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Assay of Aroma Active Components of Virgin Olive Oils from Southern Italian Regions by SPME-GC/Ion Trap Mass Spectrometry

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An SPME-GC/ion trap method was exploited to determine the chromatogram of volatile compounds of organic olive oils of southern Italian regions. The method is based on the assay of the terminal species of the "lipoxygenase pathway", which are present in the volatile fraction of the sampled compounds. Ethyl isobutanoate was used as internal standard in either the EI or CI ionization mode. The absolute concentration values of each analyte were evaluated through good-to-excellent calibration curves. Case studies on oils obtained from different cultivars or harvesting times are presented. The quantitative data for each compound were subjected to principal component analysis to characterize the different cultivars of this work.

KEYWORDS: Italian organic olive oils; olive oil chromatogram of volatile compounds; SPME-GC-ion trap assay of chromatogram

INTRODUCTION

Aroma components of products of plant origin are dependent on genetic, agronomic, and environmental factors. There are few reports (*1*−*5*) on the evaluation of the relationships between the aroma components of virgin olive oil and the metabolic pathways and varietal factors. The olive ripening process and, to some extent, the fruit growing environment affect also the composition of the volatile compounds of the oil (*6*−*11*).

The assay of secoiridoid glycosides, such as oleuropein, in virgin olive oil has been proposed as a marker of quality (*12*, *13*). In a survey of the organic olive oils produced on selected farms located in southern Italian regions, it was needed to set up affordable protocols for varietal and origin certification. With reference to the work previously mentioned, the chromatogram of volatile compounds was considered a useful target. Gas chromatographic (GC) methods have been extensively applied in this field, in connection often with mass spectrometric (MS) analysis, to detect, by the headspace method, the composition of the volatile components of foodstuffs (*14*, *15*). The number of samples to be evaluated has prompted the exploitation of the automatic solid-phase microextraction method (SPME), widely employed in food chemistry, on-line with a GC-MS ion trap mass spectrometer. The complexity of the mass chromatograms in terms of the number of components might represent a drawback when different samples are to be matched. It was decided therefore to consider chromatograms containing the minimum set of components in relation to their biogenesis (*11*). Accordingly, hexanal (**1**) and 1-hexanol (**2**), (*E*)-2-hexenal (**3**) and (*E*)-2-hexen-1-ol (**4**), and (*Z*)-3-hexenyl acetate (**5**) were chosen as markers of linoleic and linolenic acid specific lipoxygenase oxidation, respectively (paths A and B, **Scheme 1**) (*16*).

MATERIALS AND METHODS

Extraction of Olive Oil. The olive oil samples came from two different organic farms (*17*) located in the southern Italian region in Apulia and Sicily. In particular, the olive oil from Apulia came from Castellana Grotte and Villa Castelli, whereas the olive oil from Sicily came from Castelvetrano, Catania, Trapani, and Patti, respectively. For each sample 10 kg of olives was picked from three to five trees, which were homogeneous for cultivar and health, and then milled in a laboratory scale hammer mill. After 20 min of malaxation, the oil was separated by centrifugation.

Preparation of Standard Solutions. A mother solution (200 mg/kg) was prepared by dissolving 0.04 g of each analyte in 200 g of commercial seed oil. In the same manner was prepared a solution containing the internal standard. The standard solutions were prepared through serial dilutions. The commercial seed oil was checked by GC-MS using the same protocol employed in the analysis of olive oil samples. Traces of hexanal only were present, and the experimental values obtained for the olive oil samples were corrected, accordingly.

Preparation of Samples. A mother solution was prepared by dissolving 0.020 g (1.72×10^{-4} mol) of ethyl isobutanoate 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.

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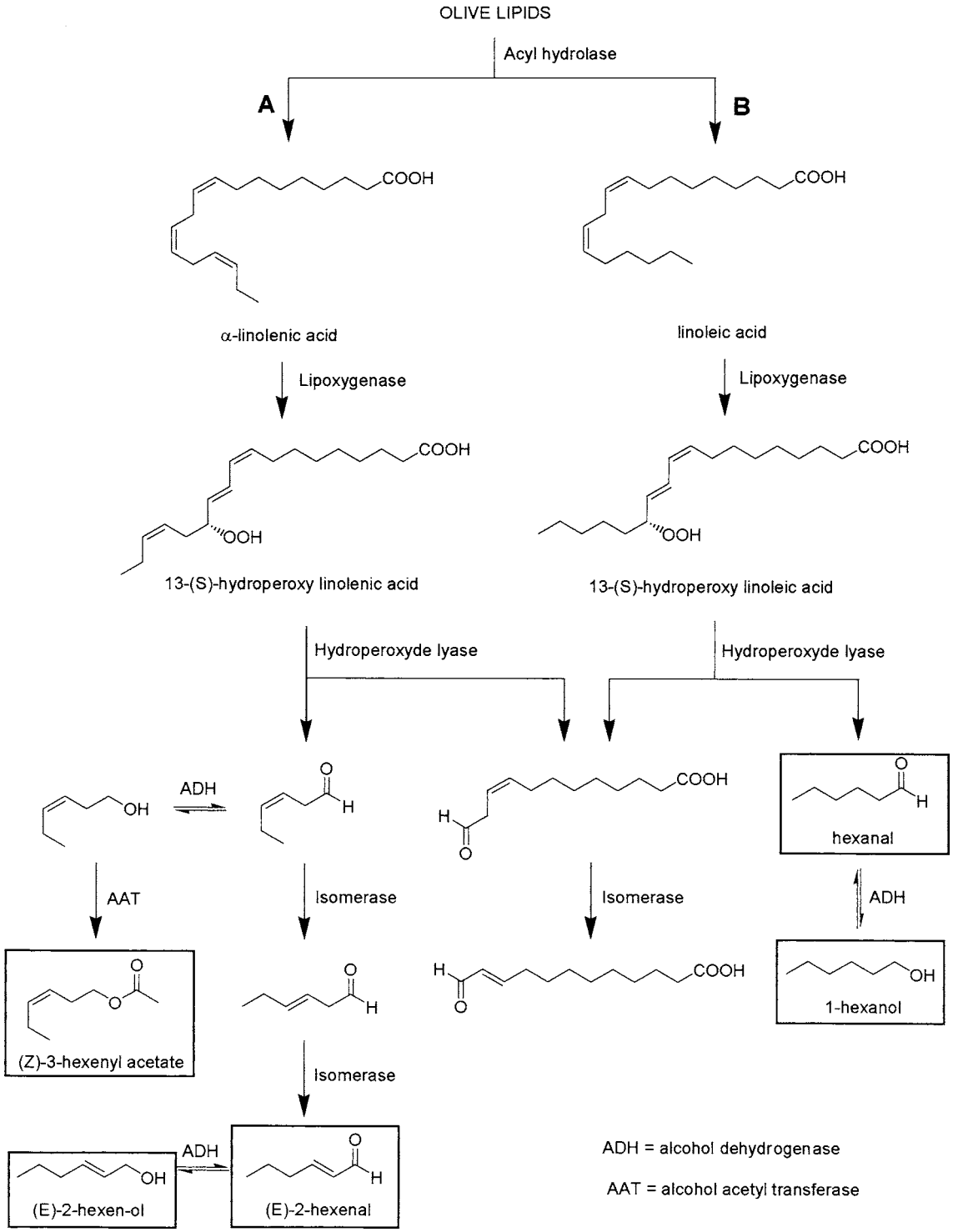
Scheme 1. Lipoxygenase Pathway



