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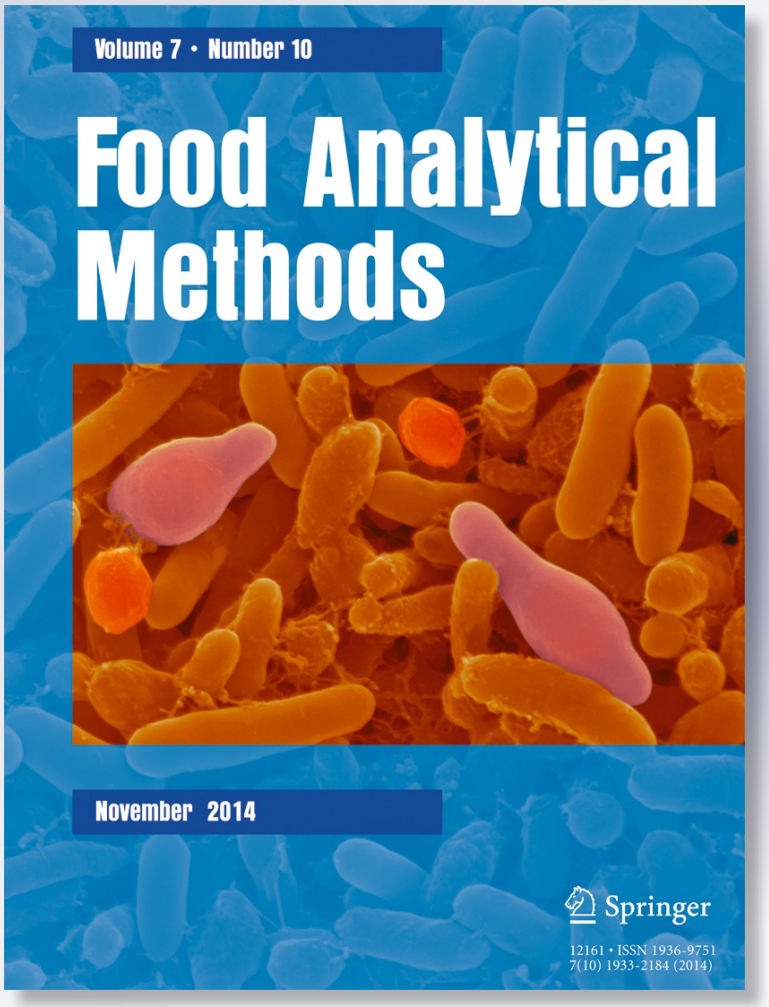
Food Analytical Methods

ISSN 1936-9751
Volume 7
Number 10

Food Anal. Methods (2014) 7:2122-2136
DOI 10.1007/s12161-014-9854-2



Springer

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Volatile Profile Characterization of Extra Virgin Olive Oils from Argentina by HS-SPME/GC-MS and Multivariate Pattern Recognition Tools

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Received: 3 December 2013 / Accepted: 26 March 2014 / Published online: 17 April 2014

Abstract The distinctive aroma of virgin olive oil is attributed to a large number of chemical compounds of different classes such as aldehydes, alcohols, esters, hydrocarbons, ketones, furans, and other volatile compounds that are not yet identified. The aim of the present study lies in the characterization of compound volatile profile of the most representative olive oil cultivars produced in Argentina. The methodology proposed was based on the development of headspace (HS)-SPME/GC-MS with the subsequent analysis of the chemical fingerprints using a discriminant analysis method and a principal component analysis data compression strategy to distinguish olive oils from different varietal origins. Carboxen/polydimethylsiloxane (75 μm) fiber was the most efficient to trap volatile compounds. Optimization of the SPME conditions was also carried out using multivariate methods. To avoid the influence of factors other than the cultivar, olive trees were cultivated under the same agronomic and pedoclimatic conditions; olive fruits were picked at the same stage of ripeness, and their oils were extracted with the same processing system. Volatile fractions from oils of the analyzed cultivars consisted of a complex mixture of more than 40 compounds. Both quantitative and qualitative differences were found among cultivars. The analytical data association with multivariate analysis constitutes a reliable analytical tool with potential ability to discriminate monovarietal extra virgin olive oil from Argentina.

Keywords GC-MS · SPME optimization · Olive oil · Varietal characterization · Multivariate analysis · Argentine

Introduction

Olive trees belong to the *Olea europea* L. family, but among them different cultivars with different characteristics can be found in the world production zones (Vaz-Freire et al. 2009). Owing to its distinctive and peculiar intense taste, extra virgin olive oil (EVOO) obtained from a single kind of variety is highly appreciated. This "monovarietal" hallmark confers an added value to the olive oil so it is protected by regulations to avoid fraud (Ruiz-Samblás et al. 2012). This fraud could be committed not only by mixing EVOO with other cheaper oils but also by mixing EVOO from several varieties, according to the International Olive Oil Council (COI 2013).

Olive growing in Argentina occupies an area of 105,000 ha (Gómez del Campo et al. 2010). In recent years, Argentina has climbed to join the ranks of the leading producers of olive oil and table olives, becoming America's top producer and exporter. The 96 % of the olive oils exported by Argentina are virgin oils, mostly belonging to the extra virgin grade; 3 % are blended and 1 % is refined. This gives Argentina a differential advantage when positioning itself as a world-standard supplier, given the extremely high quality of the oil available for export. The most important cultivars grown in Argentina are Arbequina, Coratina, Picual, Frantoio, Manzanilla, Changlot Real, Barnea, Arauco, Farga, Nevadillo, and Empeltre (F.O.A 2011) Arauco is Argentina's flagship variety, and the country has the largest Arauco acreage in the world. It has been planted in Argentina since the 1500s, when Jesuit missionaries introduced Spanish and Portuguese plants. The oil produced

Electronic supplementary material The online version of this article (doi:10.1007/s12161-014-9854-2) contains supplementary material, which is available to authorized users.

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from Arauco olives is robust, yellow greenish in color, fruity, a bitter taste characteristic, and pungent. The oils produced in Argentina are thought to possess a characteristic aroma profile, but—to our knowledge—there are not data on their composition.

Several series of chemical compounds have been used for traceability and variety characterization, volatile compounds being one of most used markers (Gómez-Rico et al. 2008; Kalua et al. 2005; Lerma-García et al. 2008; Tena et al. 2007; Tura et al. 2008; Vichi et al. 2009). The volatile and fenolic compounds are those responsible for the virgin olive oil (VOO) sensory attributes (Gómez-Rico et al. 2008; Cerretani et al. 2008). Volatiles mainly contribute to the flavor (smell and taste) and are the principal components responsible for the positive fruity attribute, characteristic of the oil from healthy, fresh fruits, both ripe or unripe (Inarejos-García et al. 2010). The volatile fractions of olive oil include aldehydes, alcohols, esters, hydrocarbons, ketones, furans, and other as yet unidentified volatiles (Pouliarekou et al. 2011; Ouni et al. 2011). The volatile compounds derived from the degradation of polyunsaturated fatty acids through a chain of enzymatic reactions known as the lipoxygenase (LOX) pathway taking place during the oil extraction process (Vichi 2010). The major volatile compounds reported in VOO are the C_6 and C_5 aldehydes, alcohols and acetylesters formed from 13-hydroxyperoxides of linoleic and linolenic acids. These compounds usually comprise 60–80 % of the total volatile compounds (TVC; Pouliarekou et al. 2011). The concentration and composition of volatile compounds in VOO are strongly dependent on the nature of the cultivar used, which are also influenced by several other factors like climatic and edaphic conditions, agricultural practices, fruit maturity stage (degree of ripeness), extraction methods, and storage conditions (Gómez-Rico et al. 2008; Kalua et al. 2005; Vichi 2010; Vinha et al. 2005; Ranalli et al. 2001; Nevado et al. 2009; Reboredo-Rodríguez et al. 2012). The influence of the cultivar on the composition of olive oil depends on the enzymatic profiling, which is genetically determined (Tena et al. 2007; Dhifi et al. 2005).

