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Analytical Methods

Prediction of the genetic variety of Spanish extra virgin olive oils using fatty acid and phenolic compound profiles established by direct infusion mass spectrometry

M.J. Lerma-García *, J.M. Herrero-Martínez, G. Ramis-Ramos, E.F. Simó-Alfonso

Department of Analytical Chemistry, Faculty of Chemistry, University of Valencia, C/Doctor Moliner 50, 46100 Burjassot, Valencia, Spain

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Abstract

The genetic varieties of Spanish extra virgin olive oils (Arbequina, Hojiblanca and Picual) were predicted by direct infusion of the samples in the electrospray ionization source of a mass spectrometer, followed by linear discriminant analysis of the spectral data. The samples were 1:50 diluted (v/v) with an 85:15 propanol/methanol (v/v) mixture containing 40 mM KOH and infused. The abundances of the $[M–H]^-$ peaks of the free fatty acids (7 peaks) and 28 phenolic compounds (20 peaks) were measured. Ratios of pairs of peak abundances were used as predictors in the construction of the linear discriminant analysis models. An excellent resolution between the three genetic varieties was achieved.
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Keywords: Fatty acids; Genetic variety; Mass spectrometry; Olive oil authentication; Phenolic compounds

1. Introduction

Olive oil is a product of significant nutritional value, and a very important ingredient of the Mediterranean diet. The beneficial effects of olive oil can be attributed not only to the high relationship between unsaturated and saturated fatty acids, but also to the antioxidant properties of its phenolic compounds (Ríos, Gil, & Gutiérrez-Rosales, 2005; Tripoli et al., 2005). Along the last years, the consumption of extra virgin olive oils has increased considerably in relation to the consumption of virgin and refined olive oils. Owing to its distinctive and peculiar intense taste, extra virgin olive oils obtained from some pure genetic varieties are highly appreciated.

Oil authentication (Lees, 1999) can be carried out by a variety of methods which have been recently reviewed (Aparicio & Aparicio-Ruíz, 2000; Aparicio & Luna, 2002). The concentration profiles of saturated and unsaturated fatty acids (Aranda, Gómez-Alonso, Rivera del Álamo, Salvador, & Fregapane, 2004; Bucci, Magri, Magri, Marini, & Marini, 2002; Caponio, Alloggio, & Gomes, 1999; D'Imperio et al., 2007; Krichene et al., 2007; Marini et al., 2004; Ranalli et al., 2002; Salvador, Aranda, Gómez-Alonso, & Fregapane, 2003; Stefanoudaki, Kotsifaki, & Koutsaftakis, 1999; Torres & Maestri, 2006), tryglycerides (Aranda et al., 2004; Stefanoudaki, Kotsifaki, & Koutsaftakis, 1997), diacylglycerols and tryacylglycerols (Nagy et al., 2005; Ranalli et al., 2002), sterols (Bucci et al., 2002; Marini et al., 2004; Nagy et al., 2005; Salvador et al., 2003), phenolic compounds (Caponio et al., 1999; Gómez-Alonso, Salvador, & Fregapane, 2002; Krichene et al., 2007; Morelló, Romero, & Motilva, 2004; Ríos et al., 2005; Saitta, Lo-Curto, Salvo, Di-Bella, & Dugo, 2002; Salvador et al., 2003; Torres & Maestri, 2006), hydrocarbons (Koprivnjak, Moret, Populin, Lagazio, & Conte, 2005), pigments (Cichelli & Pertesana, 2004; Krichene et al., 2007) and volatile components (Guadarrama, Rodríguez-Méndez, Sanz, Ríos, & De Saja, 2001; Luna, Morales,

* Corresponding author. Tel.: +34 963543176; fax: +34 963544436.
E-mail address: mlergar@alumni.uv.es (M.J. Lerma-García).