Experimental Procedure. SPME was performed with a 65 μm carbowax/divinylbenzene (DVB) fiber (Supelco, Bellefonte, PA). Equal amounts of samples (2 g) were 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. A blank analysis of the fiber did not display any peak due to the analytes under investigation.

Quantitative Analysis. The calibration curves were obtained by covering two concentration ranges: 0.2−2 mg/kg with five steps at 0.2, 0.6, 1, 1.5, and 2 mg/kg for each analyte, with 1 mg/kg of internal standard; and 5−100 mg/kg with five steps at 5, 10, 30, 60, and 100 mg/kg for each analyte, with 40 mg/kg of internal standard. Each experimental value corresponds to the average of three replicates.

The quantitative assay was performed by selecting the area of the ionic species as follows:

EI Mode. The selected ions are *m*/*z* 41, 56, 67, 72, and 82; *m*/*z* 55, 56, and 69; *m*/*z* 55, 69, 83, and 97; *m*/*z* 57, 67, and 82; and *m*/*z* 67 and 82 for analytes **1**, **2**, **3**, **4**, and **5**, respectively, and *m*/*z* 71, 88, and 116 for the internal standard.

CI Mode. The selected ions are the base peak for all analytes and internal standard.

Statistical Analysis. The quantitative data for each compound were subjected to principal component analysis (PCA) (*18*) to characterize

