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ARTICLE

Classification of Extra Virgin Olive Oils Produced at *La Comunitat Valenciana* According to Their Genetic Variety Using Sterol Profiles Established by High-Performance Liquid Chromatography with Mass Spectrometry Detection

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A method to classify extra virgin olive oils (EVOOs) according to their genetic variety using sterol profiles obtained by high-performance liquid chromatography (HPLC) with mass spectrometry (MS) detection has been developed. Sterol extracts were chromatographed on a dC_{18} Atlantis column (100 × 3 mm, 3 μm) with a gradient of acetonitrile/water (0.01% acetic acid) at a flow rate of 1.0 mL min^{-1} and positive-ion mode MS detection. Using linear discriminant analysis of the HPLC-MS data (extracted ion chromatograms), EVOO samples belonging to six genetic varieties cultivated at *La Comunitat Valenciana*, Spain (Arbequina, Borriolenca, Canetera, Farga, Picual, and Serrana), were correctly classified with an excellent resolution among all of the categories.

KEYWORDS: Extra virgin olive oil; genetic variety; high-performance liquid chromatography; linear discriminant analysis; mass spectrometry; sterols

INTRODUCTION

Extra virgin olive oil (EVOO) is a traditional Mediterranean food product, the market for which has been recently expanded due to its highly appreciated organoleptic attributes and its health and nutritional properties (*1*). During recent years, the consumption of EVOO has increased considerably in relation to the consumption of virgin and refined olive oils. Owing to its distinctive and peculiar intense taste, EVOOs obtained from some pure genetic varieties are highly appreciated.

EVOOs are mainly constituted by triacylglycerols, also containing an unsaponifiable matter which amounts to 1−3%. Within this unsaponifiable matter, sterols constitute the greatest and most studied fraction (*2*). The content of these components depends on the kind of olive oil (*3*), but can also vary due to environmental conditions, fruit quality, oil extraction system, and refining process (*4*). For these reasons, the determination of these minor components is of great value in establishing the oil genuineness and quality (*4*, *5*), having also a marked influence on typicality, flavor, aroma, and shelf life (*6*). On the other hand, sterols are supposed to decrease the cardiovascular risk of coronary heart disease (*7*),and also reduce blood cholesterol levels, showing anti-inflammatory, antibacterial, and antioxidant activities (*8*).

Official methods for the analysis of sterols involve saponification of the oil, extraction of the unsaponifiable fraction with diethyl ether, and isolation of the sterol fraction by thin layer chromatography (TLC). Quantification of the silanized sterol fraction is commonly performed by gas chromatography (GC) with flame ionization detection (FID) (*9*−*20*), but GC with mass spectrometry (MS) detection is also used (*9*, *12*, *18*, *21*−*23*). The major disadvantage of GC is the requirement of both thermally stable columns and chemical derivatization before analysis. For this reason, other methods such as capillary electrochromatography with diode array UV−vis detection (*24*, *25*), direct infusion mass spectrometry (*26*), and high-performance liquid chromatography (HPLC)-MS (*3*, *27*, *28*) have been also developed to determine sterols in vegetable oils. These HPLC-MS methods have been applied to both the analysis of olive oil samples of different qualities (*3*, *27*) and the analysis of several botanical oil varieties (*28*). To our knowledge, no study about the analysis of different EVOO genetic varieties using HPLC-MS sterol profiles has been previously reported.

On the other hand, sterol contents established by GC, followed by multivariate data treatment, have been used to distinguish different genetic varieties of EVOO (*10*, *11*, *15*−*17*, *20*). The contents of other compounds, such as *n*-alkanes (*29*), triglycerides (*30*), tocopherols (*31*), volatile compounds (*32*, *33*), fatty acids (*30*, *34*, *35*), and phenolic compounds (*31*, *35*), established by GC (*29*−*34*), HPLC (*29*−*31*, *34*), and direct infusion MS (*35*), have been also used as authentication methods for genetic varieties of EVOO.

