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High performance liquid chromatography–mass spectrometry based chemometric characterization of olive oils

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Abstract

In this study the effective discrimination of extra virgin olive oils is described using HPLC–MS, combined with chemometric evaluation. The presented method is simple since the diluted oil sample is directly injected into the system, without any preliminary chemical derivatization or purification step. Separation of diacylglycerols, triacylglycerols and sterols occurs within 20 min and is achieved using an octadecyl-silica column. Detection is performed by positive APCI mass spectrometry which provided sensitivity to detect over 50 compounds in the sample. After extraction of data, stepwise discriminant function analysis is used to select the variables with the highest discriminative power. These variables are used to perform linear discriminant analysis and classify/predict the samples. One-hundred per cent classification and 99% prediction rate was achieved for olive oils obtained from Nocellara, Biancolilla and Cerausola cultivars. Reliability of prediction was tested by cross validation.

Keywords: HPLC–MS; Olive oil; Oil cultivar; Linear discriminant analysis; Cross validation

1. Introduction

Olive oil received prominent attention in the last decades, as it is a major constituent in Mediterranean diet [1]. It enjoys the protection of several regulations and trademarks stated by the International Olive Oil Council [2] and the European Commission [3,4]. Its constituents exhibit protective effect against different types of cancer [5–7] and significantly reduce mortality caused by heart disease [6]. Resulting from its healthy effect, olive oil is more expensive than other types of oils, so it is a target for adulteration [8,9]. Since the chemical composition reflects the authenticity of the oil [10–18], development of sensitive and selective methods for olive oil analysis is desirable.

The major components of olive oil are triacylglycerols (TAGs) amounting to about 95–98% [19]. Minor components include diacylglycerols (DAGs), wax esters, free fatty acids, triterpenic alcohols, hydrocarbons, sterols, phenols, flavonoids, pigments, tocopherols and volatile compounds [20]. Several analytical approaches have been reviewed for the characterization of these constituents [19,21]. Characterization of the TAG fraction in vegetable oils is usually performed by gas chromatography (GC) [20] coupled with flame ionization detection (FID) [13,22–25] or electron ionization mass spectrometry (EI-MS) after preliminary hydrolysis of TAGs and methylation of fatty acids [13,20,22–26]. Alternatively, TAGs can be analyzed by reversed phase high performance liquid chromatography (RP-HPLC) coupled with refractive index (RI) [26] or mass spectrometric detection [25], high temperature GC [27] or nuclear magnetic resonance (NMR) [23,25,28,29]. The phenolic fraction is usually determined by ultra violet–visible spectrophotometry

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(UV–vis) [13,30] after treatment with sodium-molibdate solution, by gas chromatography–mass spectrometry (GC–MS) [18] as trimethylsilyl derivatives, by HPLC-APCI-MS [31] or by RP-HPLC-UV [18,30] after purification using solid phase extraction. Recently HPLC based methods were presented by Bendini et al. [32] and Rotondi et al. [13] for determination of phenols. A promising method was described by Bonoli et al. [33,34] for semi quantitative analysis of phenols by capillary zone electrophoresis. The unsaponifiable components of oil are usually fractioned prior to analysis using thin-layer chromatography (TLC) [21], and are analyzed as trimethylsilyl [20,21,24,35] or methyl [19] derivatives by GC. Several HPLC and GC based methods were reported for detection of sterols and tocopherols in plant oils [17,19,20,22,36,37]. Carotenes can be analyzed by HPLC [21] or TLC with colorimetric detection [17]. For the analysis of sterol oxidation products both HPLC and GC based methods were reported [21] after their saponification and solid phase extraction (SPE) [21]. Volatile fraction of plant oils can be trapped and analyzed using GC–FID [17,19,38,39], GC–MS [38,39] and also headspace mass spectrometry [9,24]. Recently electrospray ionization mass spectrometry (ESI-MS) and Fourier transform ion cyclotron resonance (FT-ICR) MS was demonstrated to analyze plant oils [40,41]. Matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) and RP-HPLC separation using acetone/acetonitrile, propionitrile or water/acetonitrile/2-propanol solvent mixtures coupled with APCI-MS were also successfully used for characterization of different plant oils [35,42,43].