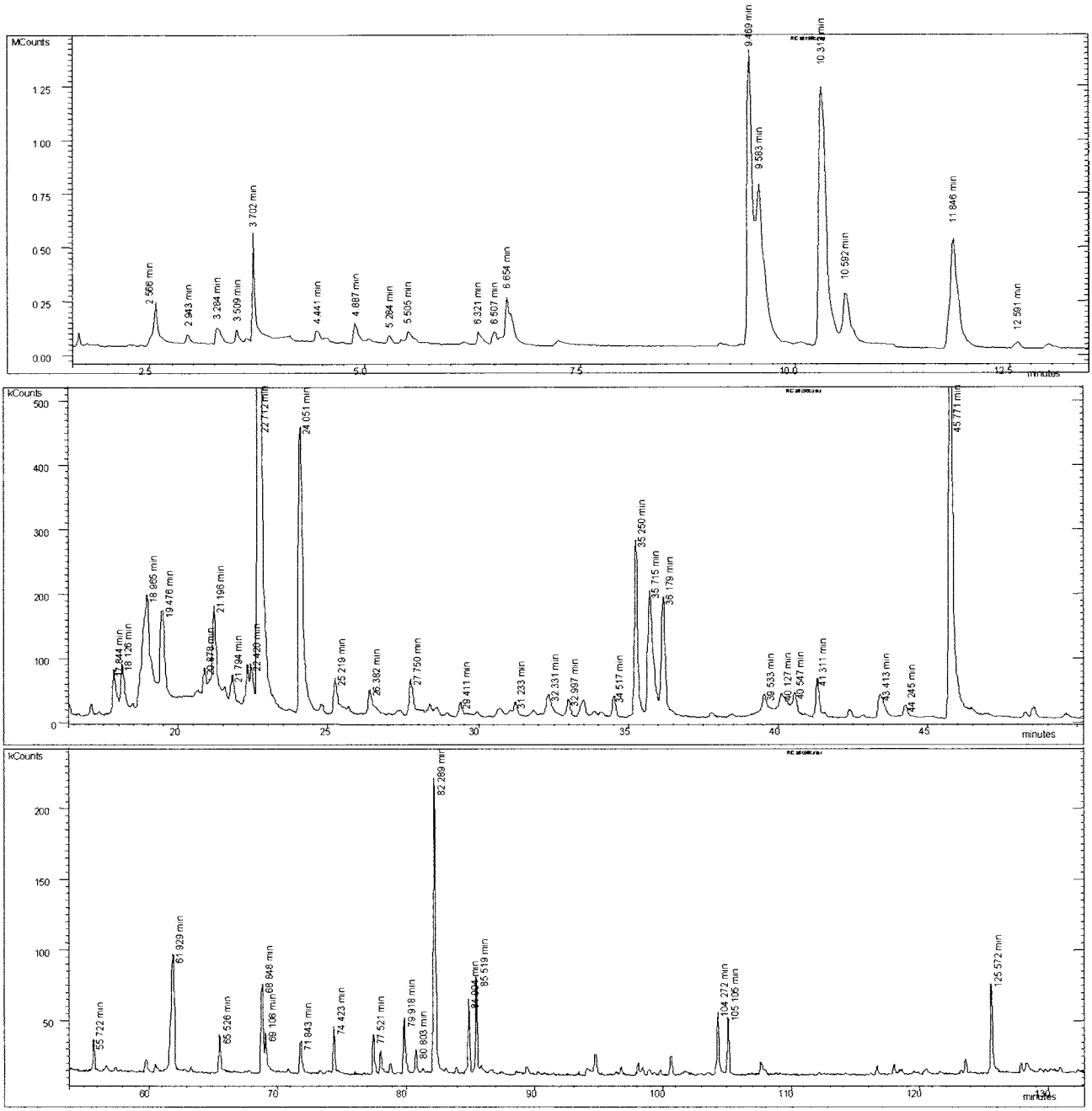


Figure 1. Typical chromatogram of volatile compounds of one of the analyzed samples.

the different cultivars of this work. The data set submitted to multivariate data analysis was formed by 36 samples of monovarietal virgin olive oil, each one described by the five measured volatile components. Multivariate data evaluation was performed by QPARVUS (*19*).

Instrumentation. Sample analyses were performed using a Varian (Walnut Creek, CA) Saturn 2000 GC-MS ion trap system in electron impact and positive chemical ionization modes, with isobutane as 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, reaction time at 50 ms, and 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 GC oven temperature was initially held at 40 °C for 3 min, then ramped at 1 °C/min to 70 °C, ramped 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 0.75 mm i.d. GC inlet liner was used.

The isobutane pressure was adjusted to produce a ratio of *m*/*z* 43 to 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; background mass, *m*/*z* 65.

RESULTS AND DISCUSSION

A typical mass chromatogram of the volatile component of one of the analyzed samples is reported in **Figure 1**. The electron (EI) and chemical [(CI) isobutane] ionization mass spectra of standards show, as expected, that the advantage of using EI is represented by the fast identification of the compounds in the commercially available databases. The EI that takes place inside the trap causes, however, the formation of $[M + H]^+$ species, such as the ions at *m*/*z* 83 and 99, in the case of compounds **4** and **3**, respectively, due to ion−molecule reactions, which account for some percentage of the total ion current. A further disadvantage of the EI method is represented

Table 1. Data of the Calibration Curves Obtained in EI Mode in the 0.2–2 mg/kg Range

compound	calibration curve	R^2	accuracy (1.2 mg/kg)
hexanal	$y = 0.772x + 0.160$	0.9911	89.43
1-hexanol	$y = 0.829x - 0.124$	0.9856	91.13
(*E*)-2-hexenal	$y = 0.989x - 0.145$	0.9934	86.02
(*E*)-2-hexen-1-ol	$y = 1.305x - 0.042$	0.9856	82.87
(*Z*)-3-hexenyl acetate	$y = 2.729x - 0.213$	0.9971	92.11

Table 2. Data of the Calibration Curves Obtained in CI Mode in the 0.2–2 mg/kg Range

compound	calibration curve	R^2
hexanal	$y = 0.367x + 0.052$	0.9972
1-hexanol	$y = 0.296x - 0.034$	0.9931
(*E*)-2-hexenal	$y = 0.811x + 0.050$	0.9955
(*E*)-2-hexen-1-ol	$y = 0.465x - 0.026$	0.9970
(*Z*)-3-hexenyl acetate	$y = 0.829x + 0.006$	0.9984

Table 3. Data of the Calibration Curves Obtained in CI Mode in the 5–100 mg/kg Range

compound	calibration curve	R^2
hexanal	$y = 0.665x + 0.005$	0.9995
1-hexanol	$y = 0.952x - 0.055$	0.9947
(*E*)-2-hexenal	$y = 1.335x + 0.008$	0.9994
(*E*)-2-hexen-1-ol	$y = 1.544x - 0.075$	0.9970
(*Z*)-3-hexenyl acetate	$y = 1.429x + 0.010$	0.9998

by the lack of structural specificity of the ions, which can be used to build up the chromatogram of the oil samples under investigation. On the contrary, the ionization in isobutane plasma provided diagnostic fragments such as $[(M + H) - H_2O]^+$ for **1**, **2**, and **4**; $[(M + H) - CH_3COOH]^+$ for analyte **5**; and the protonated molecular ion $[M + H]^+$ for the hexenal **3**.

Ethyl isobutanoate was selected as internal standard for quantitative assays. The structural features of its EI and CI spectra are similar to those of the analytes in terms of structural information and specificity of the ionic species displayed.

The widespread use of GC-MS techniques in the EI mode has suggested the evaluation of this method in the survey of the aroma markers in Italian organic olive oil, despite the drawbacks previously described. The R^2 of the calibration curves obtained in these experimental conditions and the accuracy values show that the method could be used satisfactorily for the assay of the target analytes (**Table 1**).

Better results were, however, obtained, in the CI mode, using the same internal standard. Good to excellent analytical data were obtained in the two concentration ranges (**Tables 2** and **3**).

Table 4 shows the data related to the precision and accuracy of the method.

The high precision of the method, checked six times for each analyte at 1.2 and 20 ppm, respectively, is evident from the more frequent value of 2.6% observed for all of the experiments except that which is characterized by 5%. The latter is, however, still acceptable if compared with the expected values (*20*). The accuracy, evaluated for three analytes at two different concentrations each, that is, 0.4 and 1.2 and 20 and 70 for 0.2−2 and 5−100 ppm ranges, respectively, is very satisfactory, especially for the aldehydes and the ester components.

The results obtained for all of the analyzed samples showed that the number and type of the markers selected for the survey were adequate for the characterization of olive oil as a function of the cultivars, harvesting time, and growing environment effect.

