whisker plot of the quantitative data of (E)-2-hexenal in Tunisian oils (medians, small squares; 25–75%, large bars; and min–max, lines).

Peculiar features of samples from Sidi Bouzid could be ascribed to different geographical positions in Tunisia (**Figure 2a**). In fact, the region of Sidi Bouzid lies inland, whereas Sfax and Sousse are near the coast. This fundamental difference could mean similar pedologic and climatic conditions in Sfax and Sousse regions but different to Sidi Bouzid. Furthermore, it could suppose a lower amount of linoleic acid (precursor of hexanal) in olives from Sfax and Sousse with respect to Sidi Bouzid.

Finally, the effect of irrigating farmlands was considered on the composition of volatile fraction of virgin olive oil. Seven samples from Enfidha and five samples from Sidi Bouzid, all obtained from trees grown in the irrigated farmlands, were then analyzed. In all 12 cases, it was noticed that the samples from Enfidha contained a very high amount of 1-hexanol as compared to the other samples, which also contained a considerable amount of 1-hexanol. In this case, it could be assumed that the lack of water affects to some extent the activity (or the concentration) of alcohol dehydrogenase enzymes important in the LOX pathway and, thus, results in lowering the content of (*E*)-2-hexen-1-ol and 1-hexanol. The characterization of production areas was carried out by the Wald−Wolfowitz test because, in this case, the comparison concerned two groups of samples. The statistical tool has demonstrated that hexanal was not any longer an appropriate variable ($p = 0.60270$; **Figure 9A**), since its amount was enormously increased in the sample grown in irrigated farmlands from Enfidha. The latter, therefore, has become undistinguishable from those produced from trees not grown in irrigated farmland. On the contrary, the same test applied to (*E*)-2-hexen-1-ol and 1-hexanol has proved the possibility of distinguishing sample with remarkable certainty ($p = 0.00253$; **Figure 9B**,**C**).

In conclusion, the metabolomic approach is currently considered in our and other groups as reliable means to evaluate the authenticity of foodstuff, since the number and relative concentration of components produced by any secondary metabolism pathway could store information related to the story

Table 3. Concentrations (ppm) of Selected Compounds from LOX Pathway of Samples for Areas of Tunisia[a]

sample	irrigation	origin	hexanal	(*E*)-2-hexenal	(*E*)-2-hexen-1-ol	1-hexanol	(*Z*)-3-hexen-1-yl acetate
E1	no	Enfidha	0.905	8.314	ND	ND	ND
E2	no	Enfidha	0.223	9.311	ND	ND	ND
E3	no	Enfidha	0.353	18.056	ND	ND	0.04
E4	no	Enfidha	0.885	4.036	ND	ND	ND
E5	no	Enfidha	0.669	7.398	ND	ND	ND
E6	yes	Enfidha	1.124	3.815	11.94	4.966	ND
E7	yes	Enfidha	1.492	6.008	10.73	11.28	0.024
E8	yes	Enfidha	2.203	6.225	47.38	8.854	0.029
E9	yes	Enfidha	1.962	12.12	25.81	3.82	0.024
E10	yes	Enfidha	0.821	0.507	29.63	19.01	ND
E11	yes	Enfidha	0.817	1.369	13.18	12.19	ND
E12	yes	Enfidha	0.663	1.728	7.457	4.146	ND
S1	no	Sidi Bouzid	1.121	16.48	ND	ND	0.009
S2	no	Sidi Bouzid	1.456	26.55	ND	ND	0.002
S3	no	Sidi Bouzid	1.239	4.033	ND	ND	0.022
S4	no	Sidi Bouzid	1.725	13.48	ND	ND	ND
S5	no	Sidi Bouzid	1.447	5.882	ND	ND	ND
S6	yes	Sidi Bouzid	0.185	1.157	3.262	0.227	ND
S7	yes	Sidi Bouzid	1.871	4.54	0.44	0.143	ND
S8	yes	Sidi Bouzid	1.663	13.08	0.239	ND	0.006
S9	yes	Sidi Bouzid	1.54	3.808	0.328	ND	ND
S10	yes	Sidi Bouzid	1.086	4.419	0.738	ND	0.02
T1	no	Taous	0.253	13.21	ND	ND	0.001
T2	no	Taous	0.585	15.591	0.106	ND	ND
T3	no	Taous	0.804	13.62	ND	ND	0.001
T4	no	Taous	0.597	4.674	ND	ND	0.02
T5	no	Taous	0.517	12.32	1.24	0.462	0.004
T6	no	Taous	0.794	9.06	ND	ND	0.024
T7	no	Taous	0.484	3.073	ND	ND	ND
T8	no	Taous	0.109	3.156	ND	ND	ND
T9	no	Taous	0.008	1.074	ND	ND	0.028
T10	no	Taous	0.493	3.995	ND	ND	ND
T11	no	Taous	0.542	8.742	ND	ND	ND

[a] ND, not detected.