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& Aparicio, 2006; Tura, Prenzler, Bedgood, Antolovich, & Robards, 2004) differ according to the fruit variety. Other factors as latitude, climatic conditions, irrigation regime, fruit ripening, and harvesting and extraction technologies also affect the distributions of the fatty acids (D'Imperio et al., 2007; Stefanoudaki et al., 1999; Torres & Maestri, 2006) and triglycerides (Stefanoudaki et al., 1997). The concentration profiles of other minor oil components, such as phenolic compounds (Caponio et al., 1999; Gómez-Alonso et al., 2002; Morelló et al., 2004; Salvador et al., 2003; Torres & Maestri, 2006; Tripoli et al., 2005), hydrocarbons (Koprivnjak et al., 2005) and volatile components (Tura et al., 2004), are also affected.

Authentication methods for genetic varieties of olive oils have been most frequently established using GC (Aranda et al., 2004; Bucci et al., 2002; Caponio et al., 1999; D'Imperio et al., 2007; Koprivnjak et al., 2005; Krichene et al., 2007; Luna et al., 2006; Ríos et al., 2005; Saitta et al., 2002; Stefanoudaki et al., 1999; Torres & Maestri, 2006; Tura et al., 2004) and HPLC (Aranda et al., 2004; Bucci et al., 2002; Caponio et al., 1999; Cichelli & Pertesana, 2004; Gómez-Alonso et al., 2002; Koprivnjak et al., 2005; Krichene et al., 2007; Morelló et al., 2004; Nagy et al., 2005; Ranalli et al., 2002; Ríos et al., 2005; Salvador et al., 2003; Stefanoudaki et al., 1997, 1999). The fatty acid composition of two olive oil varieties from Crete, Koroneiki and Mastoides, were studied by GC (Stefanoudaki et al., 1999). In comparison to the Mastoides variety, the Koroneiki oils were characterized by their lower concentrations of oleic and heptadecanoic acids, and higher concentrations of linoleic and palmitic acids. In the same study, oils from high-altitude locations were rich in mono-unsaturated fatty acids, whereas oils from low-altitude locations were mainly richer in saturated fatty acids. Also, the contents of both palmitic and palmitoleic acids increased with the altitude in both cultivars. In addition, the classification of olive oils according to the cultivar and geographical origin using factor analysis and linear discriminant analysis (LDA) of the fatty acid concentrations, was demonstrated. D'Imperio et al. (2007) have analyzed a large number of Sicilian extra virgin olive oils from 22 cultivars of different geographical areas and harvesting times using GC. Oleic, linoleic and palmitic fatty acids were crucial in the characterization of the olive oil varieties. Using GC and solid phase extraction followed by RP–HPLC, Krichene et al. (2007) have determined the contents of fatty acids and 14 phenolic compounds, as well as other olive oil minor components, in six Tunisian olive varieties; the concentrations of these compounds differed between the genetic varieties.

Direct infusion in the electrospray ionization source (ESI) of a mass spectrometer (MS) without prior chromatographic separation, followed by principal component analysis (PCA), were used to evaluate the adulteration of olive oils with oils of others fruits and seeds (Goodacre, Vaidyanathan, Bianchi, & Kell, 2002); using the triglyceride profile and their daughter ions, refined olive and hazelnut oils, and unrefined peanut and sunflower oils, appeared as four resolved groups. Two ion sources, ESI and atmospheric pressure photoionization (APPI), coupled to a quadrupole time-of-flight mass spectrometer, applied to the control of olive oil adulteration (Gómez-Ariza, Arias-Borrego, García-Barrera, & Beltran, 2006), were compared. Using PCA and LDA of the triglyceride profile, mixtures of olive oils with other vegetable oils were distinguished.