In this work, sterol profiles of EVOOs from six different genetic varieties produced at *La Comunitat Valenciana*, Spain, have been obtained by HPLC-MS. The normalized peak areas have been used as predictors to construct linear discriminant (LDA) models

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Table 1. Genetic Variety, Number of Samples, Geographical Origin, and Crop Season of the EVOOs Employed in This Study

genetic variety	no. of samples	geographical origin	crop season (2005−2008)
Arbequina	2	Altura (Castellón)	06/07; 07/08
	2	Maestrat *comarca* (Castellón)	06/07; 07/08
	1	Alicante	05/06
	1	Palancia *comarca* (Castellón)	07/08
Borriolenca	3	Alcalatén *comarca* (Castellón)	05/06; 06/07; 07/08
	3	La Plana *comarca* (Castellón)	05/06; 06/07; 07/08
Canetera	2	Maestrat *comarca* (Castellón)	05/06; 07/08
	2	Adzaneta (Castellón)	06/07; 07/08
	2	La Plana *comarca* (Castellón)	05/06; 07/08
Farga	2	Maestrat *comarca* (Castellón)	05/06; 06/07
	2	Alcalatén *comarca* (Castellón)	06/07; 07/08
	2	La Plana *comarca* (Castellón)	05/06; 07/08
Picual	6	Altura (Castellón)	05/06; 06/07; 07/08
Serrana	2	Altura (Castellón)	06/07; 07/08
	1	Artana (Castellón)	06/07
	1	Jérica (Castellón)	07/08
	2	Viver (Castellón)	05/06; 07/08

capable of classifying the EVOO samples according to their genetic variety.

EXPERIMENTAL PROCEDURES

Reagents and Samples. The following analytical grade reagents were used: ethanol, 2-propanol, acetic acid, acetonitrile (ACN), anhydrous sodium sulfate (Scharlau, Barcelona, Spain); diethyl ether, chloroform (J. T. Baker, Deventer, The Netherlands); KOH (Probus, Barcelona, Spain); *n*-hexane (Riedel-de Haën, Seelze, Germany); and 2,7-dichlorofluorescein (Sigma, St. Louis, MO). Glass plates for TLC, coated with silica gel without fluorescent indicador (0.25 mm plate thickness, Merck, Darmstadt, Germany) were used. Deionized water (Barnstead deionizer, Sybron, Boston, MA) was also used. The sterols used as standards were erythrodiol (Fluka, Buchs, Switzerland); β-sitosterol (mixture containing 75% β-sitosterol and 10% campesterol), ergosterol, and stigmasterol (Acros Organics, Morris Plains, NJ); and cholesterol (Aldrich, Milwaukee, WI). The EVOOs employed in this study (**Table 1**) were kindly donated by *Intercoop Olival* (Almassora, Castellón, Spain) and by the *Cooperativa de Altura* (Altura, Castellón) from different crop seasons, also specified in **Table 1**. The genetic variety, quality grade, and geographical origin of all EVOO samples were guaranteed by the suppliers.

Instrumentation and Working Conditions. An 1100 series liquid chromatograph provided with a quaternary pump (Agilent Technologies, Waldbronn, Germany) was used. Separation was carried out with a dC_{18} column (Atlantis, 3 μm, 100 × 3 mm, Waters, Milford, MA). Mobile phases were prepared by mixing ACN and water, both containing 0.01% acetic acid. A linear gradient at a flow rate of 1 mL min^{-1} from 90 to 100% ACN for 10 min, followed by isocratic elution with 100% ACN for 2 more min, was used. In all cases, 20 μL was injected. The mass spectrometer system was an HP 1100 series ion trap mass spectrometer (Agilent) equipped with an atmospheric pressure photoionization source. The MS working conditions were as follows: nebulizer gas pressure, 15 psi; drying gas flow, 12 L min^{-1} at 350 °C; vaporizer temperature, 275 °C; capillary voltage, −1.9 kV; voltages of skimmers 1 and 2, 25.9, and 6.0 V, respectively. Nitrogen was used as nebulizer and drying gas (Gaslab NG LCMS 20 generator, Equcien, Madrid, Spain). The mass spectrometer was scanned within the *m*/*z* 200−500 range in the positive ion mode. The ion trap target mass was set at *m*/*z* 397 ($[M + H - H_2O]^+$ peak of β-sitosterol). Maximum loading of the ion trap was 3×10^4 counts, and maximum collection time was 300 ms. Total ion chromatograms (TIC) and extracted ion chromatograms (EIC) were smoothed using a Gaussian filter set at 5 points.