The official procedure established by the International Olive Oil Council (IOOC) for the sensory analysis of olive oil's flavor involves notable problems related to poor repeatability and subjectivity (Pizarro et al. 2011). Therefore, it is reasonable to assume that more precise alternative strategies based on analysis of the volatile compounds are necessary in order to take advantage of the maximum amount of information present in the volatile fraction, to develop efficient classification varietal olive oil. Several analytical techniques have been used for the quantification of volatile compounds, all of them being based on the preconcentration of volatiles prior to the analysis. One reliable alternative is headspace-solid-phase microextraction (HS-SPME) and gas chromatography with mass spectrometry (GC-MS) detection (Tena et al. 2007). SPME is a sensitive, solvent-free, cost-efficient, and fast method, which allows the extraction and the concentration steps to be performed simultaneously (Pizarro et al. 2011; Ribeiro et al. 2008). The main advantage of SPME for the analysis of olive oil volatiles is to minimize sample manipulation and carry out simple and robust procedures. To optimize the efficiency and sensitivity of the method, it is necessary to identify the most suitable SPME conditions, which include, basically, the type of fiber coating, temperature, and time of extraction (Vichi 2010). Fiber is composed of a fused-silica coated with different stationary phases, such as divinylbenzene (DVB), carboxen (CAR), polydimethylsiloxane (PDMS), carbowax (CW), and their combinations. The different coatings interact with analytes through different mechanisms of adsorption and absorption (Kalua et al. 2006). This analytical tool provides a global signal of samples which can be considered a fingerprint of the total volatile profile and can be used to characterization purposes.

The aim of the present study lies in the characterization of the compound volatile profile of olive oil's most representative cultivars produced in Argentina. The methodology proposed was based on the development of HS-SPME/GC-MS with the subsequent analysis of the chemical fingerprints using a linear discriminant analysis (LDA) method and a principal component analysis (PCA) data compression strategy to distinguish olive oils from different varietal origins. Optimization of the extraction conditions was also carried out using multivariate methods; central composite design (CCD) and desirability function. This approach has been used by other researchers for the optimization of analytical methodology (Bertelli et al. 2008; Ribeiro et al. 2010; Mudiam et al. 2013). Multivariate optimization of HS-SPME has been used in olive oil for the analysis of volatile compounds from a specific family (monoterpene and sesquiterpene hydrocarbons, volatile phenols) (Vichi et al. 2006; Vichi et al. 2008), but it has never been used for the whole volatile profile.

Materials and Methods

Reagents and Materials

The SPME tested fibers were Stable Flex™ polydimethylsiloxane/divinylbenezene (PDMS/DVB, 65 μm), polydimethylsiloxane (PDMS, 100 μm), Stable Flex™ carboxen/polydimethylsiloxane (Car/PDMS, 85 μm), and Stable Flex™ divinylbenzene/carboxen/polydimethylsiloxane (Car/PDMS/DVB, 50/30 μm); SPME support and holder manual for SPME were purchased from Supelco (Bellefonte, PA, USA). Before use, fibers were conditioned following the instructions from manufacturers. Magnetic stirrer bar and 20-ml amber glass vials with PTFE-faced silicone septa were supplied by Varian (Lake Forest, CA, USA).

Stirring was made with magnetic stirrer with temperature control Ret Control Visc IKAMAG Safety Control (IKA, Wilmington, NC, USA). Ultrapure water was obtained from a RiO/Elix3-Sinergy185 purification system (Millipore, Sao Pablo, Brazil). The internal standard (IS) 4-methyl-2-pentanone, limonene, hexyl acetate, and linalool were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Methanol chromatographic grade was purchased from Merck (Darmstadt, Germany).

GC-MS Analysis

Identification of compounds was carried out by GS with a Varian CP-3800 gas chromatograph with a Saturn 2200 Ion Trap Mass Spectrometric detector (Varian, Walnut Creek, CA, USA). The system was operated by Saturn GC-MS Workstation software Version 6.41. The column was a Factor Four™ capillary column VF-5MS (50 m×0.25 mm, I.D., 0.25 μm film thickness; Varian, Lake Forest, CA, USA).

The oven temperature program was as follows: 35 °C (8 min), 4 °C min^{-1} to 200 °C (5 min), 50 °C min^{-1} to 250 °C (4 min). The 1079 injector from Varian was equipped with a glass insert for SPME, and the temperature was programmed isothermally at 240 °C. The injection was in split/splitless mode for 5 min, and then the split ratio was 1/100. The carrier gas flow-rate (Helium 6.0, Linde, Buenos Aires, Argentina) was constant at 1 ml min^{-1}. The electron ionization energy was 70 eV. Transfer line and ion trap temperature were 200 °C and manifold temperature was 40 °C. Mass spectrometry acquisition was carried out using the continuous scanning mode (5 μscan s^{-1}) from *m/z* 30 to 300. Qualitative analysis was performed by comparison of mass spectra and retention times with standard compounds and/or by comparison of the mass spectrum with those of reference compounds in the NIST Mass Spectral Search Program (NIST Version 2.0). The relative amounts of volatile compounds were expressed with respect to IS as micrograms per gram of oil.

Olive Oil Samples

Four monovarietal VOOs from Mendoza; Arauco, Arbequina, Empeltre, and Farga, have been studied. The olive samples were obtained from the same orchard located in the "East Area" of Mendoza (Rivadavia). The olive trees were cultivated under identical irrigation system and growing conditions. A randomized complete block (RCB) design was used. The olives were obtained from the four directions of the tree. Fifty kilograms of olives per sample were hand-picked at the same stage of maturity (maturity index around four). The maturity index was calculated according to the method proposed by the International Olive Oil Council, based on evaluation of the olive skin and pulp colors (COI 2013). Olive oil extractions were performed on an industrial scale using an OLIOMIO monoblock (Toscana Enológica Mori Snc, Italy). The malaxation was carried out at 25 °C for 40 min, and the oil separation was obtained using a three-phase decanter. The VOO samples were decanted, filtered, and stored at room temperature in amber glass bottles without HS until the analysis.

The oil quality was assessed by the acidity value, the peroxide number, and absorbance at 270 and 232 nm according to the International Olive Oil Council regulations (COI 2013). Sensory analysis was performed by a panel of six assessor experts.

SPME Conditions

Five grams of each olive oil sample were spiked with an IS (4-methyl-2-pentanone) solution to obtain a final concentration of 0.13 μg/ml. The sealed vial was placed in a thermoblock at 60 °C. After thermal equilibration for 20 min, the SPME needle (CAR/PDMS fiber) was inserted through the septum and the fiber exposed in the HS (3 cm) for 50 min. The sample was agitated at 750 rpm using a magnetic stirrer throughout the equilibration and extraction process. The fiber was withdrawn after 50 min of extraction, and the volatile compounds were thermally desorbed at the GC injection port at 240 °C. All the analyses were performed in triplicate.

Statistical Analysis

EVOO volatile compounds were expressed as micrograms per gram (ppm). All data were reported as the mean±SD for three replications. Comparison of the means was achieved by an analysis of variance (ANOVA). For the optimization of SPME conditions and varietal characterization of samples, data obtained were analyzed using Statgraphics Plus Version 5.0 program (Manugistic Inc., Rockville, MD, USA) and Design Expert Version 7.1.0 (Stat-Ease Inc., Minneapolis, MN, USA) chemometric program.

Results and Discussion

First, the optimal parameters for the extraction of volatile compound for SPME were obtained using univariate and multivariate statistical analysis. Then, the SPME method developed was applied to the varietal characterization of four EVOO.

Classical Quality Parameter Values of Olive Oils

The acidity, expressed as oleic acid, of all analyzed samples was below 0.8, peroxide values ranged from 4.12 to 5.21 meq O_2/kg of oil, extinction coefficients at 232 and 270 nm, respectively, were less than 2.50 and 0.25. The analytical methods used for the determination of these quality parameters were those proposed in the International Olive Oil

Council regulations (COI 2013). The values obtained for these quality parameters correspond to those specified for the category EVOO. These analytical parameters are basically affected by factors causing damage to the fruits, e.g., olives fly attacks or improper systems of harvesting, carriage, and storage of olives.