Direct infusion ESI–MS, followed by LDA, has been successfully used to classify binding media in art works (Peris-Vicente, Simó-Alfonso, Gimeno-Adelantado, & Domenech-Carbó, 2005) and midge larvae (Gama Melão, Simó-Alfonso, Ramis-Ramos, & Vicente, 2006). Direct infusion ESI–MS has been also used to classify vegetable oils according to their biological origin, and to detect the adulteration of olive oil with soybean oil (Catharino et al., 2005).

In this work, a simple and quick method based on direct infusion MS, followed by LDA of the ratios of peak pairs of both free fatty acids and a large number of phenolic compounds, capable of reliably classifying the three most common monovarietal olive oils in the Spanish market (Hojiblanca, Arbequina and Picual), was developed.

2. Materials and methods

2.1. Instrumentation and working conditions

An HP 1100 series ion trap mass spectrometer (ITMS) provided with an ESI source (Agilent Technologies, Waldbronn, Germany) was used. A syringe pump (kdScientific, Holliston, MA, USA) was used to infuse the samples at 0.3 mL h^{-1} (5 μL min^{-1}) through a 50 μm i.d. fused silica capillary. The MS conditions were: nebulizer gas pressure, 25 psi; drying gas flow, 5 L min^{-1} at a temperature of 200 °C; capillary voltage, 3.5 kV; skimmers 1 and 2 V, −26.8 V and −6.0 V, respectively. Nitrogen was used as the nebulizer and drying gas (Gaslab NG LCMS 20 generator, Equcien, Madrid, Spain). The MS was scanned within the m/z 100–800 range in the negative-ion mode. The target mass was set at m/z 281 ($[M–H]^-$ peak of oleic acid). Maximum loading of the ion trap was 3×10^4 counts, and maximum collection time was 300 ms. All samples were injected at least four times, and each time the data were averaged during 1 min. The four replicates of each sample were always performed in different days.

2.2. Reagents, samples and procedures

Methanol (MeOH) and *n*-propanol (PrOH) (Scharlau, Barcelona, Spain) and KOH (Probus, Barcelona, Spain) were used. The extra virgin olive oil samples employed in this study are indicated in Table 1. The genetic variety and geographical origin of the oils were certified by the suppliers. The oil samples were 1:50 (v/v) diluted with an 85:15 PrOH/MeOH (v/v) mixture containing 40 mM KOH. This mixture was also used to rinse and clean the

Table 1
Genetic variety, number of samples, geographical origin and brand of the extra virgin olive oil samples

Genetic variety	No. of samples	Geographical origin	Brand
Arbequina	2	Aguadulce (Sevilla)	Carbonell
	2	Vila Franca del Penedés (Barcelona)	Torrereal
	2	Les Garrigues (Lleida)	Oleastrum
	1	Estepa (Sevilla) + La Roda de Andalucía (Sevilla)	Coosur
	1	Antequera (Málaga)	Grupo Hojiblanca
	2	Huelva + Zaragoza + Palma del Río (Córdoba)	Borges
	1	Les Garrigues (Lleida)	Romanico
	1	La Puebla Nueva (Toledo)	Valderrama
	1	Sarroca de Lleida (Lleida)	Veá
	1	Mallorca	Aubocassa
	1	La Rioja	Rihuelo
Hojiblanca	2	Estepa (Sevilla)	Carbonell
	2	Luque (Córdoba)	Coosur
	3	Puente Genil (Córdoba)	Borges
	5	Fuente de Piedra (Málaga)	Grupo Hojiblanca
	1	Santaella (Córdoba)	Columela
Picual	2	Martos (Jaén)	Carbonell
	2	Villanueva del Arzobispo (Jaén) + Porcuna (Jaén)	Coosur
	1	Quesada (Jaén)	Borges
	1	Montoso (Córdoba)	Grupo Hojiblanca
		Tabernas (Almería)	Castillo de Tabernas
		Canena (Jaén)	Castillo de Canena