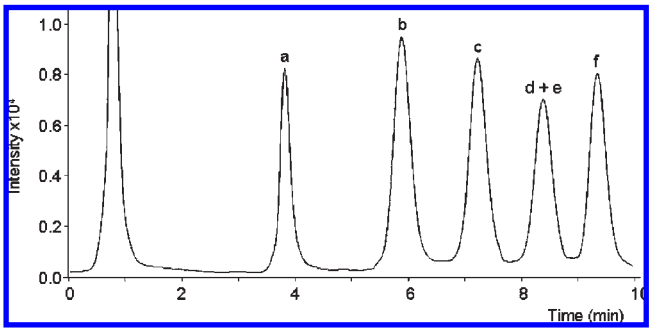

Figure 1. TIC of a standard solution of sterols (ca. 100 mg L^{-1}) using a linear gradient from 90 to 100% ACN for 10 min, followed by isocratic elution with 100% ACN for an additional 2 min. Peaks: (a) erythrodiol; (b) ergosterol; (c) cholesterol; (d) campesterol; (e) stigmasterol; (f) β-sitosterol.

Table 2. Peak Labeling, Retention Time (t_R), and *m*/*z* Value of the Sterols Studied in This Work

peak	analyte	t_R (min)	*m*/*z*[a]
1	erythrodiol	3.90	425
2	uvaol	3.90	425
3	brassicasterol	6.10	381
4	cholesterol	7.25	369
5	Δ^7-avenasterol	7.25	395
6	Δ^5-avenasterol	7.25	395
7	campesterol	8.30	383
8	campestanol	8.30	385
9	stigmasterol	8.40	395
10	$\Delta^{5,24}$-stigmastadienol	9.30	395
11	Δ^7-stigmastenol	9.40	397
12	β-sitosterol	9.40	397

[a] *m*/*z* value corresponding to the $[M + H - H_2O]^+$ peak.

Sample Preparation. The sterol fraction of the EVOO samples was obtained following the procedure established by EC Regulation (*36*). Accordingly, 5 g of oil was saponified by refluxing with 2 M ethanolic KOH for 20 min; 50 mL of distilled water was added, and the nonsaponifiable fraction was extracted three times with diethyl ether. The three ether extracts were introduced into a separating funnel and washed with distilled water (50 mL each time) until neutral reaction. The organic extracts were dried with anhydrous sodium sulfate and filtered. These extracts were evaporated to dryness using a rotatory evaporator. The remaining unsaponifiables were dissolved in 2 mL of chloroform, and then the sterol fraction was separated by TLC using a plate-developing chamber, which contained hexane/diethyl ether 60:40 (v/v). After TLC separation, the silica plate was sprayed lightly and uniformly with 2,7-dichlorofluorescein. The sterol band was removed from the silica plate using a spatula. This material was dissolved in 10 mL of diethyl ether and filtered through a Whatman no. 1 paper using a Büchner funnel. A rotatory evaporator was used to remove this solvent, and the residue was dissolved in 500 μL of 2-propanol and stored at −20 °C in amber vials. These solutions were properly diluted with the mobile phase and injected two times.

