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ORIGINAL PAPER

Classification of Almond Cultivars Using Oil Volatile Compound Determination by HS-SPME–GC–MS

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Abstract Protected designations of origin "Alicante" and "Jijona" nougats are manufactured products produced using raw materials from Eastern Spain. In order to avoid adulteration practices, determination of volatile compounds from three different almond cultivars (Spanish Guara and Marcona and, from the USA, Butte) was performed to obtain a set of parameters for discrimination between Spanish and American cultivars. Factorial experimental design was applied for the development of a headspace solid-phase microextraction–gas chromatography–mass spectrometry (HS-SPME–GC–MS) analytical method for isolation and determination of the volatile compounds in almond oils. Main HS-SPME variables optimized were extraction temperature, extraction time, and stirring speed. Several volatile compounds including aldehydes, alkanes, alcohols and aromatic hydrocarbons were identified. Multivariate techniques were applied for classification and discrimination of the different almond cultivars studied. Specifically, cluster and stepwise linear discriminant analysis (LDA) were used, with LDA showing the best performance. The results obtained demonstrated that the proposed method combined with multivariate statistical analysis can be successfully applied for discrimination among different almond cultivars.

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Introduction

In recent years, food and health aspects have been receiving special attention, and food authenticity and origin determination have become a crucial issue in food quality control and safety [1]. Nuts and especially almonds are very important food products from a nutritional point of view mainly due to their high content of numerous beneficial nutritive and bioactive compounds [2, 3]. Almonds are grown as orchard crops, are highly nutritious and have a high fat content, but they also have a high cost. The fruit is highly valued for its dietetic, cosmetic and pharmaceutical properties [4]. Health benefits have been linked to their fatty acid composition (associated with a reduction in the risk of coronary heart disease, diabetes and cancer) and the presence of minor compounds with antioxidant activity [5–7] (tocopherols, polyphenols) and cholesterol lowering effects of phytosterols.

Alicante and Jijona nougats are typical Spanish confectionery food products manufactured from toasted almonds, sugar and honey in a traditional way. They are produced using raw materials from Eastern Spain and are protected by the Regulation Council of the Protected Designations of Origin Jijona and Alicante Nougat [8]. In recent years, almond production in Spain has decreased considerably compared with the USA, where cultivation has increased due to the improvement of agricultural techniques and a selection of new almond cultivars [9]. To avoid adulteration practices, some studies have focused on

the characterization of different almond cultivars and criteria for almond quality evaluation. Fatty acid profile, triglyceride and tocopherol composition have been used as discriminant parameters in order to differentiate among different almond cultivars [6, 10].

Lipid oxidation is the main cause of off-flavor development in almonds due to their high content of unsaturated fatty acids and the presence of riboflavin which acts as a photosensitizer in photo-oxidation [2]. As a result, several volatile compounds are formed which are essential for the overall food flavor but they become undesirable if concentrations exceed certain limits. HS-SPME (headspace solid-phase microextraction) is a fast, simple, solventless, sensitive and economical method for sample preparation prior to GC–MS analysis and it has been successfully used for the determination of volatile flavor profiles in oil samples [11, 12]. Optimization of variables affecting HS-SPME is necessary for a better extraction efficiency and sensitivity. In this regard, the superiority of factorial designs is well recognized compared with the classical "one variable at a time" optimization [13].

Only a few studies of aroma profiles of almond cultivars have been reported in the literature. L. Vázquez-Araújo et al. used simultaneous steam distillation and extraction (SDE) followed by GC–MS for analysis of volatile compounds in different toasted almonds and nougats [9, 14–16]. Volatile organic compounds (VOCs) emission of almonds and their relationship with navel orangeworm have been also studied using a Tenax collection system [17] or HS-SPME [18] and identified via GC–MS analysis. Finally, HS-SPME–GC–MS has been applied to the determination of hexanal and other volatile compounds in γ-irradiated raw unpeeled almonds [19]. However, an optimization of VOC extraction conditions and application of discriminant parameters for classification of different almond cultivars have not yet been studied.

The aim of the present work was the development of an analytical method based on HS-SPME coupled with GC–MS for rapid analysis and characterization of volatile compounds in three different almond cultivars in order to obtain discriminant parameters that can be useful to differentiate the studied cultivars avoiding adulteration practices in food products.

Materials and Methods

Reagents and Materials

Analytical grade petroleum ether was purchased from Panreac (Barcelona, Spain) and it was used as the almond oil extraction solvent.

The SPME fiber used was divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 μm, StableFlex, 1 cm long, mounted to a SPME manual holder assembly by Supelco (Bellefonte, PA, USA). Prior to use, the fiber was conditioned following the manufacturer's recommendations.

Standards of 3-methyl-3-pentanol, octane, 1,3-dimethylbenzene, nonane, 5-hexen-2-ol, 2-methylpentane, decane, octanal, undecane, nonanal, dodecane, decanal, 2-decenal, nonanoic acid, tridecane, undecanal, 2-undecenal, tetradecane, dodecanal and *n*-hexadecanoic acid in the available purest form were acquired from Sigma–Aldrich Inc (St. Louis, MO, USA). Tetradecanal (GC standard grade) was obtained from ChemService Inc (Teknokroma, Barcelona, Spain).