Table 2
$[M–H]^-$ peaks of the selected fatty acids and phenolic compounds

Compound (acronym)	m/z
Myristic acid (C14:0)	227
Palmitoleic acid (C16:1)	253
Palmitic acid (C16:0)	255
Linolenic acid (C18:3)	277
Linoleic acid (C18:2)	279
Oleic acid (C18:1)	281
Stearic acid (C18:0)	283
Tyrosol	137
p-Hydroxybenzoic acid	137
Cinnamic acid	147
p-Hydroxyphenylacetic acid	151
p-Anisic acid	151
Hydroxytyrosol	153
Gentisic acid	153
Protocatechuic acid	153
Coumaric acid	163
Vanillic acid	167
Gallic acid	169
Caffeic acid	179
Homovanillic acid	181
Ferulic acid	193
Syringic acid	197
Sinapic acid	223
Elenolic acid	241
Dialdehydic form of deacetoxy ligstroside	303
Deacetoxy ligstroside aglycone	303
Dialdehydic form of deacetoxy oleuropein	319
Deacetoxy oleuropein aglycone	319
Pinoresinol	357
Dialdehydic form of ligstroside	361
Ligstroside aglycone	361
Dialdehydic form of oleuropein	377
Oleuropein aglycone	377
10-Hydroxyoleuropein aglycone	393
1-Acetoxypinoresinol	415

capillary at 10 bar between successive infusions. Rinsing for 5 min was enough to achieve a satisfactory reduction of the background noise and reproducible oil profiles. The LDA models were constructed using the SPSS software (v. 12.0.1, SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Optimization of the ITMS working conditions

To optimize the ITMS working conditions, a sample of Hojiblanca oil was used.

The peaks of seven fatty acids and those of 28 phenolic compounds selected according to Ríos et al. (2005) and Tripoli et al. (2005) were used as variables. The selected compounds, and the m/z values of the corresponding $[M–H]^-$ peaks, are indicated in Table 2. Owing to the coincidence of the m/z values of several phenolic compounds, these were jointly measured; then, a total of 20 peaks were obtained to be used as variables. Most peaks showed a slight intensity increase when the drying gas temperature was increased from 150 to 200 °C. No further improvement was observed at higher temperatures up to 350 °C, then 200 °C was selected. Most peak intensities also improved by increasing the nebulizer pressure from 5 to 25 psi, but a plateau was reached at higher pressures; then, 25 psi was selected. The influence of the drying gas flow was negligible, thus 5 L min^{-1} was selected. The stability parameter was set at 75%, since no further sensitivity improvement was observed at lower values.

3.2. Construction of the data matrices

The peak profiles of oils of the three genetic varieties are compared in Fig. 1. In this Figure, and in order to make comparison easier, the spectra were standardized by dividing each peak abundance by the abundance of C16:0 peak; further, the peak of C18:1 (oleic acid), which is the most intense signal in all the genetic varieties, was tailored at 1.2. In this way, the differences between the fatty acid profiles of the three genetic varieties were enhanced. The C18:1 peak intensities, which cannot be appreciated in the Figure due to the cut off, followed the decreasing order: Hojiblanca > Picual > Arbequina. In comparison to oleic acid, the signals of palmitic, linoleic and stearic acids showed intermediate intensities. The stearic acid peak (C18:0) was