Effect of Olive Ripeness. Organic olive oils produced from the cultivar Nocellara del Belice grown in Castelvetrano (Sicily, **Figure 2**) show a deep variation of the distribution of the aroma markers with olive ripeness (Jaén method) (*21*). The oil produced in the first period is particularly rich in alcohols, and 1-hexanol is the most abundant. The concentration of the latter decreases considerably, as does, to a lesser extent, that of (*E*)-2-hexen-1-ol, with harvesting time, whereas an increase of the aldehyde component, particularly that of (*E*)-2-hexenal, is observed.

This behavior cannot be generalized. Oils produced from Coratina cultivar in Andria (Apulia, **Figure 3**) show, in fact, the opposite trend. (*E*)-2-Hexenal is always the dominant component of the aroma, whereas hexanol and (*E*)-2-hexen-1-ol slowly increase with the ripening of olives.

Different cultivars differ also significantly in the composition of the aroma, even if the oil is produced from drupes with identical ripening indices.

Organic oils obtained from drupes of the Nocellara del Belice cultivar, grown at Castelvetrano, and the Nocellara Etnea cultivar, grown at S. Maria di Licodia (Sicily), with the same ripening index value of 0.18 show an enormous difference in the composition of their aroma markers (**Figure 4**). Both products are very rich in 1-hexanol, whereas (*E*)-2-hexenal, which is very abundant in most Calabrian and Apulia oils, represents a minor component. The most impressive differences are represented (i) by the absolute amount of (*E*)-2-hexen-1-ol, which is 1.8 mg/kg in the oil from cv. Nocellara del Belice and practically absent in the Etnea analogue, and (ii) by the relative amount of hexenyl acetate.

When other Sicilian oils such as Ogliarola Messinese, grown at Patti (Sicily), and Cerasuola, grown at Trapani (Sicily), produced from drupes with identical ripening indices, are compared, important differences are disclosed (**Figure 5**). 1-Hexanol, only, is present in similar quantities in both oils, whereas Cerasuola displays a distribution centered on (*E*)-2-hexen-1-ol (6.51 mg/kg) and Ogliarola Messinese has a flat distribution of markers.

Figure 6 shows the chromatogram of volatile compounds of the Ogliarola Salentina cultivar, grown at Villa Castelli (Apulia),

Table 4. Precision and Accuracy of the Method

	precision (CV%)		accuracy			
compound	1.2 mg/kg	20 mg/kg	0.4 mg/kg	1.2 mg/kg	20 mg/kg	70 mg/kg
hexanal	2.5	2.0	104.2	112.5	97.3	102.1
1-hexanol	2.3	5.0	108.5	118.7	73.2	82.8
(*E*)-2-hexenal	1.7	2.9	99.3	106.6	97.0	103.1
(*E*)-2-hexen-1-ol	3.3	3.4	110.4	115.8	80.3	98.3
(*Z*)-3-hexenyl acetate	3.1	2.9	101.9	109.3	99.0	100.5

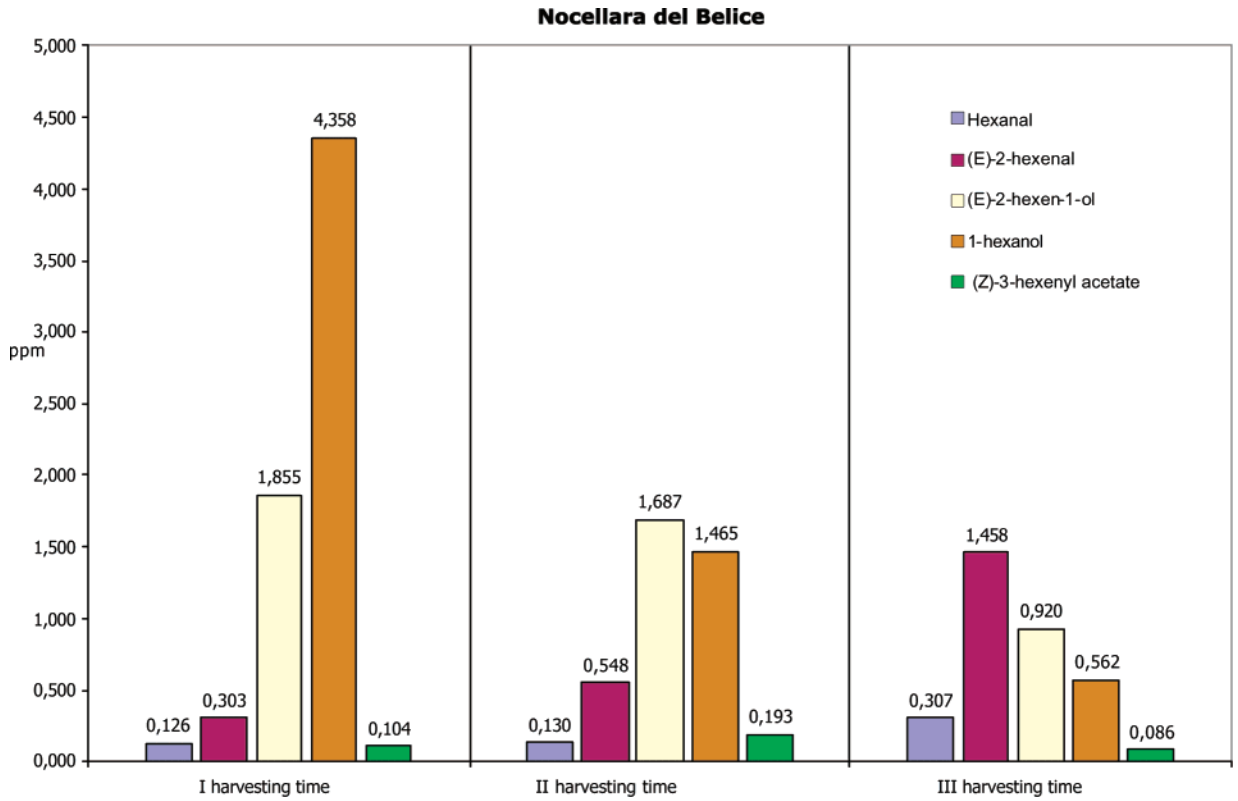


Figure 2. Variation of the distribution of the aroma markers with harvesting time in the olive oils produced from the Nocellara del Belice cultivar.