Distinction between different vegetable oils was reported using GC–MS, GC–FID, HPLC-UV [20], HPLC–MS [42,43], NMR [28], FT-ICR [40] and infrared spectroscopy (IR) [8] techniques in combination with chemometric data evaluation. Discrimination of oils originated from different plant sources is relatively easy, as the composition difference between oils obtained from different vegetables is usually large [20,43]. Distinction between mixed and pure oils is also possible [20,35] based on their unsaponifiable minor lipid composition, however hyphenated techniques are required to evince the differences. Distinction between different cultivars, detection of inadequate harvesting, storage, processing parameters and characterization of biological activity requires measurement of a wide variety of constituents and detection of fine differences among the samples. This challenge usually requires the combined application of several analytical techniques [13,23,26,44–46] often involving chemical derivatization and the results even in that case may not be unequivocal [26,29,39,44–47].

In this study, we aimed to demonstrate the usefulness of a recently developed fast HPLC–MS approach for the efficient characterization of very similar extra virgin olive oils obtained from different cultivars. The presented method does not require any previous fractionation or purification step. Data obtained by the presented method were evaluated using chemometric approaches such as PCA, discriminant function analysis (DFA) and LDA.

2. Experimental

2.1. Chemicals

HPLC grade methanol, water, *n*-hexane, acetic acid and sitosterol were purchased from Sigma-Aldrich GmbH (Steinheim, Germany).

2.2. Samples

Olives collected for oil production were hand harvested, avoiding foliage and wood contamination. The harvesting was conducted in the months of November and December, 2003, analysis was performed in January 2004. Olives were processed in different plants with the same technology (olive washing, cold mill, cold (about 40 °C) mixing with water and finally cold centrifugal separation). Extra virgin oil samples were collected immediately after centrifugal separation production at room temperature and stored in PTFE sealed vials. Seventeen oil samples were studied. With three exceptions, all samples originated from Sicily (one sample originated from Umbria, one from Toscana and one from Greece). Before analysis oils were diluted to 0.01% in methanol, and studied without any sample purification or derivatization.

2.3. HPLC instrumentation and conditions

The HPLC system used consisted of a binary solvent delivery system (two isocratic Perkin-Elmer Series 200 LC Pumps connected via a Scientific Systems, Inc. high pressure mixer device), and a Perkin-Elmer Series 200 Autosampler (Norwalk, CT, USA) equipped with a 10 μL sample loop. A Purospher Star RP-18 e (55 mm × 2 mm I.D., particle size 3 μm) column was used, purchased from MERCK KGaA (Darmstadt, Germany). Experiments were carried out at room temperature, no column thermostat was applied. All solvents were degassed in an ultrasonic bath prior to use.

The following HPLC elution was applied according to Table 1: column was conditioned by pumping methanol/water 90/10 (v/v%) containing 0.2% acetic acid (solvent A) through the column for ten minutes using a flow-rate of 200 μL/min. Then 10 μL sample was injected. A stepwise gradient

Table 1
Elution scheme used for HPLC separation of TAG and sterol components of extra virgin olive oil

Time [min]	Mobile phase		Flow rate [μL/min]	Elution curve
	A [%]	B [%]		
−10	100	0	200	–
0	100	0	200	–
1	100	0	200	Immediate switch
10	0	100	200	linear
11	0	100	700	–
20	0	100	700	–

program was used switching from solvent A to solvent B (methanol/*n*-hexane 90/10 (v/v%), also acidified by 0.2% acetic acid) 1 min after sample injection. Flow rate was 200 μL/min. From 10 to 11 min flow rate was increased to 700 μL/min and then was kept constant until 20 min. After each analysis the column was re-equilibrated with mobile phase A at 200 μL/min for 10 min. Note that this flow gradient was obtained at a relatively low pressure (ca. 80 bar) so gradients with higher flow rates may easily be applied if needed.