Method Development

Fiber Coating Selection

The nature of the fiber coating strongly affects the effectiveness of HS-SPME sampling, and thus the fiber should be chosen taking into account the chemical nature of the analytes (Bicchi et al. 2000). Four fiber types (PDMS, PDMS/DVB, Car/PDMS/DVB, and Car/PDMS) were evaluated. For all the cultivars studied, it was determined which fiber extracted most effectively volatile compounds (expressed as the amount of retained compounds by the different fibers). Twenty grams of olive oil of each cultivar was partitioned in four vials containing 5 g each. The samples were spiked with an IS solution to obtain a final concentration of 0.13 μg ml^{-1}. SPME conditions were as follows: equilibration and extraction temperature, 45 °C; equilibration time, 30 min; and extraction time, 40 min. Samples were stirred at 750 rpm throughout equilibration and extraction time. The fiber was exposed to HS at 3 cm. After the extraction, the fiber was introduced into the injection port and the volatiles were thermally desorbed at 240 °C. The whole procedure was performed in triplicate.

Figure 1 shows the chromatograms corresponding to the different fibers tested. The amount of retained compounds of olive oils in the HS sampled by HS-SPME (efficiency) for the different fibers was compared. The total amount of compounds extracted with PDMS and PDMS/DVB was lower in relation to the amount extracted with CAR/PDMS and CAR/PDMS/DVB under the same extraction conditions (Fig. 2). These results are similar to those reported previously by other authors in other foodstuffs (Dufour et al. 2001; Garcia-Esteban et al. 2004).The volatile components identified include: alcohols, aldehydes, ketones, esters, terpenes, hydrocarbons, furans, and ethers. These groups of volatile compounds basically agree with those reported for olive oil by different authors using SPME (Vichi et al. 2003; Cecchi and Alfei 2013). Considering that the proportion of retained compounds of each chemical family mentioned were similar for Car/PDMS and Car/PDMS/DVB fibers (Fig. 3), for this study,

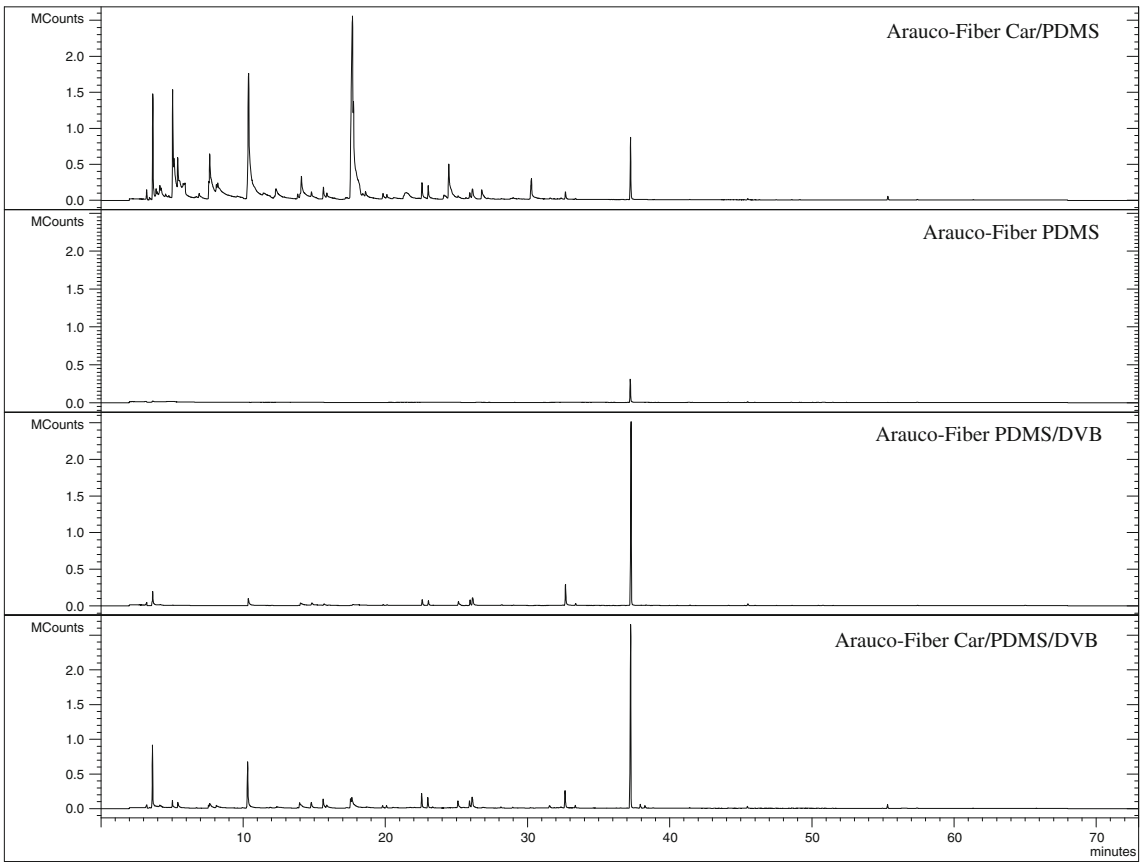

Fig. 1 Gas chromatograms of volatile compounds of olive oils from Arauco cultivar extracted by HS-SPME with different types of fibers

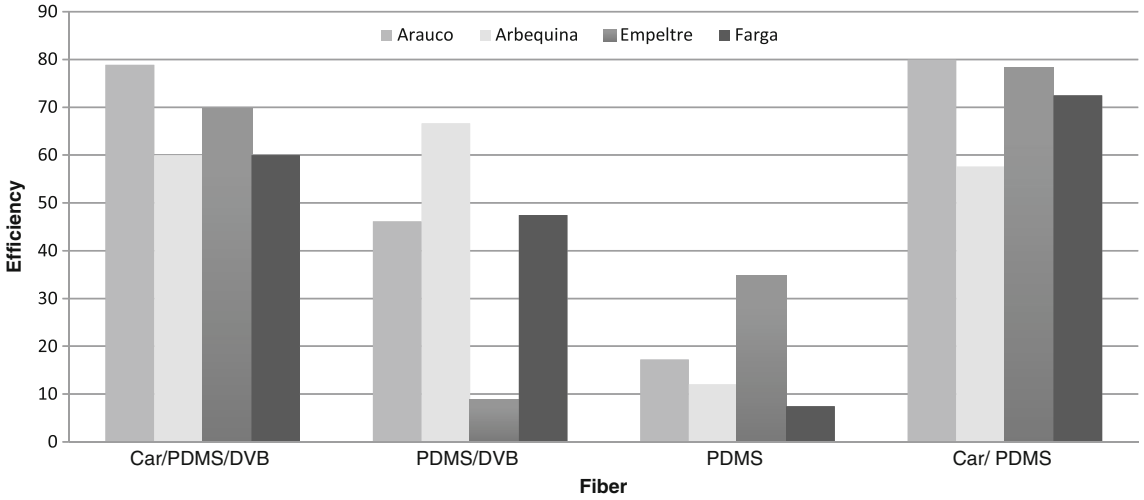

Fig. 2 Number of compounds detected by each fiber type in different cultivars olive oil by HS-SPME

the CAR/ PDMS fiber was selected as optimal, because it showed a slightly higher efficiency for three of the four samples tested. The CAR coating showed a high sensitivity for small volatile molecules. This is a porous carbon with a high surface area (around 1,200 m^2 g^{-1}), which has been described as adequate for extraction of low boiling point compounds, although it allows the adsorption of a wide range of compounds due to the presence of different types of pores (micro-, meso-, and macropores). Conversely, PDMS showed very high sensitivity to nonpolar compounds (Kalua et al. 2006; Garcia-Esteban et al. 2004). The condition of the fiber has to be frequently checked by carrying out blanks and visual fiber examination. The main drawback encountered in the SPME extraction derives from the heterogeneity of fiber lots (Escuderos 2011). In this study, we used the same fiber to assure the good repeatability needed for chemometrics.