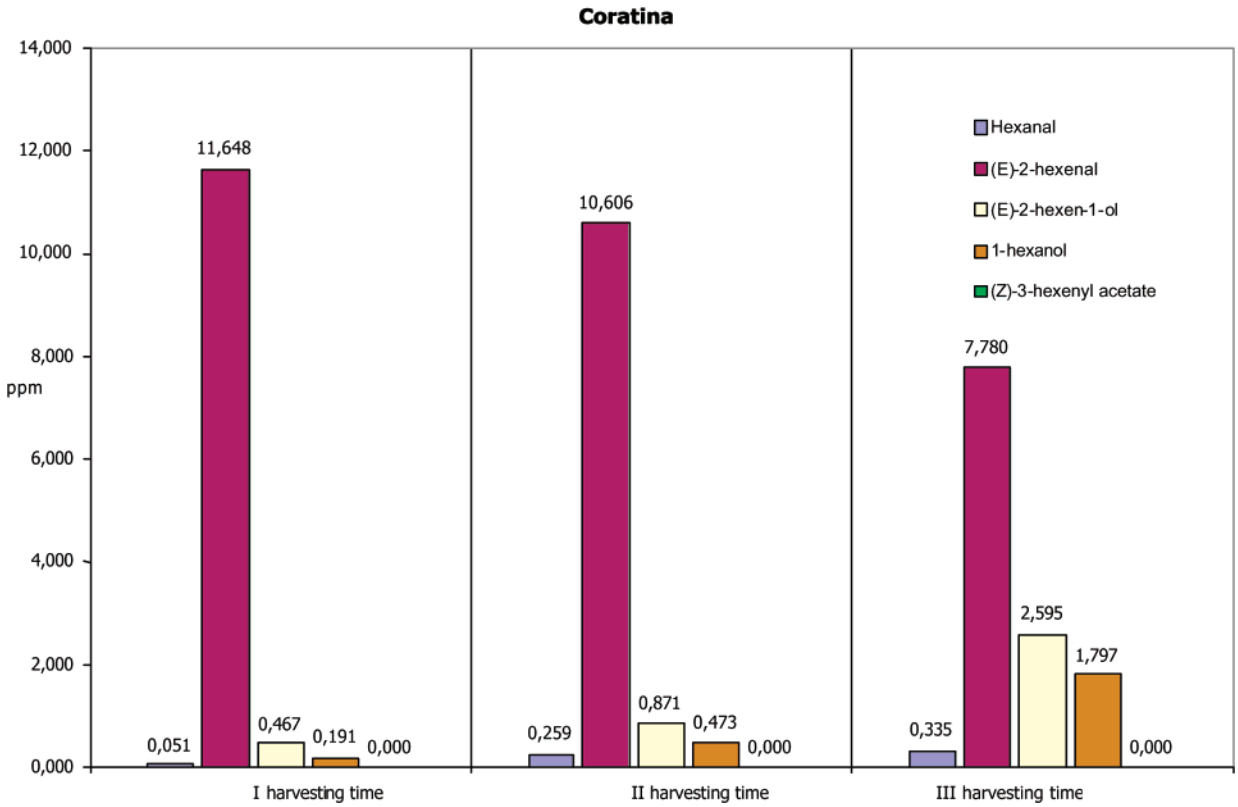


Figure 3. Variation of the distribution of the aroma markers with harvesting time in the olive oils produced from the Coratina cultivar.

and the Ogliarola Barese cultivar, grown at Conversano (Apulia). A preliminary observation concerns the relative amount of (*E*)-2-hexenal, always the main component, which points out a characteristic of the Apulian oils with respect to the others produced in southern Italy, that, their typical richness in the aldehyde components. Moreover, the ratios (*E*)-2-hexen-1-ol/(*E*)-2-hexenal and 1-hexanol/hexanal in the oil produced from the Ogliarola Salentina cultivar probably depend on the activity of alcohol dehydrogenase enzymes, which seems to be higher for the saturated than for the unsaturated substrate. The relatively