2.4. Mass spectrometry

A Perkin-Elmer SCIEX API 2000 triple quadrupole mass spectrometer (Toronto, Canada) was interfaced to the HPLC system using an atmospheric pressure chemical ionization (APCI) source in positive mode. APCI corona probe current was 4 μA. Vaporizer temperature was 500 °C. High-purity nitrogen was used as nebulizer (60 psi), auxiliary (60 psi) and curtain (40 psi) gases. Mass spectra were acquired in the m/z = 100–1000 Th range at an orifice voltage of −86 V. The quadrupole filter was operated in unit resolution mode, scan time was 2 s, step size was 0.2 Th. Using methanol/*n*-hexane solvent mixture may be regarded as an explosive hazard using high temperature APCI. However, note that a high flow of inert (nitrogen) nebulizer and auxiliary gas is added to the solvent vapor in APCI which cools the solvent vapor and reduces the risk of ignition. Addition of methylene chloride to the eluent can further minimize this risk.

2.5. Data analysis

HPLC–MS data were acquired using the program Analyst 1.4 (Applied Biosystems, MDS Sciex). Peak intensities of 52 compounds (variables) in the case of the 17 samples were put into a tabular form using Excel XP (Microsoft Corporation). In the case of each sample the peak intensities were normalized by dividing them with the molecular ion intensity of the most abundant component (triacylglycerol abbreviated as OOO, observed at m/z = 885.6 Th peak at 13.4 min retention time). This way the TAG containing three oleic acids served as an internal standard and errors caused by dilution and sampling could be minimized. All chemometric calculations were conducted on a Pentium IV personal computer using the Multivariate Exploratory Techniques functions of the Statistica 6.0 (StatSoft Inc., Tulsa, OK, USA) program running under Windows XP Professional operating system.

3. Results and discussion

Extra virgin olive oil samples were diluted and directly injected onto an HPLC column without any preliminary chemical derivatization or purification. This simple sample preparation technique is advantageous with respect to the other methods reported in the literature (see Section 1), since majority of these does involve chemical derivatization and/or purification step. Separation and detection was achieved using a recently described, efficient RP-HPLC–MS method [48], detailed in the Section 2.

A typical total ion chromatogram (TIC) of an extra virgin olive oil sample is shown in Fig. 1. Separation time is relatively short (less than 20 min) and this fact in combination with the simple sample preparation makes this method feasible for screening purposes. The main peaks in the chromatogram appear in the 12–15 min retention time window and are due to various TAGs. Under the applied circumstances