SPME Optimization

To evaluate suitable extraction conditions for the determination of volatile fraction, a CCD was performed (Table 1). The following variables were tested: equilibration time (14–56 min), extraction time (14–56 min), and extraction temperature (36–64 °C). These ranges were selected based on prior knowledge about the system under study and previous studies (Pouliarekou et al. 2011; Vichi et al. 2008; Vichi et al. 2006; Reboredo-Rodríguez et al. 2012; Dierkes et al. 2012; Cajka et al. 2010). The amount of sample was not studied because in

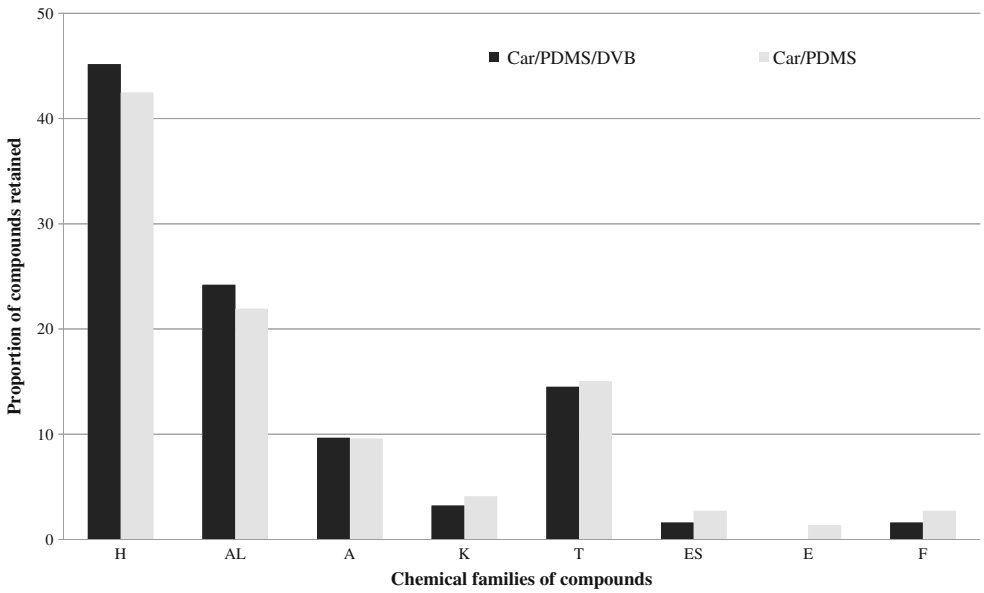

Fig. 3 Proportion of compounds of each chemical family retained in HS-SPME by Car/PDMS and Car/PDMS/DVB

Table 1 Codex matrix of the central composite design (CCD), factors, and levels

STD	Run	Block	Equilibration time (min)	Extraction time (min)	Temperature (°C)
5	1	1	20	20	60
9	2	1	35	35	50
4	3	1	50	50	40
8	4	1	50	50	60
2	5	1	50	20	40
6	6	1	50	20	60
3	7	1	20	50	40
12	8	1	35	35	50
10	9	1	35	35	50
1	10	1	20	20	40
11	11	1	35	35	50
7	12	1	20	50	60
15	13	2	35	14	50
16	14	2	35	56	50
14	15	2	56	35	50
13	16	2	14	35	50
17	17	2	35	35	36
18	18	2	35	35	64
19	19	2	35	35	50
20	20	2	35	35	50

the case of lipid matrices, it does not significantly affect the mass of analyte absorbed by the SPME coating (Vichi et al. 2008). The factorial design consisted in 20 experiments in two blocks (two consecutive days) to remove the expected variation caused by some change during the course of the experiment. One hundred grams of olive oil sample was partitioned in 20 vials containing 5 g each. Then, olive oil sample were spiked with an IS solution to obtain a final concentrate on of 1 μg ml^{-1}. Finally, the vials were capped and stored in the dark at 4 °C until analysis. SPME analysis was performed using the previously selected fiber (Car/PDMS coating). The sample was placed under agitation (750 rpm) to temperatures set for different periods of time of equilibration and extraction. Then, the fiber was immediately introduced into the GC injection port to allow thermal desorption of the analytes at a temperature of 240 °C. Chromatographic responses (peak area) of 1-penten-3-one, copaene, hexanal, limonene, and hexane-1-methoxy were monitored. These volatile compounds were selected as they represent different families of chemical compounds present in olive oil. Qualitative analysis was performed by comparison of mass spectra and retention times with those of standard compounds and/or by comparison of the mass spectrum with those of reference compounds in the NIST Mass Spectral Search Program (NIST Version 2.0). Chromatographic responses for all the compounds tested were fitted to linear model (Eqs. 1, 2, 3, 4, and 5).

$$\text{copaene} = -2.57982e^5 + 6879.05034 \times \text{temp.extr.} \quad (1)$$

$$\text{hexane-1-methoxy} = -2.99295e^6 + 27,322.48434 \times \text{time extr.} + 60,342.08893 \times \text{temp.extr.} \quad (2)$$

$$\text{hexanal} = -1.13637e^6 + 10,562.50224 \times \text{time extr.} + 27,986.78523 \times \text{temp.extr.} \quad (3)$$

$$\text{limonene} = -27,687.73839 + 257.85906 \times \text{time extr.} + 557.70134 \times \text{temp.extr.} \quad (4)$$

$$\text{1-penten-3-one} = 3.30161e^5 + 13523.25615 \times \text{time extr.} \quad (5)$$

Once, outliers were removed by analyzing the difference between fitted values test (DFFITS). This test measures the influence each point has on the predicted value, computing a standardized value, which can be interpreted as the number of standard deviation units owed to experimental data exerts disproportionate influence on the model (Vera-Candioti et al. 2008; Jofré et al. 2010). The model coefficients were calculated by backward multiple regression and validated by the ANOVA. As can be seen in Table 2, linear model are those

Table 2 Statistical value when applying ANOVA of lack of fit and regression of the selected models

Response	Model	*p* value[a] Model	*p* value* Lack of fit	R^2 adj.
1-penten-3-one	Linear	0.0452	0.8281	0.2157
Copaene	Linear	<0.0001	0.0013	0.6041
Hexanal	Linear	<0.0001	0.4805	0.8570
Limonene	Linear	0.0230	0.1423	0.3761
Hexane-1-metoxi	Linear	<0.0001	0.9729	0.7878

*p value <0.05, significant level

which better explain the behavior of studied responses factors, although irrelevant main terms were maintained to fit hierarchical models. The coefficients of variation were lower than 20 % in all cases. The p value showed that at 95 % confidence level, the extraction temperature affects the peak area of copaene, hexane-1-methoxy, hexanal, and limonene. For the last three compounds, extraction time was also significant. In the case of 1-penten-3-one, just the extraction time was significant. The lack of fit test is not significant (p value >0.05) in all cases except for copaene. In the case of this analyte, a significant lack of fit test was observed for the several models studied, making it impossible to predict the responses (peak area) for other extraction times and temperatures (Bogusz Junior et al. 2012). Consequently, copaene was excluded from the analysis of desirability function.

When a single response is being analyzed, the model analysis indicates areas in the design region where the process is likely to give desirable results, which is a relatively easy task. However, a function of more than one response can be used: the desirability function, which includes the researcher's priorities and desires on building the optimization procedure. In this methodology, the desirable combination of k response variables, each of which depends upon a set of p design variables, is obtained through a desirability function. This function transforms each estimated response variable $\hat{y}_i$, calculated by the fitted response surface associated with the CCD experimental design used in this work, into a desirability value d_i, using the following equation:

$$d_i = \begin{cases} 0 & \widehat{y}_i \leq y_{i\min} \\ \left[\dfrac{\widehat{y}_i - y_{\min}}{y_{i\max} - y_{i\min}}\right] & y_{i\min} < y_i < y_{i\max}, \text{for} = 1, 2, \ldots, k \\ 1 & y_i \geq y_{i\max} \end{cases} \quad (6)$$

where the values $y_{i\min}$ and $y_{i\max}$ are the minimum and maximum acceptable value of $\hat{y}_i$, respectively (Ribeiro et al. 2010) Its application involves creating a function for each individual response d_i and finally obtaining a global function D that should be maximized choosing the best conditions of the designed variables. The latter function varies from 0 (value totally undesirable) to 1 (all responses are in a desirable range simultaneously) and can be defined by Eq. (7):

$$D = \left(d_1^{r1}\, x\, d_2^{r2} x \ldots d_m^{rm}\right)^{1/\sum rj} = \left(\prod_{j=1}^{m} d_j^{rl}\right)^{1/\sum rj} \quad (7)$$

where, d_1, …, d_m correspond to the individual desirability function for each response being optimized, m is the number of responses in the measure and r is the importance relative of each response over the other responses (Ribeiro et al. 2010; Khodadoust et al. 2013; Zolgharnein et al. 2013). Following the mentioned procedure, five responses were simultaneously optimized by using the desirability function. The selected criterion was to maximize the individual responses (peak area) assigning a relative importance to each response, giving more importance to the analytes with lower smaller area (limonene) and in decreasing order of importance for the rest of the analytes. The desirability function presented in Fig. 4 was obtained for a pair of factors (temperature of extraction vs. time of extraction), maintaining the time of equilibration in their optimal value. The figure shows that the desirability function increases when temperature and time of extraction increases. Figure 5 shows the individual and combined desirability obtained for the tested compounds. The experimental conditions corresponding to one maximum in the desirability function (D=0.756) are: time of equilibration, 20 min; time of extraction, 50 min; and temperature, 60 °C. Temperatures above 60 °C were not considered to avoid thermal volatile alterations and oxidative degradation of the oil matrix, not inducing the formation of artifacts in the samples (Reboredo-Rodríguez et al. 2012; Vichi et al. 2006; Vichi et al. 2008). Therefore, these conditions were used for the final analysis of the olive oil samples.