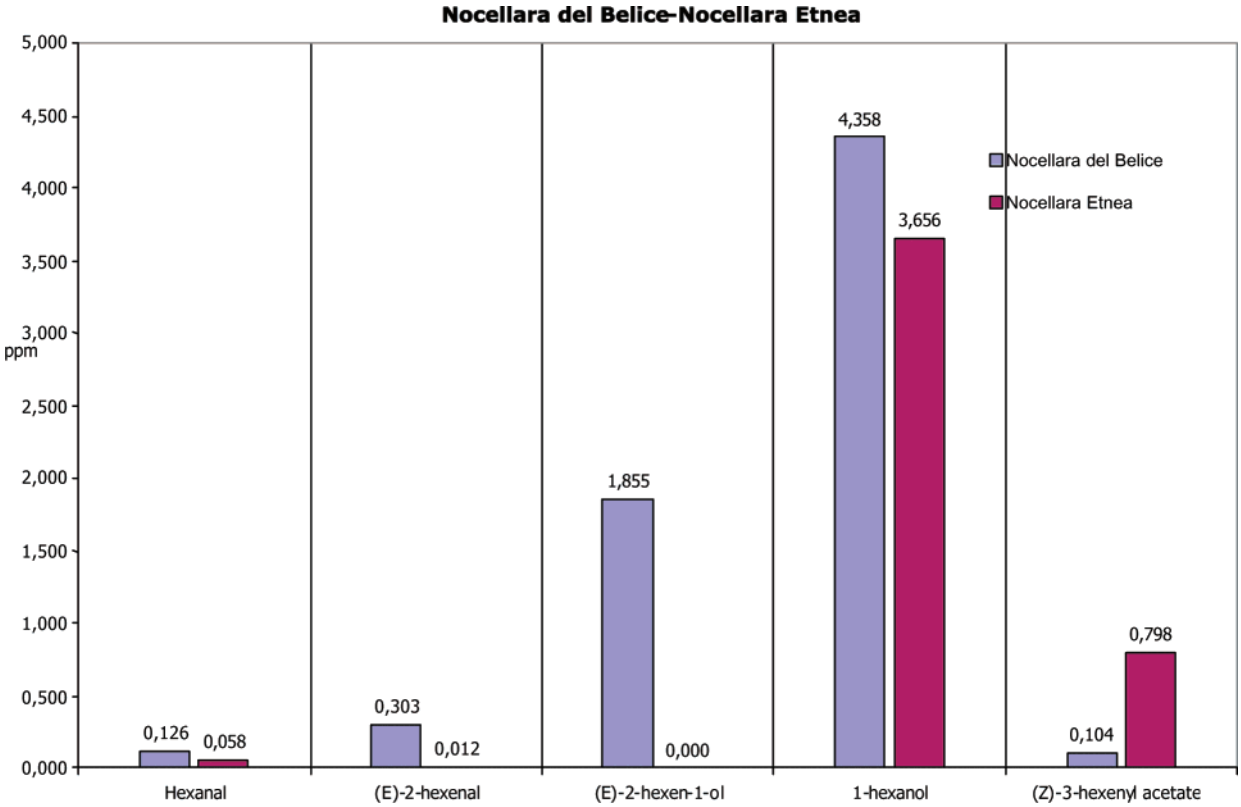


Figure 4. Distribution of the aroma markers in the olive oils produced from drupes of Nocellara del Belice and Nocellara Etnea cultivars with the same ripening index.

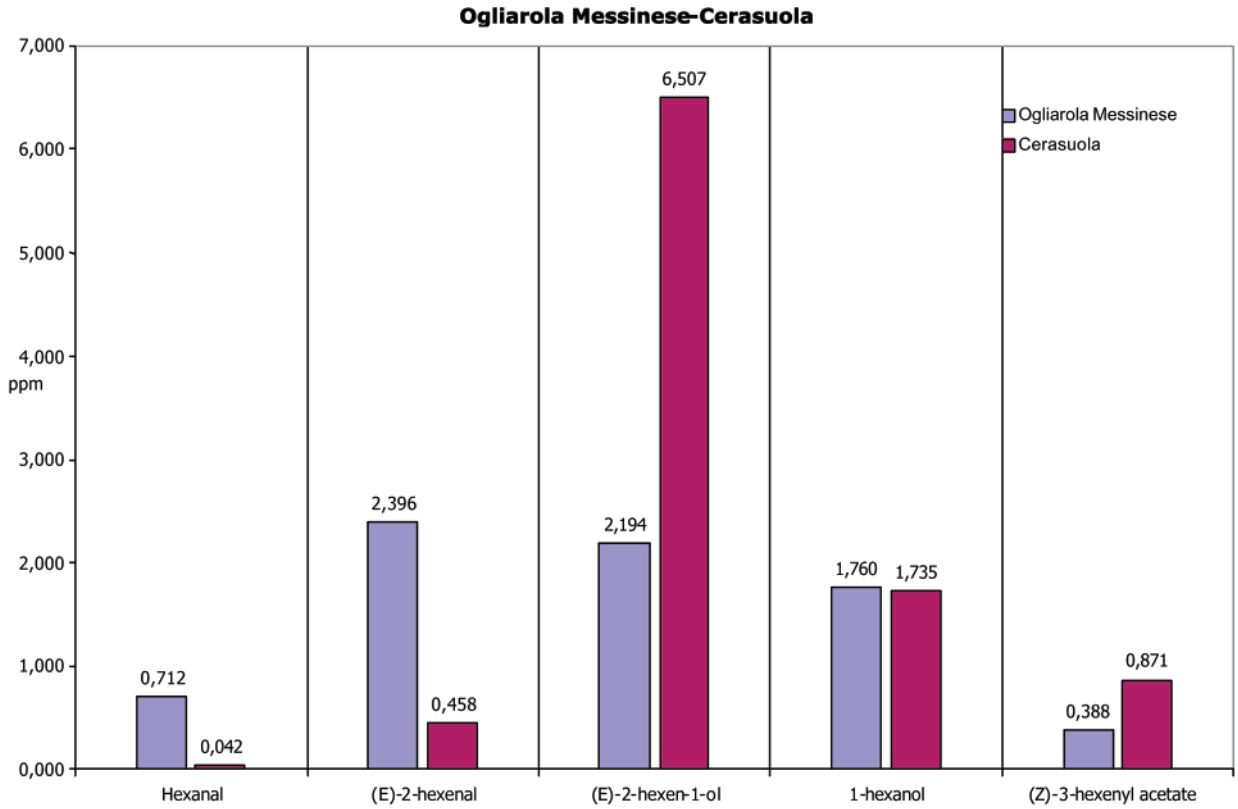


Figure 5. Distribution of the aroma markers in the olive oils produced from drupes of Ogliarola Messinese and Cerasuola cultivars with the same ripening index.

high concentration of (*E*)-2-hexen-1-ol (0.8 mg/kg), in the case of the Ogliarola Barese cultivar, is probably due to an enhanced activity of the enzyme specific for this process.

The PCA was applied to confirm the hypothesis that the five selected markers of specific lipoxygenase oxidation could be used to differentiate the various cultivars (vide infra).