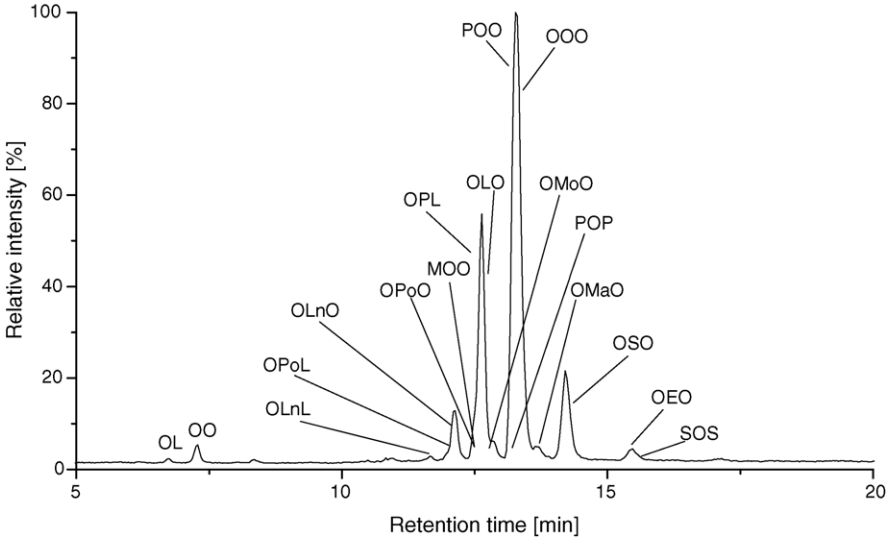

Fig. 1. HPLC–MS total ion current obtained from diluted extra virgin olive oil sample. Most of the major peaks correspond to TAGs. Structure is indicated by fatty acid composition (e.g. OOO for trioleicglycerol) using the following abbreviations: M-myristic acid, Po-palmitoleic acid, P-palmitic acid, S-steraic acid, O-oleic acid, L-linoleic acid, Ln-Linolenic acid, E-eicosanoic acid, G-gadoleic acid, Ma-margaric acid, Mo-heptadecenoic acid.

Table 2
The major DAGs and TAGs detected in diluted extra virgin olive oil using HPLC-APCI-MS in positive mode

Number	Name	Protonated nominal molecular mass [Da]	Fragment 1 [Th]	Fragment 2 [Th]	Fragment 3 [Th]	Retention time [min]
1	OL	619	601	337	339	6.7
2	OO	621	603	339	–	7.3
3	OS	623	339	341	–	8.05
4	PLnP	829	573	551	–	12.1
5	MOO	831	549	603	–	12.5
6	POP	833	551	577	–	13.2
7	OPoL	855	573	575	601	12.0
8	OPoO	857	575	603	–	12.5
9	OPL	857	577	575	601	12.6
10	POO	859	603	577	–	13.3
11	OMoO	871	589	563	–	12.9
12	OMaO	873	591	603	–	13.7
13	OLnL	879	597	599	601	11.7
14	OLnO	881	599	603	–	12.1
15	OLO	883	601	603	–	12.6
16	OOO	885	603	–	–	13.3
17	OSO	887	605	603	–	14.2
18	SOS	889	605	607	–	15.5
19	OGO	913	631	603	–	14.2
20	OEO	915	633	603	–	15.4

Structure is indicated by fatty acid composition (e.g. OOO for trioleicglycerol) using the following abbreviations: M-myristic acid, Po-palmitoleic acid, P-palmitic acid, S-steraic acid, O-oleic acid, L-linoleic acid, Ln-Linolenic acid, E-eicosanoic acid, G-gadoleic acid, Ma-margaric acid, Mo-heptadecenoic acid.

these compounds yield abundant protonated $[M + H]^+$ pseudo molecular ions and often also fragments of significant intensity. The latter provide additional information beside the molecular mass, thus the structure of TAGs can be identified [49,50]. The TAGs detected this way in olive oil samples are given in Table 2 and are in good agreement with those listed in the literature [35]. The TAG content is a prime characteristic of olive oils and is well suited to identify extra virgin olive oil in confront to other vegetable oils [20,35,51]. Even different grade olive oils (such as extra virgin, virgin, refined virgin, etc.) can be routinely distinguished based on TAG content [52]. The TIC traces of the various extra virgin oil samples were very similar, close to that shown in Fig. 1. This suggests that distinction among these extra virgin olive oils based on the major TAG content alone is not expedient.