Once the optimal SPME parameters were established, CAR/PDMS and DVB/CAR/PDMS fibers were tested. The results were analogous to those obtained for fiber selection (Fig. 2).

Multivariate optimization methods can assess the statistical significance of the independent variable effects under studied, as well as to evaluate their interaction effects between two or more variables. In our study, significant interaction effects were observed among the tested factors, which would not have been detected using univariate optimization.

Performance of the Analytical Method

Analytical quality parameters were evaluated to assess the performance of the HS-SPME/GC-MS procedure with the selected conditions. Thus, representative compounds found at low, medium, and high levels distributed along the sample chromatogram were selected. The standards used were

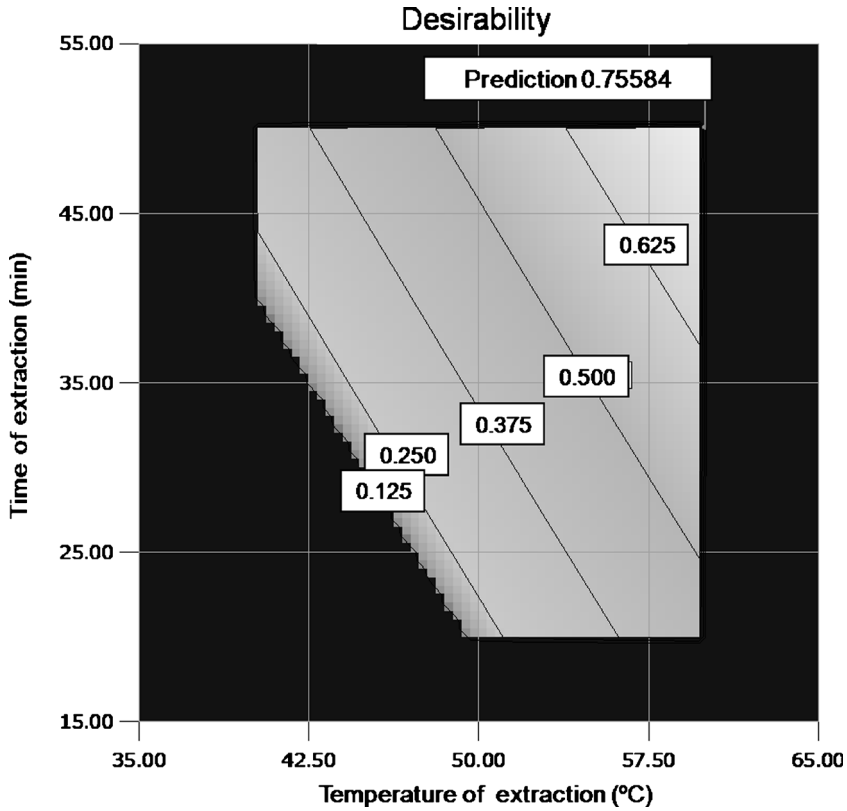

Fig. 4 Response surface plots corresponding to the desirability function when optimizing the extraction time and temperature while maintaining constant the time of equilibration at their optimum values (20 min)

limonene, hexyl-acetate linalool, hexanal, and 3-hexen-1-ol. Repeatability of the SPME method was determined by five replicate analyses of same olive oil spiked at levels of 0.01, 0.1, and 1.0 mg kg^{-1}. Linearity testing was carried out using the same olive oil spiked at levels of 0.01–1.0 mg kg^{-1}. The linearity was evaluated by plotting the ratio of each analyte area to IS area against concentration. IS (4-methyl-2-pentanone) concentration was maintained at 0.13 mg kg^{-1}. LOD and LOQ were calculated as LOD=3 *s*/*m* and LOQ=10 *s*/*m*, respectively, where *s* is the standard deviation of the baseline noise and *m* is the slope of the calibration curve.

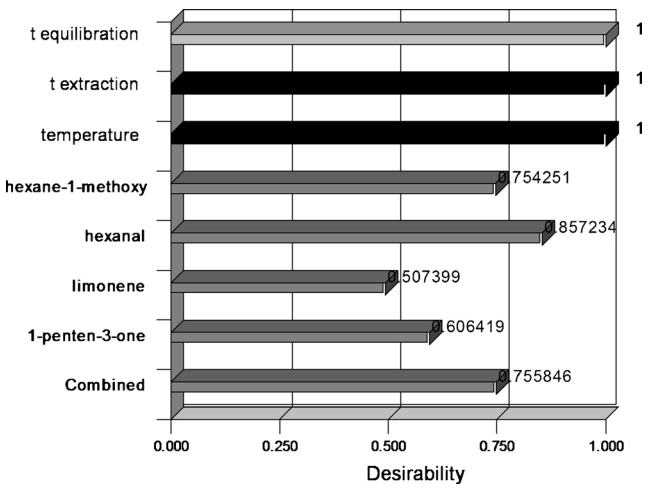

Fig. 5 Individual and combined desirability

The resulting performance characteristics are summarized in Table 3. Linearity of the analysis and extraction procedure of oil samples showed good correlation values for the compounds studied. The relative standard deviations (RSDs) for samples spiked with 0.01, 0.1, and 1.0 mg kg^{-1} were always below 8 %.

Varietal Characterization of Samples Olive Oils

Samples of olive oil of the selected cultivars, Arauco, Arbequina, Empeltre, and Farga, were analyzed. Forty-four different volatile compounds were identified and quantified belonging to various compound classes: hydrocarbons, aldehydes, ketones, alcohols, esters, ether, terpene, and furans (Table 4). Comparison of the typical chromatograms corresponding to four cultivars is shown in Fig. 6.

PCA and LDA were used to highlight the data structure and to find the relationships between volatile compounds and olive oil variety. PCA, applied in the first stage of the data

Table 3 Resulting performance characteristics

	Repeatability RSD (%; n=4)			Linearity R^2 (0.01–1 μg g^{-1})	LOD (ng g^{-1})	LOQ (ng g^{-1})
	0.01 μg g^{-1}	0.1 μg g^{-1}	1 μg g^{-1}			
Limonene	0.02	0.63	0.94	0.999	0.003	0.011
Hexyl acetate	0.20	2.43	7.72	0.993	0.015	0.049
Linalool	0.02	4.77	2.50	0.999	0.011	0.038
Hexanal	1.56	2.78	3.82	0.998	0.130	0.400
3-hexen-1-ol (E)	2.78	3.75	4.74	0.999	0.249	0.700