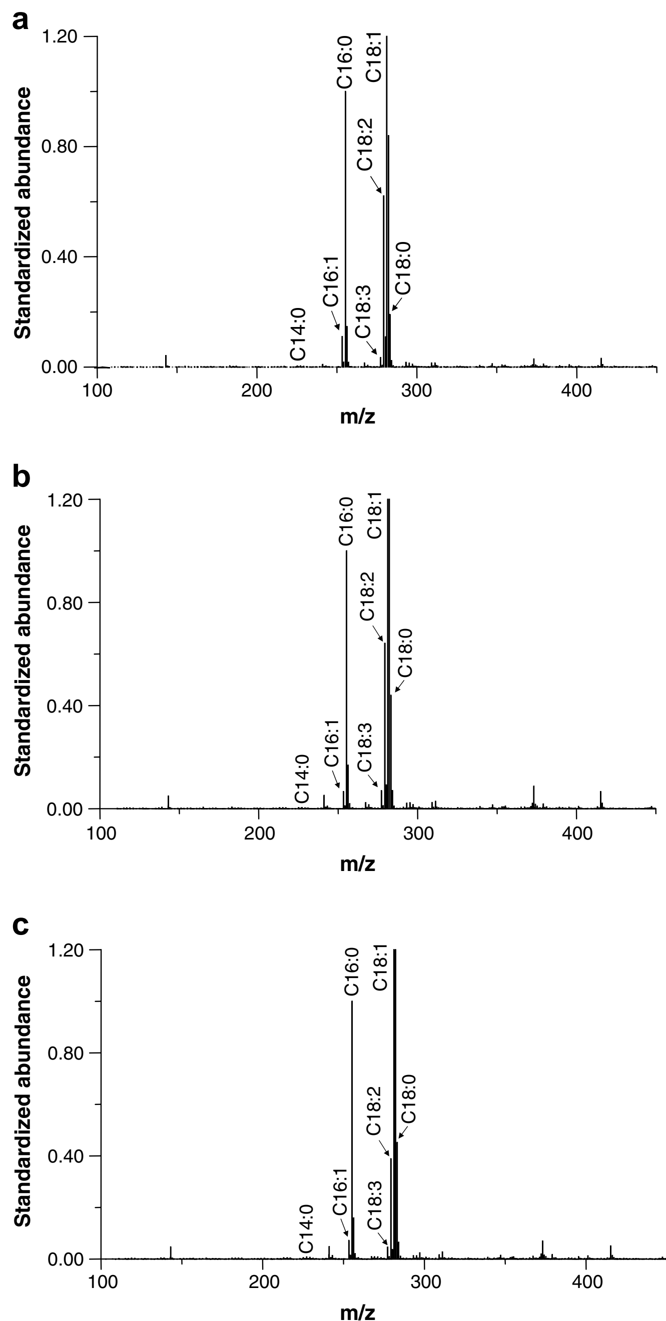

Fig. 1. Standardized mass spectra of olive oils of the three genetic varieties: (a) Arbequina, (b) Hojiblanca and (c) Picual. The intensity of all peaks was divided by the intensity of the C16:0 peak; also the C18:1 peak was tailored at 1.2.

lower for Arbequina than for the Hojiblanca and Picual oils, and the intensities of the linoleic acid peak (C18:2) showed the decreasing order: Arbequina > Hojiblanca > Picual. The peaks of many phenolic compounds were also identified in the MS spectra; however, the intensity of these peaks was several orders of magnitude lower than that of the fatty acids. The spectrum of a Picual oil, with an expanded vertical axis, is shown in Fig. 2. Similar spectra, showing small differences among the peak profiles, were obtained for the Arbequina and Hojiblanca oils.

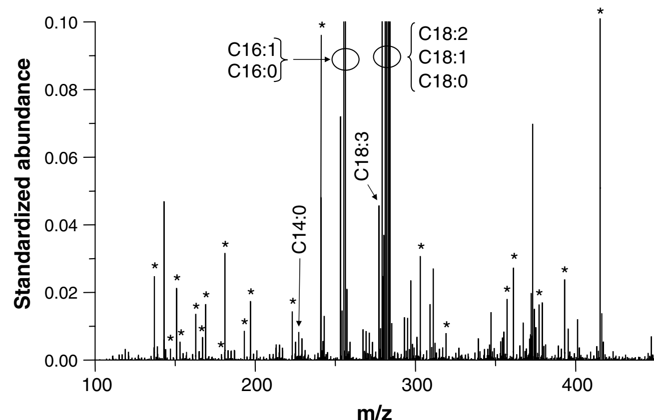

Fig. 2. Standardized mass spectrum of a Picual olive oil sample with the vertical axis tailored at 0.1. The peaks of the phenolic compounds of Table 2 are indicated with an asterisk.