Beside the main components which are shown in the TIC trace, over thirty other compounds were identified in the same chromatogram by plotting various ion chromatograms. This observation suggests that the present method alone can be used for the detection of various compound classes at the same time, while in the literature usually combined application of different methods is required to achieve this (see

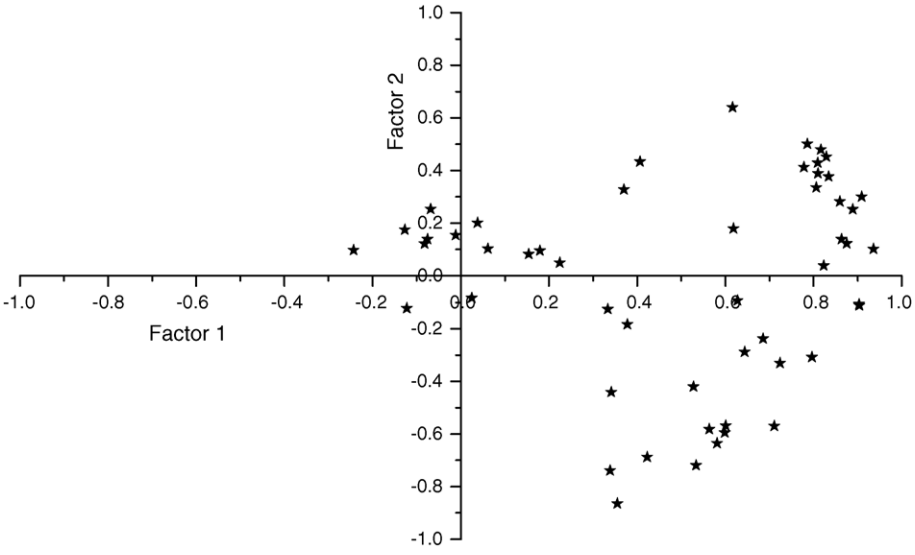

Fig. 2. Results of PCA analysis of the 52 variables extracted from the measurements of extra virgin olive oils. The positions of the variables in the plane of the first two principal factors represent their similarity.

Section 1). The intensity of the observed trace-compounds showed significant differences among the studied samples. This suggests that the applied method is sensitive [48] and is well suited for efficient discrimination of extra virgin olive oils. The minor TAGs and DAGs useful for classification (see later) were identified based on their protonated molecular ion, expected retention time and specific fragments. Presence of sitosterol was confirmed by comparing its mass spectrum and retention time with the data obtained from the standard compound. Presence of gamma-tocotrienol is also suggested on the base of the protonated molecular ion. For characterizing olive oils repeatability is an important issue. Repeatability of the method was checked by analyzing the same sample five times on one day and four times on different days. Intraday RSD of retention times of the five major TAGs was 0.8%, RSD of signal heights was 6.4%. Interday RSD of retention times of the five major TAGs was 1%, RSD of signal heights was 10%.

For classification of olive oils it is expedient to use mathematical/statistical procedures, as frequently done [9,23–26,35,44,46,47,53]. In the present study first, PCA was applied to get an overall impression about the correlation among the large number of variables (peak intensities of the measured compounds). In simple cases, clustering can be observed and redundant variables can be identified. However, in our case no clustering was observed among the variables,