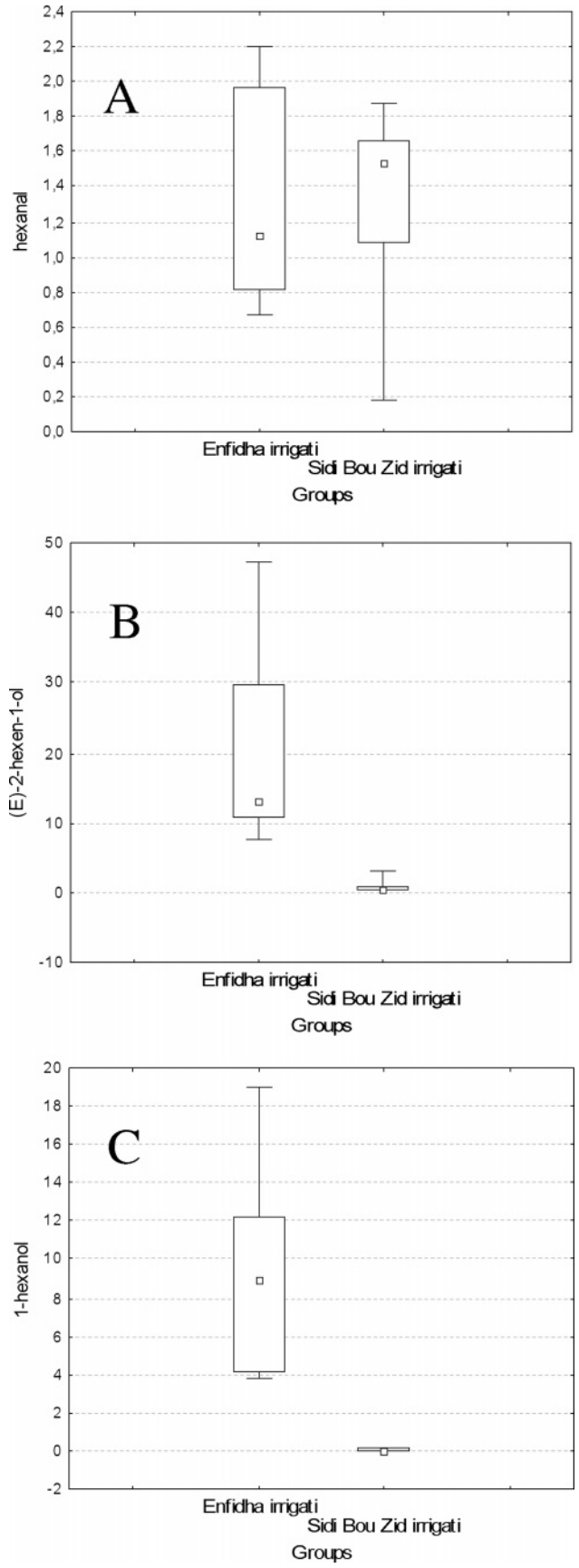

Figure 9. Box and Whisker plot of the quantitative data of hexanal (**A**), (*E*)-2-hexen-1-ol (**B**), and 1-hexanol (**C**) in Tunisian oils obtained from olive fruits of irrigated trees (medians, small squares; 25–75%, large bars; and min–max, lines).

of the aliment. In the case here examined, we have been able to distinguish olive oils produced in very close areas of the same region. This result opens the opportunity of building-up useful databases to recognize the origin of this important foodstuff. Moreover, it represents the first step for the solution of the old problem of olive oil authenticity, which is associated, nowadays, to the bottling sites only.

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Received for review October 12, 2006. Revised manuscript received December 14, 2006. Accepted December 18, 2006. CRA-Isol project "RIOM" is greatly acknowledged. F.H. and A.L. thank CNR-MAE (Italy) for a mobility grant.

JF062929U