In order to reduce the variability associated with the infusion in the mass spectrometer, and consequently minimize the influence of sources of variance non-associated to the origin and history of the oils, normalized ion abundances were employed for data analysis. Owing to the large differences between the peak intensities, the peaks were divided in two groups, fatty acids and phenolic compounds, and two normalization procedures were independently applied to each group. First, for each spectrum, the peak abundance of each fatty acid was divided by the sum of the peak abundances of the seven fatty acids, and the peak abundance of each phenolic compound was divided by the sum of the 20 peak abundances of the phenolic compounds (normalization procedure A). Second, the peak abundance of each fatty acid was divided by each one of the peak abundances of the other six fatty acids, and the peak abundance of each phenolic compound was divided by each one of the abundances of the other 19 peaks of the other phenolic compounds (normalization procedure B). To avoid perfectly correlated variables, each pair of peak abundances was divided only once. Then, for the fatty acids, 7 and 21 normalized variables were obtained by normalization procedures A and B, respectively. Similarly, for the phenolic compounds, 20 and 190 normalized variables were, respectively obtained.

3.3. Prediction of the genetic variety by LDA

LDA, a supervised classificatory technique, is widely recognized as an excellent tool to obtain vectors showing the maximal resolution between categories. In LDA, vectors minimizing the Wilks' lambda, λ_w, are obtained. This parameter is defined as:

$$\lambda_w = S_{intra}/(S_{intra} + S_{inter})$$

where S_{intra} is the sum of squares of data points belonging to the same category, and $S_{intra} + S_{inter}$ is the total sum of squares (Vandeginste et al., 1998). Values of λ_w approach-

ing zero are obtained when all the categories are well resolved, whereas overlapped categories make λ_w to approach one. Up to N-1 discriminant vectors are constructed by using LDA, being N the lowest value of either the number of predictors or the number of categories. The 38 samples of the monovarietal oils of Table 1 were used to construct LDA models capable of classifying olive oil samples according to their genetic variety.

Selection of the variables during model construction was made with the stepwise algorithm of SPSS. Using this algorithm, the predictor causing the most significant decrease of λ_w is first introduced in the model, this criterion being successively applied to all the predictors. Acceptation of the predictors is performed with an *F*-test. In order to avoid the introduction of variables with reduced discriminant capability, an entrance threshold, F_{in}, is adopted. However, the entrance of a new predictor modifies the significance of the predictors which are already present in the model. Then, in the stepwise algorithm, a rejection threshold, F_{out}, is used to resolve if a predictor should be removed from the model. The process terminates when there are no predictors entering or being eliminated from the model.

To construct the data matrices, each oil sample was injected in two different days, and each day four replicates were accomplished. Two matrices containing 304 injections, and a total of 27 or 211 predictors (according to normalization procedures A and B, respectively) were constructed. A response column, containing the three categories corresponding to the three genetic varieties, was added to the matrices. These matrices were used both to construct LDA training matrices and to provide evaluation sets. Only the means of the replicates of the samples were included in the training matrices; in this way, the internal dispersion of the categories was reduced, which was important to also reduce the number of variables selected by the stepwise algorithm during model construction. In the evaluation set, all the individual injections of the samples were included.