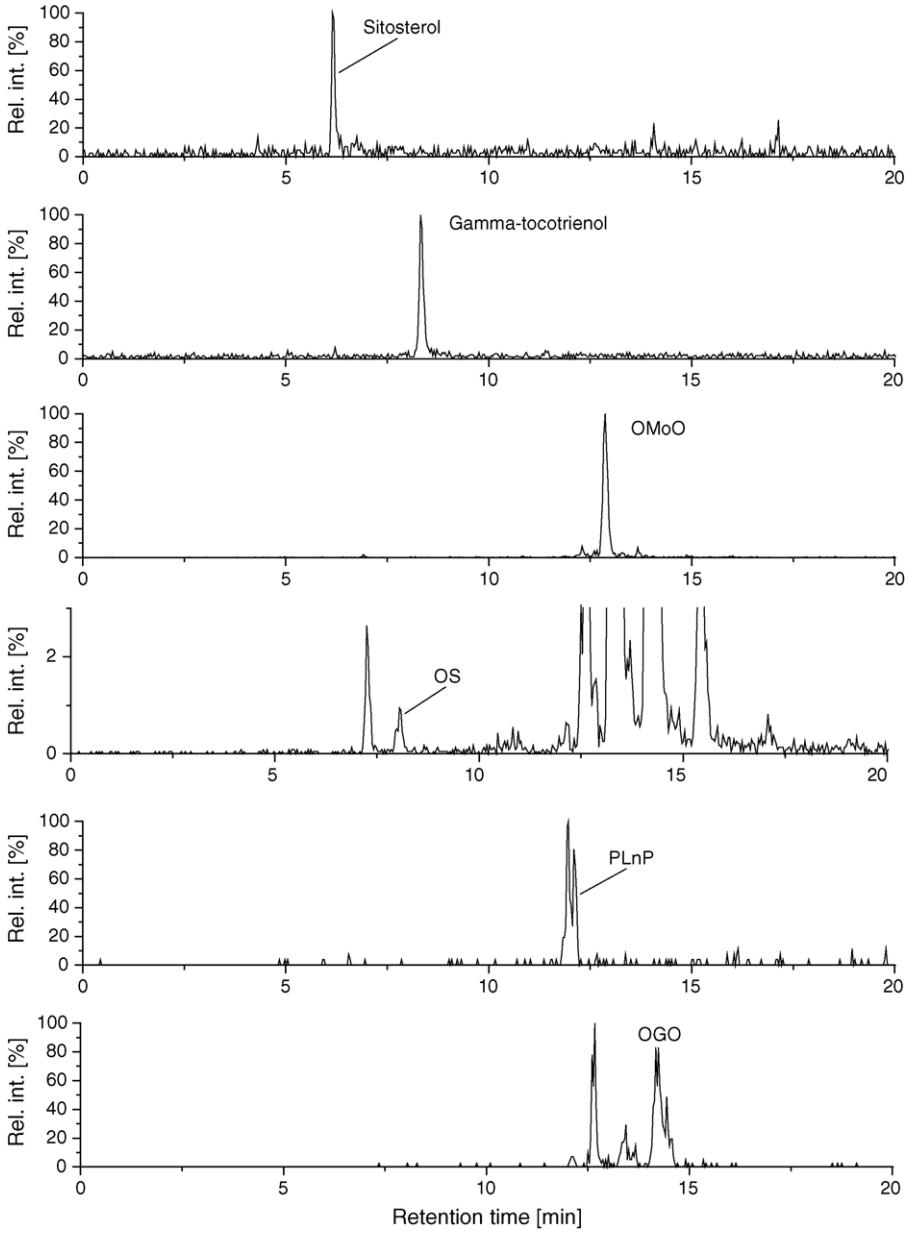

Fig. 3. Ion chromatograms of the six most discriminating variables selected by discriminant function analysis. Note that most of these compounds are minor or trace constituents of the extra virgin olive oil.

Table 3
List of the most discriminating variables with the *F* values, corresponding retention times, recorded ion channels, suggested molecular masses and compounds

	Calculated *F* value	Calculated *p* value	*m/z* used for characterization [Th]	Retention time [min]	Nominal MW [amu]	Proposed structure
1	171233	0.000006	605.4	8.19	622	OS
2	29609	0.000034	913.4	14.30	912	OLA
3	11832	0.000085	397.2	6.28	414	Sitosterol
4	3121	0.000320	411.4	8.49	410	Gamma tocotrienol
5	414	0.002408	829.6	12.03	828	PLnP
6	325	0.003065	589.3	12.93	870	OMoO
7	73	0.013555	883.6	10.40	–	–

(–): Unknown compound.

as shown in Fig. 2. Since classification based on PCA is unfeasible, in the next step LDA was performed. In LDA the number of variables should be significantly less than that of the samples, thus the size of the dataset was reduced to keep only the most important variables.