LOD limit of detection (in ng g^{-1}), *LOQ* limit of quantification (in ng g^{-1})

processing, represents one of the most frequently used chemometric tools. One of its attractive features is the possibility to project (in a relatively easy way) particular data from a higher to a lower dimensional space and then reconstruct them without any preliminary assumptions about their distribution (Cajka et al. 2010). PCA permitted a reduction of all volatile compounds found in olive oil to three principal components (PCs; with eigenvalues of >1). These three PCs were extracted explaining 96.4 % of the total variance of EVOO samples. The first principal component (PC 1) represented about 50.8 %, and the next PCs, 27.2 and 18.4 %, respectively. The PCA was calculated using the concentrations of the 44 volatile compounds tested and assigning value 0 to those listed as not detected. PCA results of complete aroma profile emitted by EVOO showed a marked difference among cultivars distributing EVOO samples into groups represented their type (Fig. 7). The formed clusters indicated a large variation in the profiles of olive oil volatiles between the four cultivars. To display more clearly these differences, volatile compounds were grouped into different classes (hydrocarbons, terpenes, aldehydes, furans, ketones, alcohols, esters, compounds of five straight-chain carbons (C_5 compounds), compounds of six straight-chain carbons (C_6 compounds), and total volatile compounds (TVC)). The C_6 and C_5 compounds, especially C_6 aldehydes and alcohols and the corresponding esters are the most important compounds in the VOO aroma, from either a quantitative or a qualitative point of view (Angerosa et al. 2004; Sánchez-Ortiz et al. 2012). C_6 and C_5 compounds are enzymatically produced from polyunsaturated fatty acids through the so-called LOX pathway, and their concentrations depend on the level and the activity of each enzyme involved in this LOX pathway (Angerosa et al. 2004). As can be seen in Table 5, there are significant differences ($p \leq 0.05$) among cultivars for all classes of volatile compounds studied. The TVC ranged from 4.27 mg kg^{-1} for Arbequina cultivar to 7.09 mg kg^{-1} for Empeltre cultivar. The biosynthesis of VOO volatile compounds depends on the availability of substrates to be catabolized through the LOX pathway during the process to obtain this oil. This availability seems to be cultivar-dependent, and it is comparatively lower in Arbequina fruits (Sánchez-Ortiz et al. 2012). C_6 compounds ranged from 6.50 mg kg^{-1} for the cultivar Empeltre to 2.01 mg kg^{-1} for the cultivar Arauco, representing 92 and 33 % of TVC, respectively. In this sense, there are references in the literature on the interference of many of the components of the fruits, mainly phenolic compounds, on LOX activity (Dohi et al. 1991). Studies carried out by our research group, indicate that Arauco oils have a higher content of total phenolic compounds determined by Folin–Ciocalteu (above 500 ppm expressed as caffeic acid). Indeed, the analysis of 11 phenolic compounds by dispersive-liquid liquid micro-extraction-capillary zone electrophoresis (DLLME-CZE) demonstrated that the sum of phenolic compounds concentration was the highest in Arauco samples (Monasterio et al. 2013). This could explain the lower concentration of C_6 compounds in cultivar Arauco. Values for compounds of C_5 compounds were 0.63 mg kg^{-1} for the Arbequina cultivar and 0.09 for Farga cultivar. For cultivars Arbequina, Empeltre, and Farga, the main compounds are aldehydes, being hexanal the most abundant for Arbequina and 2-hexenal for the other two cultivars. Hexanal is the oxidation product of linoleic acid deriving from either the action of LOX or from chemical oxidation; it has been related to grassy, green-sweet and green-apple odor notes (Kiritsakis 1998; Kalua et al. 2007). Conversely, 2-hexenal provides the typical "green note" of olive oil, the product of the LOX pathway, inversely related to the maturity and degree of oxidation of VOO (Pouliarekou et al. 2011). Arauco is represented by a high content of hydrocarbons and alcohols. The most important hydrocarbon found in this cultivar is 7-hexadecene. This compound was found in all oils tested in this study, however its origin and relevance is still unknown. It was previously found in olive oils from traditional Portuguese varieties (Vaz-Freire et al. 2009). Among the alcohols, 3-hexen-1-ol and 5-hexen-2-ol

Table 4 Concentration of volatile compounds (μg g^{-1}) determined by HS-SPME/GC-MS analysis in monovarietal olive oil samples from Mendoza, Argentine

ID	Compounds	RT	Class	ARB μg g^{-1a}	ARAU μg g^{-1}	FAR μg g^{-1}	EMP μg g^{-1}
a1	1,3-pentadiene(z)	4.2	H	0.0549±0.0051	0.0081±0.0004	0.0169±0.0009	0.0170±0.0008
a2	Cyclopentane methyl	4.8	H	nd	nd	nd	0.0010±0.0001
a3	Butanone, 4-acetyloxy	5.6	K	nd	0.1279±0.0237	nd	nd
a4	Butanal, 2-methyl	6.8	A	nd	nd	0.0040±0.0002	0.0173±0.0011
a5	4-ethyl-4-methyl-1-hexene	7.5	H	nd	0.1748±0.0002	0.1489±0.0207	nd
a6	1-penten-3-one	7.6	K	0.3447±0.0288	nd	nd	0.1210±0.0203
a7	Pentane, 3,3-dimethyl	7.9	H	nd	nd	0.0021±0.0000	nd
a8	Furan, 2-ethyl	8.1	F	nd	nd	0.0384±0.0058	nd
a9	Cyclohexanol, 4-methyl	8.2	AL	nd	0.0699±0.0004	nd	0.0122±0.0010
a10	2-pentenal	11.4	A	nd	nd	0.0134±0.0003	0.0322±0.0005
a11	2-penten-1-ol	12.2	AL	0.2362±0.0623	0.1752±0.0226	0.0591±0.0003	0.0532±0.0094
a12	1-hexene, 3,5-dimethyl	12.8	H	nd	nd	0.0150±0.0019	0.0086±0.0001
a13	Hexane, 2,4-dimethyl	13.3	H	0.0572±0.0012	0.0259±0.0024	0.0714±0.0102	0.0882±0.0106
a14	Hexanal	13.6	A	2.9609±0.2773	0.1368±0.0024	1.0851±0.0622	0.9573±0.2439
a15	Hexane, 2,3,5-trimethyl	14.4	H	nd	0.0106±0.0006	nd	nd
a16	Hexane, 1-methoxy	14.9	E	nd	0.3222±0.0094	nd	nd
a17	5-hexen-2-ol	15.1	AL	nd	0.4292±0.0156	nd	nd
a18	2,2-dimethyl-1-heptene	15.5	H	0.0062±0.0015	nd	nd	0.0068±0.0001
a19	2-hexenal	16.7	A	0.1250±0.0110	0.3199±0.0316	4.5336±0.4052	4.9159±0.2128
a20	3-hexen-1-ol(E)	16.8	AL	nd	0.5935±0.1999	nd	nd
a21	3-ethyl-1,5-octadiene	18.3	H	0.1484±0.0266	0.0750±0.0010	nd	nd
a22	3-decyne	18.6	H	0.1484±0.0251	0.0792±0.0004	0.0387±0.0074	nd
a23	2-hexanone, 4-methyl	18.7	K	nd	nd	0.0158±0.0028	nd
a24	Cyclohexanol 2-methyl	19.1	AL	nd	nd	0.0262±0.0056	nd
a25	2,4-hexadienal (e-e)	19.9	A	nd	nd	0.1396±0.0025	0.0923±0.1029
a26	2(5h)furanone, 5-ethyl	22.1	F	nd	nd	0.1933±0.0401	0.1129±0.1086
a27	4-hexenyl acetate	23.9	ES	nd	nd	0.0636±0.0043	0.3958±0.0400
a28	Hexyl acetate	24.2	ES	nd	nd	0.0351±0.0036	0.0425±0.0205
a29	*p*-cymene	24.8	H	nd	nd	0.0045±0.0008	0.0026±0.0008
a30	Limonene	25.0	T	nd	0.0088±0.0004	0.0046±0.0013	0.0065±0.0011
a31	1,6-anhydro-2,3-dideoxy αthreo hexopiranosa	25.6	O	nd	0.0270±0.0010	nd	nd
a32	*o*-cymene	25.7	T	0.0566±0.0056	nd	0.0508±0.0110	0.0044±0.0000
a33	Tetradecane	27.8	H	nd	nd	0.0036±0.0007	0.0031±0.0005
a34	Linalool	28.0	T	nd	nd	nd	0.0068±0.0014
a35	3-nonen-1-ol	28.2	AL	nd	nd	0.0519±0.0218	0.0600±0.0069
a36	Thymol	28.3	T	0.0047±0.0005	0.0058±0.0003	nd	nd
a37	Cyclohexane 2-ethenyl 1,1-dimethyl, 3-methylene	28.4	H	nd	0.3723±0.0254	0.0515±0.0076	0.0514±0.0052
a38	7-hexadecene	31.9	H	0.1217±0.0237	3.0945±0.0668	0.0875±0.0234	0.0641±0.0078
a39	α-cubenene	37.2	T	nd	0.0073±0.0000	nd	0.0028±0.0006
a40	(+)cyclosativene	38.1	T	0.0011±0.0001	0.0024±0.0001	nd	0.0003±0.0001
a41	α-copaene	38.3	T	0.0048±0.0002	0.0680±0.0033	0.0015±0.0002	0.0126±0.0022
a42	Caryophyllene	39.8	T	nd	0.0021±0.0001	nd	0.0010±0.0002
a43	Cedran-diol-8s-14	41.9	T	0.0010±0.0001	0.0022±0.0000	nd	nd
a44	δ-cadinene(+)	42.7	T	nd	nd	nd	0.0009±0.0001