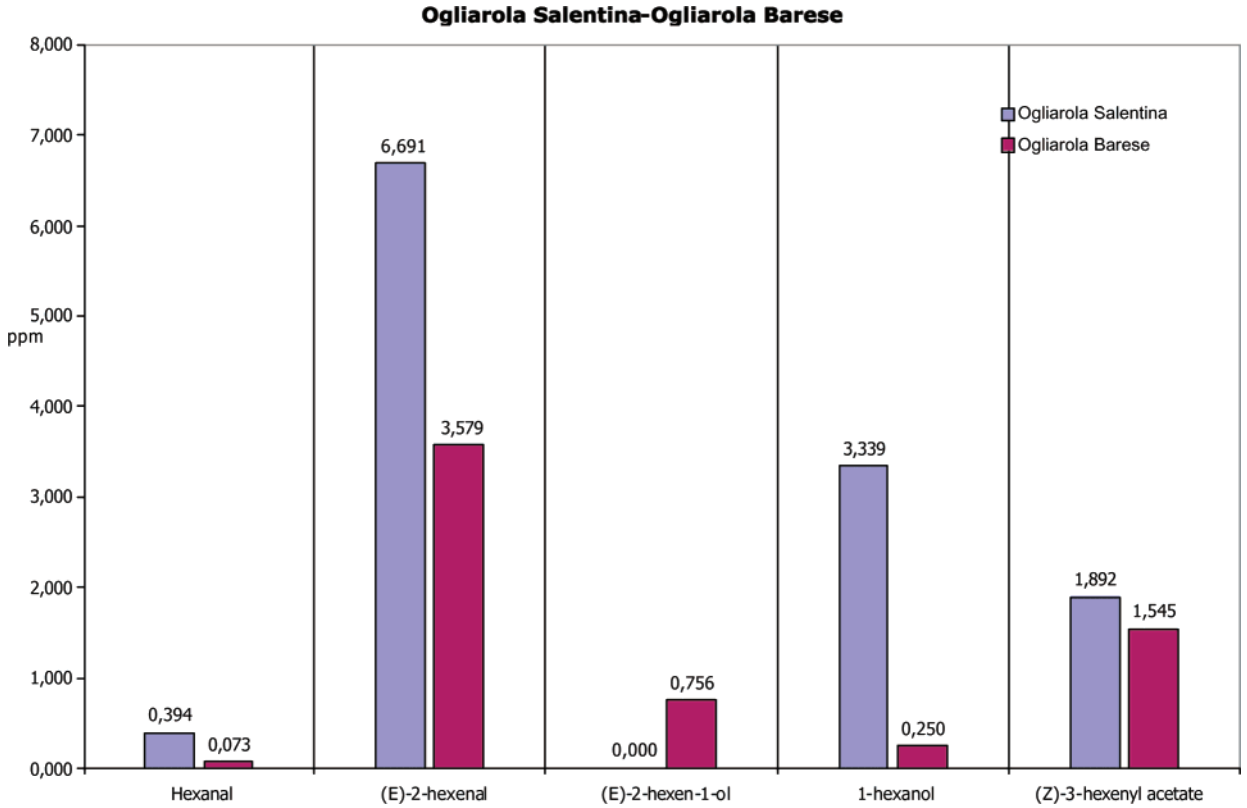


Figure 6. Distribution of the aroma markers in the olive oils produced from drupes of Ogliarola Salentina and Ogliarola Barese cultivars with the same ripening index.

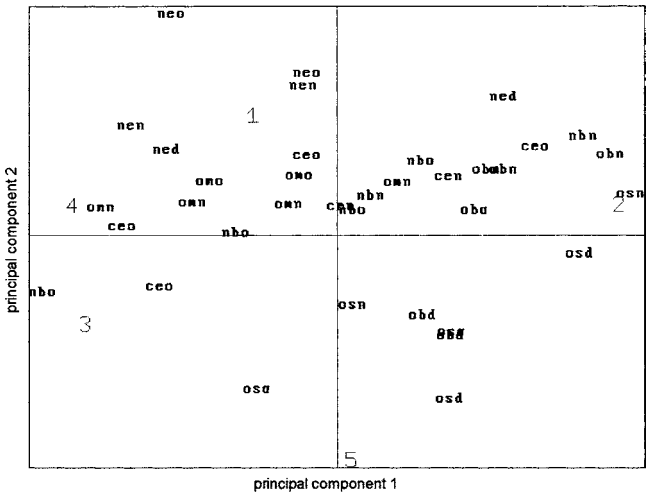


Figure 7. Score and loading plot. Samples are plotted by selected letters [first and second letters: nb, cv. Nocellara del Belice (Sicily); ne, cv. Nocellara Etnea (Sicily); ce, cv. Cerasuola (Sicily); om, cv. Ogliarola Messinese (Sicily); ob, cv. Ogliarola Barese (Apulia); os, cv. Ogliarola Salentina (Apulia); the third letter indicates the month of sampling: o, October; n, November; d, December; g, January].

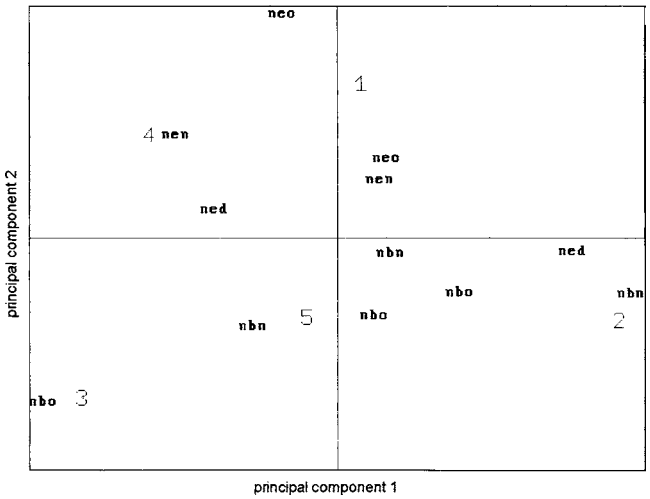


Figure 8. Score and loading plot. Samples are plotted by selected letters [first and second letters: nb, cv. Nocellara del Belice (Sicily); ne, cv. Nocellara Etnea (Sicily); the third letter indicates the month of sampling: o, October; n, November; d, December; g, January].