This was performed using forward stepwise discriminant function analysis (DFA). The statistical significance (discriminating power) of variables can be characterized by the *F* value (ratio of between-groups variance to within-group variance). It is a measure of the contribution of a variable for prediction of group membership. The higher the *F* value, the better the discriminating power of the variable. Another parameter, *p* (representing the probability of a wrong classification, which is inversely proportional to the *F* value) is also used to characterize variables and guide the variable reduction process. Samples were divided into groups (corresponding to Nocellara, Cerasuola and Biancolilla cultivars) and forward stepwise DFA was performed as described above. The most discriminating variables were selected based on the $p < 0.05$ criterion, and are listed in Table 3. From these seven variables only those six with suggested identity were used for further calculations, as from the chemical standpoint it is not straightforward to use a variable with unknown identity. Ion chromatograms of the corresponding *m/z* channels are shown in Fig. 3.

Classification of the samples was performed by LDA using only the selected six variables. Using such a small number of variables avoids problems with over-fitting and makes the developed method suitable for prediction purposes as well. The obtained results are shown in Fig. 4, which depicts very tight clustering of the Nocellara, Biancolilla and Cerasuola cultivars, obtaining 100% classification rate. The "tightness" of clustering may be characterized by squared Mahalanobis distances, given in Table 4. In this case these numbers represent the distance of a given sample from the middle of a certain cultivar. If this number is small, then it is highly probably that the sample belongs to that cultivar. In addition to "pure" samples, two extra virgin oil samples produced from a mixture of Nocellara and Biancolilla olives were classified as unknown samples and evaluated by the same model. The results are very encouraging, shown in Fig. 4 placing these mixed samples correctly between the Nocellara and Biancolilla cultivars.

To check the usefulness of the method for prediction purposes cross validation was performed. Using the so-called leave-*n*-out method, all olive oil samples but one were used for calculating discriminant functions, and then the omitted sample was used as an unknown, and classified. The procedure was repeatedly performed for all samples. Classification rate was 100%, prediction rate was 99%. This suggests that

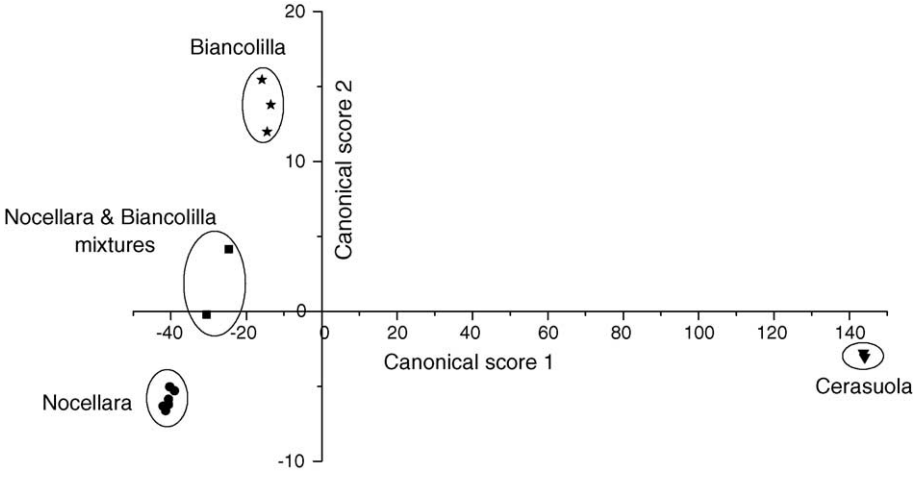

Fig. 4. Classification results of linear discriminant analysis using the six selected variables. The ion chromatograms of the six ions corresponding to these variables are shown in Fig. 3.