Initially, the capability for the classification of the studied olive oils according to their genetic variety, using fatty acids or phenolic compounds, was tested. Both normalization procedures were tried. Normalization procedure B, which led to a better separation of the categories, was selected in further studies. Then, an LDA model constructed exclusively with the fatty acids ratios showed a clear separation between the three genetic varieties ($\lambda_w = 0.396$). Using the SPSS default probability values of $F_{in} = 0.05$ and $F_{out} = 0.10$, the model was constructed with 7 variables and mainly with the variables 279/283 and 279/281. All samples were correctly classified. On the other hand, the phenolic compounds were used to construct another LDA model. In this case, 22 variables were selected ($\lambda_w = 0.236$), but with an apparent lower separation between categories. The variables showing higher weight in the model construction were 303/241, 303/193 and 393/241. Then, both variables sets were jointly used to construct another LDA model. A model with 30 predictors was obtained. Thus, in order to reduce the entrance of predictors in the model, the probability values $F_{in} = 0.01$ and $F_{out} = 0.20$ were used. Now, only 11 predictors were included in the model. An excellent resolution between the three categories was obtained ($\lambda_w = 0.237$). A score plot on the plane of the two discriminant functions is shown in Fig. 3. The predictors selected by the SPSS stepwise algorithm, and the corresponding standardized coefficients of the model, which indicate the discriminant capabilities of the predictors, are given in Table 3. The model was used to classify the samples of the evaluation set. Using a 95% probability, all the objects were correctly assigned; thus, the prediction capability was 100%.

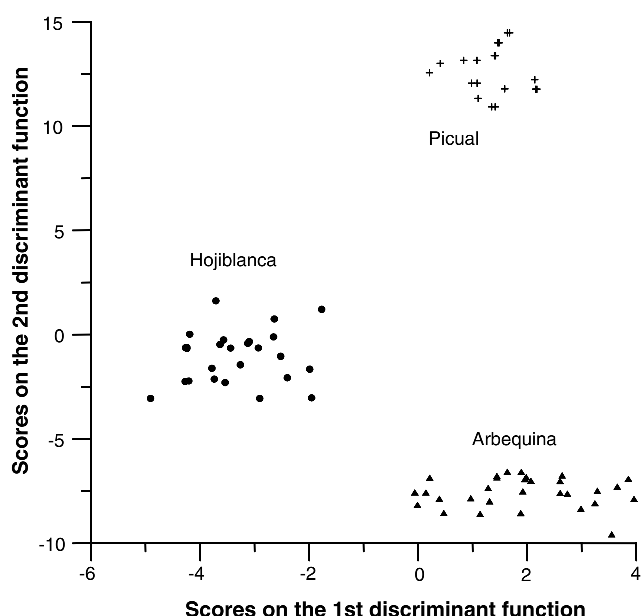

Fig. 3. Score plot on the plane of the two LDA discriminant functions obtained to predict olive oil varieties after data normalization by procedure B.

Table 3
Predictors selected and corresponding standardized coefficients of the LDA model constructed to predict the genetic variety of extra virgin olive oils

Compounds	Predictors[a]	f_1	f_2
Fatty acids	227/279	−0.4	2.78
	227/281	0.63	−1.98
	255/279	−0.32	0.45
	277/279	−1.21	0.16
	279/281	0.35	0.7
	281/283	0.81	1.08
Phenolic compounds	193/167	0.83	0.22
	223/181	0.67	−0.02
	303/197	−0.27	−0.82
	415/197	0.87	0.52
	361/223	−0.28	−0.7

[a] *m*/*z* values of the ratios of abundances of peak pairs.

4. Conclusions

With the jointly use of the peaks of both fatty acids and phenolic compounds, direct infusion MS spectra of monovarietal extra virgin olive oils provide the necessary information to predict the genetic variety with a high reliability. For this purpose, the use of LDA models constructed using the intensity ratios of peak pairs as predictor is recommended. The MS intensities of the free fatty acids and many phenolic compounds can be measured after a simple dilution of the oil samples with a miscible alkaline solvent mixture. In this way, the conventional laborious extraction protocols commonly reported for phenolic compounds are avoided, being only necessary to rinse the spectrometer for 5 min between successive infusions to achieve low backgrounds and reproducible oil profiles. Thus, an extremely quick and simple procedure, which can be easily automatized, has been described.

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