Data Treatment and Statistical Analysis. The peak area of each sterol was measured from the smoothed EIC, and a data matrix was constructed using the areas of all peaks as original variables. After

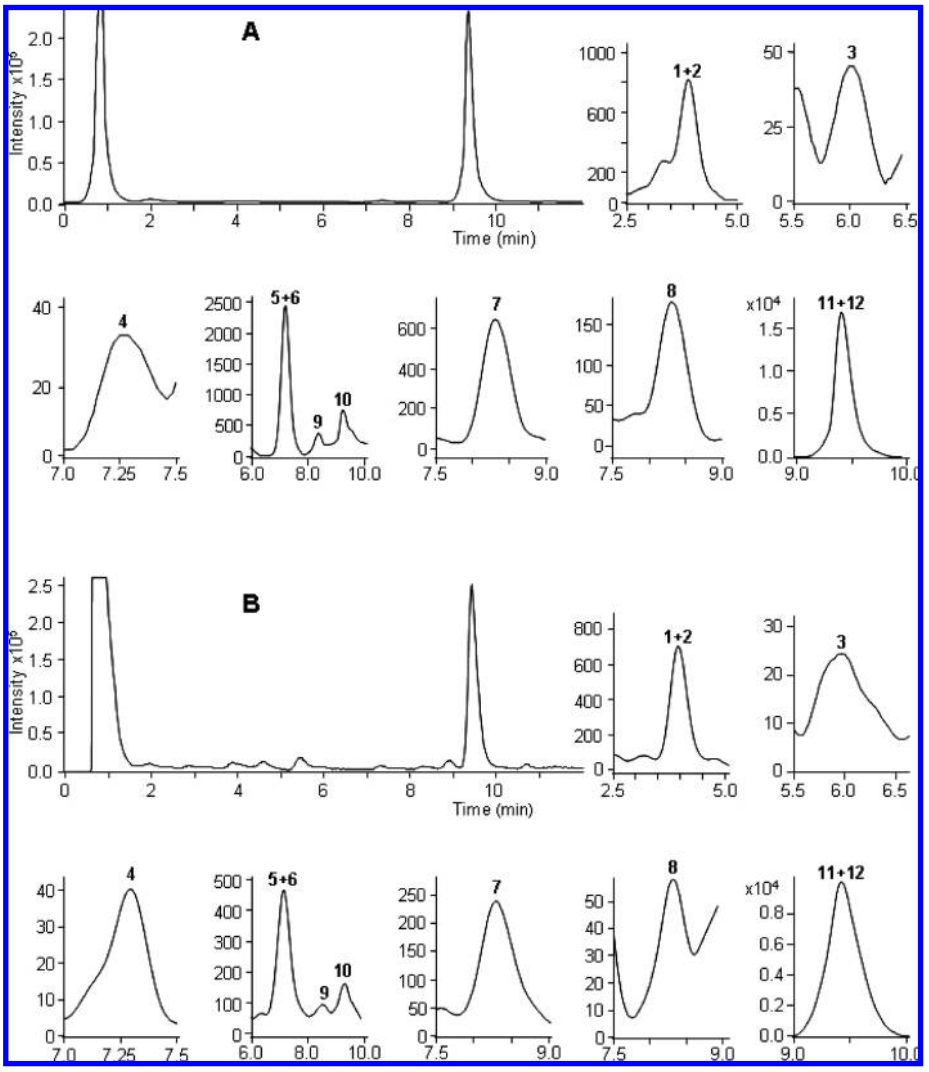

Figure 2. TIC and EICs of Canetera (**A**) and Serrana (**B**) EVOO sterol extracts. EICs were obtained at the *m/z* values indicated in **Table 2**. Peak labeling is as indicated in **Table 2**. Other experimental conditions were as in **Figure 1**.