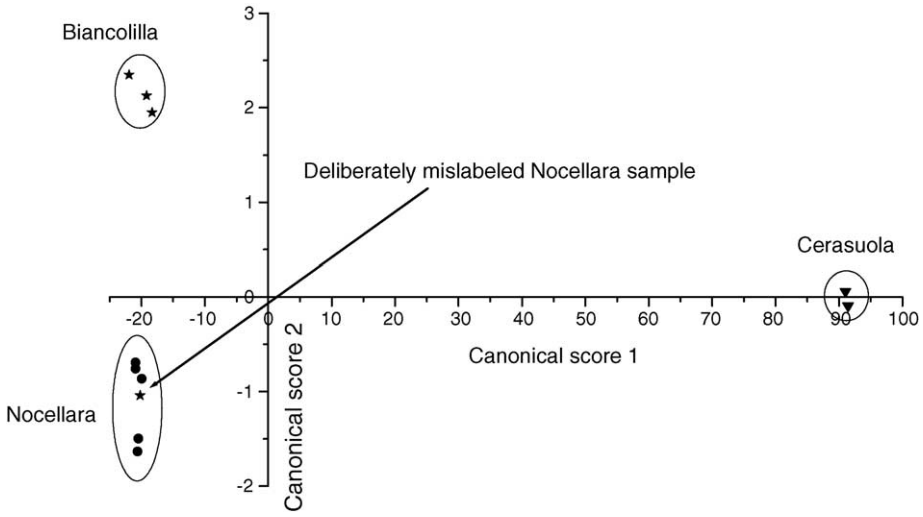

Fig. 5. Two dimensional score plot of linear discriminant analysis using the six selected variables and after deliberately putting one Nocellara sample to the Biancolilla cultivar. The ion chromatograms of the six ions corresponding to these variables are shown in Fig. 3.

the presented method may be a potential choice for checking olive oil quality, origin and adulteration. The reliability of the prediction based on the selected variables was further tested by deliberately labeling one sample erroneously into a wrong cultivar and then performing classification. The incorrectly labeled sample was always classified into the right cultivar by the procedure. A typical example is shown in Fig. 5, where even though one Nocellara sample was deliberately mislabeled and put into the Biancolilla cultivar, the calculation classified it (correctly) into the middle of the Nocellara cultivar. This supports further the strong predictive potential of the model and suggests that the chosen variables represent very well the difference among the cultivars. The effective discrimination achieved among extra virgin olive oils, based on their sterol and minor TAG composition, could be a helpful tool for studying the actual influence of olive origin on the biological activity of extra virgin olive oils.

Table 4
Squared Mahalanobis distances, representing the distance of a given sample from the middle of a certain cultivar

Sample name	Mahalanobis distances		
	Nocellara	Biancolilla	Cerasuola
Nocellara1	5	1159	34583
Nocellara2	0	1076	34056
Nocellara3	4	962	33478
Nocellara4	2	1013	33926
Nocellara5	7	1069	34060
Nocellara6	6	1140	34362
Biancolilla1	1010	5	25291
Biancolilla2	1073	5	25853
Biancolilla3	1130	5	25055
Cerasuola1	34135	25454	4
Cerasuola2	34020	25343	4
Mixture1	137	449	30418
Mixture2	362	197	28455

4. Conclusions

The present study describes the application of an RP-HPLC–MS method in combination with chemometric evaluation for efficient characterization of extra virgin olive oils. The advantages of the proposed method are (a) simplicity: no derivatization or sample purification is needed; (b) speed: separation occurs within 20 min; (c) robustness: repeatability of retention times is 1%, repeatability of signal heights is 10%; (d) sensitivity: more than 50 compounds were detected in the methanolic solution of olive oil; (e) price: no chemicals or consumables tools are needed for sample preparation, in addition solvent consumption is lower than compared to monolithic columns while providing similar elution times. The main advantages of the combination of chemometric evaluation with HPLC–MS are threefold: first, stepwise discriminant function analysis can be used to select the most discriminative variables detected during the HPLC–MS experiment. Second, application of linear discriminant analysis in combination with HPLC–MS can be used to classify extra virgin olive oil samples, e.g. originating from different cultivars. Third, cross-validation is an invaluable tool to give confidence in the obtained results and to indicate the predictive potential of the HPLC–MS technique developed.

These advantages make this method a practical choice both for research and for quality control purposes.

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