RT retention time, *H* hydrocarbon, *K* ketone, *A* aldehyde, *F* furan, *AL* alcohol, *E* ether, *ES* esther, *T* terpene, *O* other, *nd* not detected

Means±standard deviation of the concentration of volatile compounds for each cultivar in micrograms per gram in relation to the concentration of IS

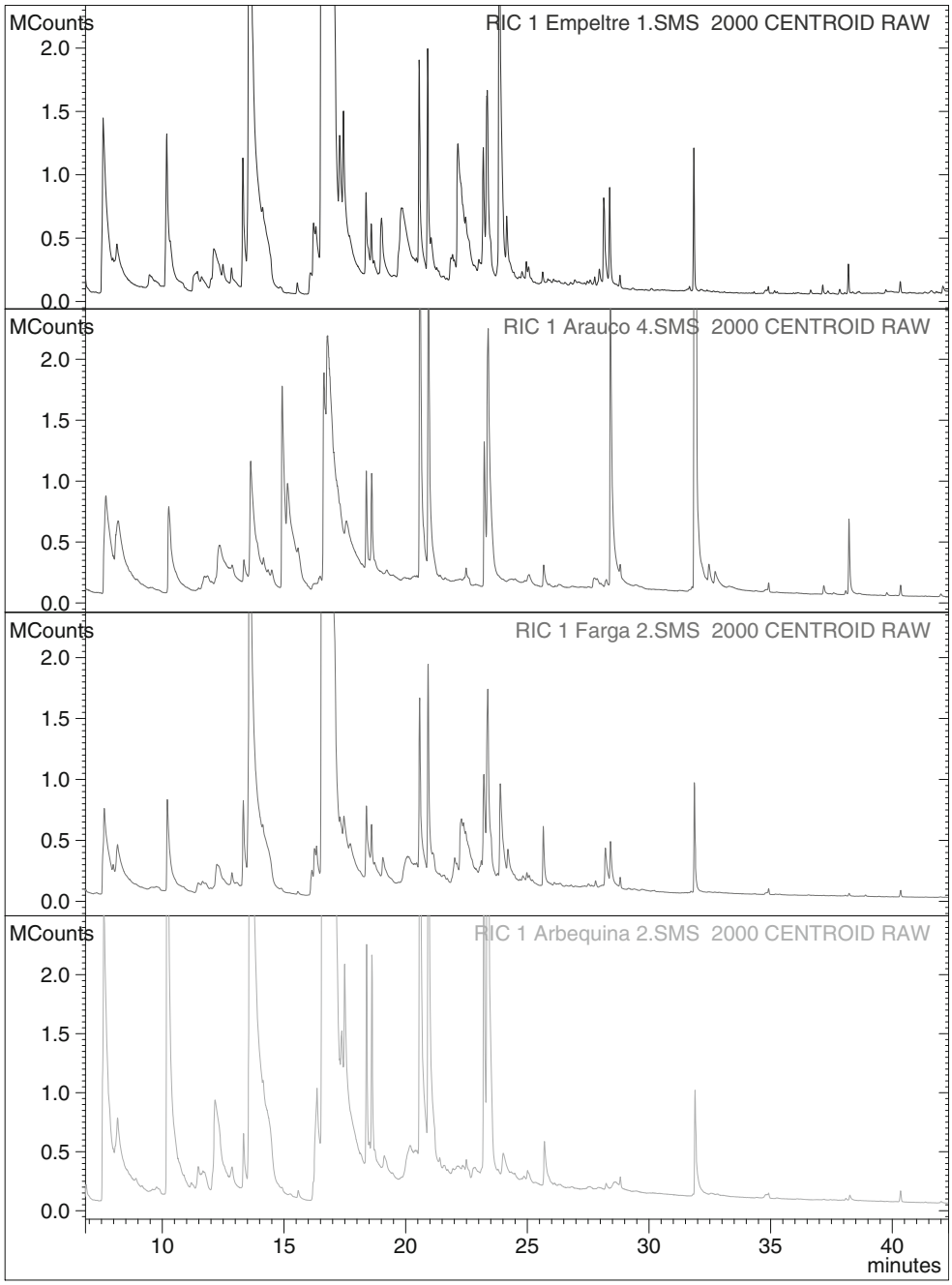

Fig. 6 HS-SPME/GC-MS chromatograms of volatile profile obtained by analyzing EVOO from four different cultivars

are the most important; this latter was identified for the very first time in an VOO. Arauco cultivar showed overall amounts of C_6 alcohols higher than the sum of C_6 aldehydes and C_6 esters. These results may be explained by differential activity of the enzyme alcohol dehydrogenase (ADH), which reduces the C_6 aldehydic compounds in the corresponding alcohols. Alcohols have less sensory significance than aldehydes because of their higher odor threshold values, their sensory descriptions being associated with fruity, soft green and aromatic sensory notes (Luna et al. 2006). A secondary metabolic pathway of LOX is active on the linolenic acid substrate, leading to the production of C_5 volatile compounds, which are also present in the VOO aroma (Angerosa et al. 2000). EVOO samples contain varied concentrations of C_5 compounds, among which are hydrocarbons (1,3-pentadiene; pentane 3,3-dimethyl), aldehyde (2-pentenal), alcohol (2-penten-1-ol), and ketone (1-penten-3-one). They are thought to derive from the hydroxylation or dimerization of pentene radicals originated by β-scission of alkoxy radicals formed from 13-hydroperoxides by an enzyme-mediated mechanism

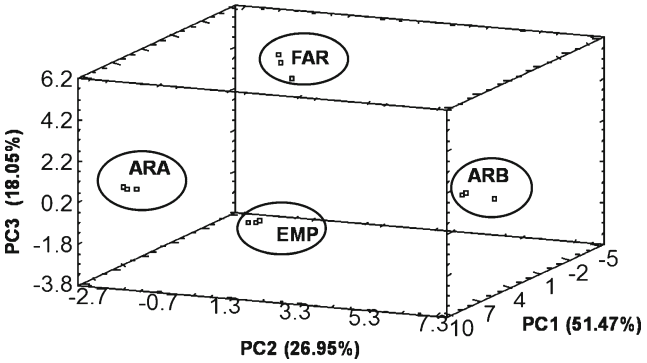

Fig. 7 Three-dimensional PCA plot applied to TVC from samples of olive oils four different cultivar

(Angerosa et al. 1998). The highest concentration of C_5 compounds was found in Arbequina cultivar, being 1-penten-3-one and 2-penten-1-ol the most important. The 1-penten-3-one has been mostly associated with fruity, sweet, and pleasant attribute as tomato and strawberry (Luna et al. 2006; Angerosa et al. 2000; Aparicio and Luna 2002; Morales et al. 1995). Just as in other studies (Baccouri et al. 2008), the 1-penten-3-one levels did not exceed 1 mg kg^{-1}. On the other side, the 1-penten-3-one is characterized by a very low odor threshold (0.7–50 μg kg^{-1}) so its contribution to whole aroma can be considered important. Factors such as volatility, hydrophobic character, size, shape, and conformational structure of the odorous molecule as well as type and position of functional groups seem to affect the odor intensity more than its concentration (Ghanbari et al. 2012). Esters consisted of (Z-)3-hexenyl acetate and hexyl acetate deriving from the LOX pathway. These compounds were only detected in Farga and Empeltre cultivars, representing a 1.5 and 6.2 % of total volatiles compounds respectively. Hexyl acetate contributes significantly to sweet, fruity notes, whereas 3-hexenyl acetate contributes to the green and banana pleasant notes (Pouliarekou et al. 2011; Kalua et al. 2007). Among the furans, furan-2-ethyl was detected only in Farga cultivar and 2(5H)furanone-5-ethyl was found in Farga and Empeltre cultivars. Ethyl furan is usually associated with sweet and ethereal sensory (Luna et al. 2006; Kanavouras et al. 2005; Ghanbari et al. 2012). According to literature, polyunsaturated fatty acids are cited as the most important furan precursors (Owczarek-Fendor et al. 2010). Some compounds were found in HS-SPME of VOO for the very first time, to the best of our knowledge, based on an extensive literature search. These compounds are the 5-hexen-2-ol and 3-decyne. However, these compounds have been found in the volatile composition of other plant species. 5-hexen-2-ol has been previously found in the essential oil of *Casimiroa pringlei*, an aromatic shrub commonly known as zapotillo (Ponce-Monter et al. 2008). Conversely, 3-decyne was an important volatile compound identified in peel and flesh of cucumber (*Cucumis sativus* L.) (Guler et al. 2013). The presence of such compounds could be used to trace the geographical origin and the genotypical characterization of VOOs