normalization of the variables, statistical data treatment was performed using SPSS (v. 15.0, Statistical Package for the Social Sciences, Chicago, IL). LDA, a supervised classificatory technique, is widely recognized as an excellent tool to obtain vectors showing the maximal resolution between a set of previously defined categories. In LDA, vectors minimizing Wilks' lambda, λ_w, are obtained (*37*). This parameter is calculated as the sum of squares of the distances between points belonging to the same category divided by the total sum of squares. Values of λ_w approaching 0 are obtained with well-resolved categories, whereas overlapped categories made λ_w approach 1. Up to N − 1 discriminant vectors are constructed by LDA, *N* being the lowest value for either the number of predictors or the number of categories. The selection of the predictors to be included in the LDA models was performed using the SPSS stepwise algorithm. According to this algorithm, a predictor is selected when the reduction of λ_w produced after its inclusion in the model exceeds F_{in}, the entrance threshold of a test of comparison of variances or *F* test. However, the entrance of a new predictor modifies the significance of those predictors that are already present in the model. For this reason, after the inclusion of a new predictor, a rejection threshold, F_{out}, is used to decide if one of the other predictors should be removed from the model. The process terminates when there are no predictors entering or being eliminated from the model. The values of F_{in} and F_{out}, 3.84 and 2.71, respectively, were adopted.

RESULTS AND DISCUSSION

Optimization of the Sterol Separation. For each sterol standard peak, two *m/z* values, corresponding to $[M + H]^+$ and $[M + H - H_2O]^+$ ions, were observed. However, and as previously reported (*3*, *25*, *28*), the $[M + H - H_2O]^+$ peaks showed higher intensities than the respective $[M + H]^+$ peaks. For this reason, the intensities of the $[M + H - H_2O]^+$ peaks were used for identification and classification. Different gradient elutions using ACN/water mixtures, both containing 0.01% acetic acid, at a constant flow rate of 1.0 mL min^{-1} were tried. As a result of this study, the linear gradient that provided the best separation/analysis time ratio was achieved with 90−100% ACN for 10 min, followed by isocratic elution with 100% ACN for an additional 2 min. **Figure 1** shows a chromatogram of sterol standards using this gradient elution. As observed, the total analysis time was 10 min, which was much lower than that reported for sterol separation using GC-FID. On the other hand, an overlapping between campesterol and stigmasterol peaks was observed in all of the gradient elutions tried. This finding was in agreement with some previous HPLC results (*38*).

Then, all EVOO extracts were injected using these optimal conditions. To identify other sterol peaks present in the samples, the EICs at the *m/z* values of **Table 2** were performed. A total of 9 peaks, corresponding to 12 possible sterols, were identified in < 10 min. The TIC and EICs of two EVOO extracts of different genetic varieties, Canetera and Serrana, are shown in panels **A** and **B**, respectively, of **Figure 2**. Several differences between the peak profiles of both varieties were evidenced. The quantitative results of sterols in the vegetable oils analyzed are shown in

Table 3. Proportions of Sterols Found in Total Sterol Fraction of EVOO of Different Genetic Varieties

sterol	Arbequina	Borriolenca	Canetera	Farga	Picual	Serrana
erythrodiol + uvaol	0.1−0.3	0−0.09	2.0−4.0	0.0−0.4	0.2−0.6	5.0−7.7
brassicasterol	0.08−0.11	0.06−0.09	0.06−0.12	0.09−0.12	0.1−0.2	0.08−0.15
cholesterol	0.3−0.6	0.3−0.5	0.1−0.2	0.1−0.5	0.4−0.5	0.2−0.4
Δ^7- + Δ^5-avenasterol	12.1−14.9	7.0−9.9	11.5−12.3	7.8−9.5	4.3−13.2	5.1−6.4
campesterol	3.7−4.0	3.2−3.7	3.0−3.5	3.2−3.6	2.5−3.1	1.8−2.4
campestanol	0.2−0.4	0.2−0.3	0.5−0.9	0.2−0.3	0.2−0.3	0.3−0.6
stigmasterol	0.7−1.5	1.2−2.8	1.5−2.0	1.1−1.9	0.9−1.5	1.0−2.5
$\Delta^{5,24}$-stigmastadienol	0.7−1.9	0.6−1.0	3.0−3.7	0.8−1.0	0.9−1.3	1.0−1.5
Δ^7-stigmastenol + β-sitosterol	76−80	79.0−82.3	75.0−77.8	78.7−90.8	77.3−90.0	81.0−82.7