The first step of the PCA was represented by the obtainment of the best display of this multivariate data set after its autoscaling to assign the same numerical weight to each variable.

The scores of the samples and the loadings of the variables on the two first principal components are plotted in **Figure 7**: the information retained is 41.95% of the total. In general, the Apulian cultivars Ogliarola Barese (ob letters) and Ogliarola Salentina (os letters) have the lowest scores on component 2 [high contents of (*Z*)-hexenyl acetate], whereas the Sicilian cultivars Nocellara del Belice, Nocellara Etnea, Cerasuola, and Ogliarola Messinese have highest scores on the same component (high contents of hexanal). However, the second component divides samples of Nocellara del Belice from samples of Nocellara Etnea.

Nocellara del Belice versus Nocellara Etnea. The scores of the samples and the loadings of the variables on the two first principal components are plotted in **Figure 8**: the information retained is 37.16% of the total. The Nocellara del Belice cultivar (nb letters) has the lowest scores on the second principal component [high contents of (*Z*)-hexenyl acetate], whereas cultivar Nocellara Etnea has the highest scores on the same component (high contents of hexanal).

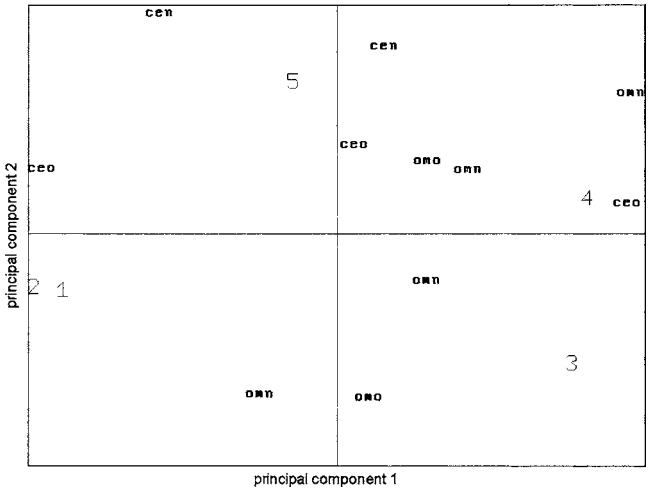


Figure 9. Score and loading plot. Samples are plotted by selected letters [first and second letters: ce, cv. Cerasuola (Sicily); om, cv. Ogliarola Messinese (Sicily); the third letter indicates the month of sampling: o, October; n, November; d, December; g, January].

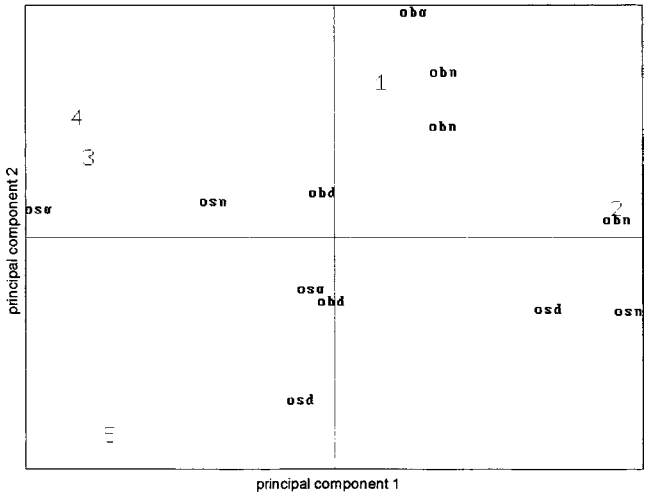


Figure 10. Score and loading plot. Samples are plotted by selected letters [first and second letters: ob, cv. Ogliarola Barese (Apulia); os, cv. Ogliarola Salentina (Apulia); the third letter indicates the month of sampling: o, October; n, November; d, December; g, January].

Cerasuola versus Ogliarola Messinese. The scores of the samples and the loadings of the variables on the two first principal components are plotted in **Figure 9**: the information retained is 56.81% of the total. Unfortunately, the samples of Cerasuola and Ogliarola Messinese overlap in score and loading plot of the first two principal components.

Ogliarola Barese versus Ogliarola Salentina. The scores of the samples and the loadings of the variables on the two first principal components are plotted in **Figure 10**: the information retained is 52.81% of the total. The Ogliarola Salentina cultivar (os letters) has the lowest scores on the second principal component [high contents of (*Z*)-hexenyl acetate], whereas cultivar Ogliarola Barese has the highest scores on the same component (high contents of hexanal).

Conclusions. The analytical approach for the evaluation of the chromatogram of volatile compounds of virgin olive oils here proposed has many advantages over similar methods. The protocol of analysis is fully automated, the only manual procedure consisting of sampling the oil into the vials; the method is reliable in terms of sensitivity, precision, and accuracy and is not affected by the presence of solvent residue, which is a typical feature of SPME technology. Finally, the method is affordable in terms of costs because commercial and relatively economical instrumentation is required.

The five markers selected as active components of the chromatogram provide important information on the cultivar and ripening phase of the drupes. In fact, on the grounds of the results obtained from statistical analysis, they allow the distinction of olive oils produced in Apulia and Sicily, respectively. Finally, statistical analysis also confirms the usefulness of volatile components to distinguish some monovarietal olive oils. Therefore, the combination of simple and relatively inexpensive analytical parameters such as volatile components and chemometric techniques represents a useful tool to characterize and classify olive oils.

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