Table 5 Family of volatile compounds determined by HS-SPME/GC-MS analysis in monovarietal EVOO samples from Mendoza, Argentina

Classification	ARB		ARAU		FAR		EMP	
	μg g^{-1a}	%	μg g^{-1a}	%	μg g^{-1a}	%	μg g^{-1a}	%
Hydrocarbons	0.5369±0.0799 b[b]	12.6	3.8404±0.0939 a	62.8	0.4400±0.0486 b	6.5	0.2425±0.0217 c	3.4
Ketones	0.3447±0.0288 a	8.1	0.1279±0.0237 b	2.1	0.0158±0.0028 c	0.2	0.1210±0.0203 b	1.7
Aldehydes	3.0859±0.2728 b	72.2	0.4567±0.0292 c	7.5	5.7757±0.4650 a	85.5	6.0149±0.3315 a	84.8
Furans	nd		nd		0.2318±0.0343 a	3.4	0.1129±0.1086 b	1.6
Alcohols	0.2362±0.0623 b	5.5	1.2679±0.1617 a	20.7	0.1372±0.0273 b	2.0	0.1253±0.0161 b	1.8
Esthers	nd		nd		0.0988±0.0078 b	1.5	0.4383±0.0589 a	6.2
Terpenes	0.0682±0.0061 b	1.6	0.0966±0.0035 a	1.6	0.0569±0.0123 b	0.8	0.0353±0.0036 c	0.5
C_6 compounds[c]	3.1432±0.2723 b	73.6	2.0130±0.1489 c	32.9	6.1081±0.4851 a	90.4	6.5005±0.3955 a	91.7
C_5 compounds[d]	0.6358±0.0607 a	14.9	0.1833±0.0222 b	3.0	0.0915±0.0005 c	1.4	0.2233±0.0252 b	3.1
TVC	4.2719±0.3909 c	100	6.1117±0.2284 b	100	6.7561±0.5290 ab	100	7.0902±0.3921 a	100

TVC total volatile compounds

[a] Means±standard deviation of the concentration of volatile compounds for each cultivar in micrograms per gram in relation to the concentration of IS

[b] Means with different letter in the same file are significantly different (Tukey's test, $p \leq 0.05$)

[c] Hydrocarbons, aldehydes, ketones, alcohols, ether, and esther

[d] Hydrocarbons, aldehydes, ketones, and alcohols

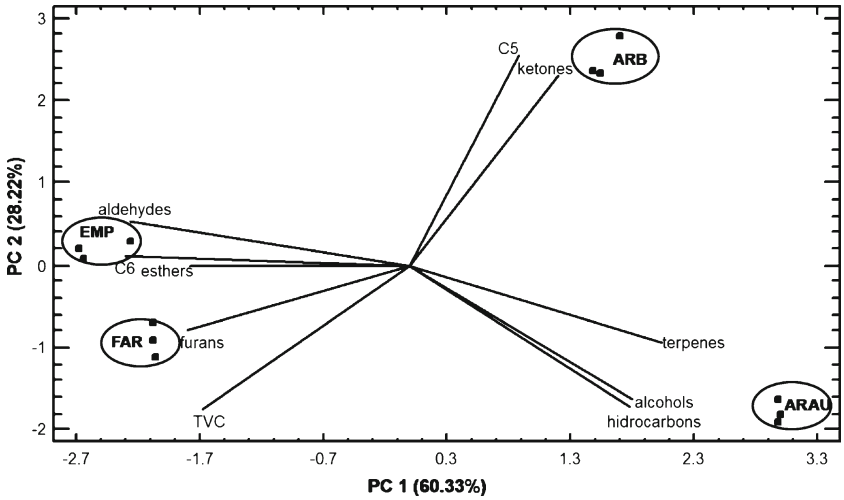

Fig. 8 PCA loading and score plot applied to different families of volatile compounds by samples of olive oils of four different cultivars

When performing a PCA of the different families of volatile compounds, two principal components having eigenvalues greater than one were obtained. The first principal component (PC) accounted for 60.3 % of variance, whereas the second PC contributed 28.2 %. The most important PCs contributed to 88.5 % of total variance. The interpretation of the results of PCA is usually carried out by visualization of the loadings plot (Fig. 8). This approach allowed the proper cultivar characterization of the EVOO samples. High concentration of terpenes, hydrocarbons, and alcohols characterized Arauco samples. Ketones and C_5 compounds were typical indicators for Arbequina cultivar. The samples of Empeltre were characterized by high concentrations of aldehydes, C_6 compounds and esthers. Furans concentration was high in Farga cultivar.

Then, LDA was performed on the volatile profiles (concentration) to extract only the variables (compounds) with the highest discriminant ability between the cultivar studied. This supervised technique is widely recognized as an excellent tool to obtain vectors showing the maximal resolution between a set of previously defined categories (Lerma-García et al. 2011). Three discrimination functions that accounted jointly for 100 % of the total variance were obtained by LDA ($p<0.05$). Function 1 accounts for 94.06 % of the total variance, function 2 accounted 5.83 %, and function 3 accounted 0.10 %. These functions showed Wilks' Lambda values of 1.08×10^{9}, 2.56×10^{-5}, and 3.75×10^{-2}, respectively, indicating a satisfactory discrimination. The variables having the highest discrimination power were 1-penten-3-one, hexane 2,4-dimetil, 5-hexen-2-ol, 3-decyne, (+)cyclosativene, cedran-diol-8S-14, and δ-cadinene(+). All the samples were 100 % correctly classified. It has to be pointed out that hydrocarbons and terpenes have already been demonstrated as markers of the genetic and/or geographic origin of VOOs (Vichi et al. 2006).

Conclusions

The optimization and application of HS-SPME/GC-MS to the simultaneous analysis of the whole volatile profile in VOO allowed the characterization of monovarietal EVOO from Argentina. To our knowledge, this is the first approach to characterize volatile compounds of varietal olive oils produced in Argentina, with special emphasis in Arauco olive oil (Argentina's flagship variety). Arauco EVOO were characterized by the lowest content of C_6 compounds as aldehydes (hexanal) and esters, compounds give the typical notes associated with "green" and "fruity," with respect to the other cultivars studied. Olive oil obtained from Arauco showed high concentration of alcohols, hydrocarbons, and terpenes. The most important hydrocarbon found in this cultivar was 7-hexadecene.

The analytical SPME/GC-MS data association with PCA and LDA demonstrated the ability to classify monovarietal EVOO from Argentina. The following compounds were decisive for oil varietal characterization 1-penten-3-one, hexane 2,4-dimethyl, 5-hexen-2-ol, 3-decyne, (+)cyclosativene, cedran-diol-8S-14, and δ-cadinene(+).

The study of a larger number of samples from the same cultivars and other geographical areas would further support the results obtained in the present study.

Acknowledgments This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Laboratorio de Aromas y Sustancias Naturales, Estación Experimental Agropecuaria Mendoza, Instituto Nacional de Tecnología Agropecuaria (INTA), and Facultad de Ciencias Agrarias, Universidad Nacional de Cuyo (Mendoza, Argentina). The authors are particularly grateful to Enrique Tittarelli for providing the equipment for the extraction of olive oil and assistance in the technical aspects of olive tree cultivation.

Conflict of Interest María de los Angeles Fernández declares that she has no conflict of interest. Mariela Assof declares that she has no conflict of interest. Viviana Jofre declares that she has no conflict of interest. María Fernanda Silva declares that she has no conflict of interest. This article does not contain any studies with human or animal subjects.

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