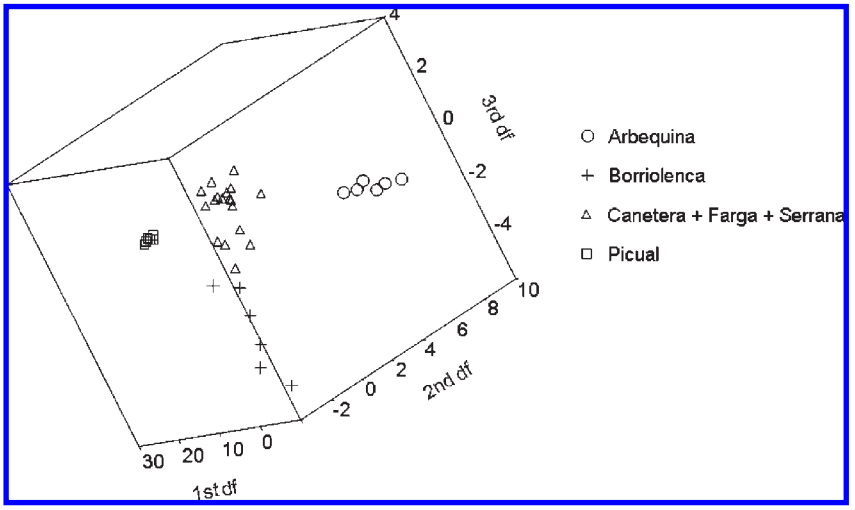

Figure 3. Score plot on an oblique plane of the three-dimensional space defined by the three discriminant functions of the LDA model constructed to resolve the Arbequina, Borriolenca, Picual, and Canetera + Farga + Serrana categories.

Table 3. In general, the levels of sterols found in these samples are in good agreement with data reported in the literature (*39*). As shown in this table, several differences between the contents of the different genetic varieties were found. Similar sterol separation performance was observed compared to the literature (*28*), but lower analysis times were obtained (*3*, *27*, *28*).

Normalization of the Variables for LDA Classification. To reduce the variability associated with the total amount of sterols recovered from the oil samples and to minimize other sources of variance also affecting the sum of the areas of all peaks, normalized rather than absolute peak areas were used. To normalize the variables, the area of each peak taken from the corresponding EIC was divided by each of the areas of the other eight peaks (also taken from their EICs); in this way, and taking into account that each pair of peaks should be considered only once, $(9 \times 8)/2 = 36$ nonredundant peak ratios were obtained to be used as predictors.

Construction of the Data Matrices and LDA Models. Using the normalized variables, LDA models capable of classifying the EVOO samples according to their respective genetic varieties were constructed. First, from the 36 samples of **Table 1**, a matrix containing 72 injections (all samples were injected two times) and 36 predictors, was constructed. A response column, containing the six categories corresponding to the six genetic varieties of the EVOOs, was added to this matrix. This matrix was used as an evaluation set. To construct LDA training matrices, only the means of the replicates of the samples were included (36 objects); in this way, the internal dispersion of the categories was reduced, which was important to reduce the number of variables selected by the SPSS stepwise algorithm during model construction.

To classify the EVOOs according to the six genetic varities of **Table 1**, an LDA model was constructed. With this model, the categories Arbequina, Borriolenca, and Picual appeared to be clearly resolved from each other and were also well separated from the other three categories (Canetera, Farga, and Serrana), which overlapped (data not shown). For this reason, a new LDA model was constructed in which these three categories were grouped into a single one. An excellent resolution between these four categories (Arbequina, Borriolenca, Picual, and the one formed by the other three categories) was obtained (**Figure 3**, $\lambda_w = 0.290$). The variables selected by the SPSS stepwise algorithm, and the corresponding model standardized coefficients, showing the predictors with large discriminant capabilities, are given in **Table 4**. All of the points of the training set were correctly classified by leave-one-out cross-validation. The evaluation set, containing the 72 original data points, was used to check the prediction capability of the model. Using a 95% probability, only three objects corresponding to replicates of different samples (one Borriolenca, one Canetera, and one Farga) were not correctly assigned; thus, the prediction capability was $>95\%$.

Next, the Arbequina, Borriolenca, and Picual categories were removed from the training set, and the remaining categories (Canetera, Farga, and Serrana) were used to construct another LDA model. Now, the three categories were separated with excellent resolution (**Figure 4**, $\lambda_w = 0.209$). The variables selected and the corresponding model standardized coefficients are also given in **Table 4**. All of the points of the training set were correctly classified by leave-one-out cross-validation. To estimate the prediction capability of this model, the evaluation set, constituted now by 36 original data points, was used. Using a 95% probability, only two objects, which corresponded to replicates of

Table 4. Predictors Selected and Corresponding Standardized Coefficients of the Two Sequential LDA Models Constructed

	Arbequina/Borriolenca/Picual/Canetera + Farga + Serrana			Canetera/Farga/ Serrana	
predictor[a]	f_1	f_2	f_3	f_1	f_2
p1 + 2/3	−1.65	0.48	0.02	−2.08	1.32
p1 + 2/5 + 6	2.62	−1.12	−0.95	3.86	−3.08
p1 + 2/7	3.82	7.47	−0.12	2.13	−1.44
p1 + 2/9	−3.30	5.65	−1.29	−5.16	11.45
p1 + 2/10	−12.61	−5.83	6.49	12.83	−0.58
p1 + 2/11 + 12	24.16	2.47	−5.37	−15.50	1.49
p11 + 12/3	−0.80	1.44	2.45	3.60	−1.00
p11 + 12/5 + 6	−2.08	0.74	−0.36	−3.59	0.99
p11 + 12/7	−4.47	−1.08	−0.13		
p11 + 12/9	6.36	−2.86	5.84	4.63	−11.39
p11 + 12/10	2.00	0.06	−1.84	3.18	4.81
p3/5 + 6	3.27	−2.38	7.39		
p3/7	0.83	−7.68	0.54		
p3/9	0.93	−0.36	−3.68		
p3/10	−20.81	6.40	−6.09		
p5 + 6/9	−4.79	−1.77	−0.78		
p5 + 6/10	2.79	1.58	0.40		
p9/10	4.19	1.25	−0.39	6.51	1.91

[a] *m*/*z* values of the ratios of sterol peaks.

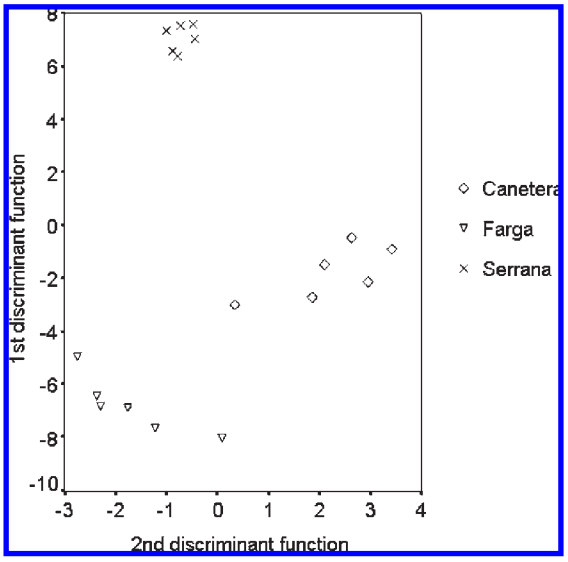

Figure 4. Score plot on the plane of the two discriminant functions of the LDA model constructed to resolve the Canetera, Farga, and Serrana categories.

different samples (one Canetera and one Farga), were not correctly assigned; thus, the prediction capability was >88%. Therefore, the possibility of classifying EVOOs according to their genetic variety by using sterol profiles obtained by HPLC-MS has been demonstrated, when a sequential application of two LDA models was performed. Thus, the proposed method is of interest to control the genetic origin of the olives used to obtain the EVOOs.

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