Almond Samples

Twenty-four samples from three different almond cultivars were used in this work eight Marcona (M), eight Guara (Gu) and eight Butte (Bu) samples. Marcona was selected because it is a Spanish cultivar of recognized organoleptical quality. Spanish Guara cultivar was included in the analysis due to its morphological similarity to Marcona that leads to the replacement of this latter cultivar in the production of Spanish nougat. Finally, American Butte is sometimes included as an ingredient in the manufacture of nougat because it is one of the most widely grown cultivars in the world [20], easier to obtain and cheaper than the other Spanish cultivars.

All samples were acquired unshelled from cultivars grown in the same crop year. Marcona and Guara cultivars were grown and collected from different Spanish geographical areas i.e. Xixona (Alicante), Pinoso (Alicante), Velez Rubio (Almería), Alcañiz (Teruel), Sella (Alicante), Tobarra (Albacete) and Barracas (Castellón). All of them were supplied by "Colefruse S.A" (San Juan, Alicante, Spain). Butte samples were grown in California and were obtained from a Spanish importer (Almendras Llopis, San Vicente Del Raspeig, Alicante, Spain).

The shells of the unshelled almonds were immediately removed by using a hammer. The seeds obtained were then stored at a temperature of 7 °C in order to keep them fresh until oil extraction.

Almond Oil Extraction

Almond seeds were ground in a domestic electric grinder just before oil extraction. The seed fragments were passed through a 1.5 mm sieve and stored in a desiccator.

For the oil extraction, a previously developed analytical method was applied [6]. Five grams of the ground sample was extracted by using a commercial fat extractor (Selecta, Barcelona, Spain) with 40 ml of petroleum ether for

90 min. According to the instrument's manufacturer, the temperature of the heating module was set at 135 °C, i.e., roughly two times the boiling point of the extraction solvent. However, the actual temperature of the extraction process was fixed at 60 °C because of the boiling point of the petroleum ether.

The oil obtained was a mixture of 12 independent fat extractions and it was dried under a nitrogen current and kept sealed in an amber vial in a freezer at −21 °C until required for analysis.

HS-SPME Conditions

In order to evaluate suitable extraction conditions for the determination of volatile compounds in almond oils, sample 4 of the Butte cultivar was selected. Three variables were studied by a two-level factorial experimental design (2^3) extraction temperature, extraction time and stirring speed. This design consists of eight experiments which were performed in duplicate plus one central point which was replicated four times; and allows us to study the main effects and factor interactions. All experiments were run randomized.

The type of fiber was not considered as a variable in the optimization process. A DVB/CAR/PDMS fiber was selected based on previous studies where different solid-phase microextraction fibers were used and compared [21]. The best results for target compounds were obtained using a DVB/CAR/PDMS fiber, which can be explained not only by its nature, but by slightly larger capacity for the analytes. In addition, this fiber maintains its performance at well above 100 extractions with between-day precision below 10%.

A preliminary study to optimize the amount of sample was conducted. For this purpose, a set of experiments using 0.5, 1 and 2 g were carried out. An increase from 1 to 2 g in the extraction efficiency for almost all the analytes was observed. As the amount of sample increases, the headspace volume is minimized, as Pawliszyn had established in previous studies [22]. In contrast, an increase in the memory effect of the fiber was also observed; in accordance with previously reported results due to the Carboxen phase that is characterized by very small pores and long desorption times [12]. On the other hand, by using 0.5 g almond oil a decrease in the extraction efficiency was observed, with a lower number of previously detected compounds. On this basis, the amount of sample was fixed at 1 g as a good extraction efficiency was obtained, avoiding high memory effects.

Table 1 lists the upper and lower values for each factor which were selected considering instrumental limitations. For the maximum extraction temperature, this factor was set to 60 °C in order to avoid the oxidation of the almond oil. On the other hand, 60 min was considered as the maximum time in order not to have extremely long experimental extraction times.

HS-SPME analyses were carried out by weighing 1 g of almond oil into a 20-mL dark glass vial sealed with an aluminium crimp cap provided with a polytetrafluoroethylene/silicone septum. Then, the vial was placed into a water bath where the sample was maintained under magnetic stirring. After 5 min of sample conditioning, the needle of the SPME device was inserted into the vial through the septum, and the fiber was exposed to the vial headspace for different time periods (20, 40 or 60 min). After that, the fiber was retracted into the needle assembly, removed from the vial, transferred to the injection port of the GC unit and immediately desorbed.

GC–MS Analysis

Analysis of volatile compounds was performed by using a Perkin Elmer TurboMass Gold GC–MS equipped with a split/splitless injector, a SPB-5 capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) (Supelco, Bellefonte, PA, USA) and a quadrupole mass spectrometer operating in electronic impact (EI) ionisation mode (70 eV). Helium was used as the carrier gas (1 mL/min). The column temperature was programmed from 50 °C (hold 10 min) to 280 °C (hold 5 min) at 4 °C/min heating rate. Ion source and detector temperatures were 180 and 300 °C, respectively. The injector temperature was 270 °C and the time for fiber desorption into the injection port was fixed at 10 min in splitless mode (1.5 min splitless-period). After every run, the SPME fiber was conditioned for 30 min at 270 °C in the injector of the gas

Table 1 Factor levels and design matrix of experiments for optimization of HS-SPME factors

Factor			Levels			Run								
Variable	Key	Units	Low (−)	High (+)	Middle (0)	1	2	3	4	5	6	7	8	9
Stirring speed	A	rpm	0	110	55	−	+	−	+	−	+	−	+	0
Time	B	min	20	60	40	−	−	+	+	−	−	+	+	0
Temperature	C	°C	35	60	47.5	−	−	−	−	+	+	+	+	0

chromatograph followed by a blank analysis to avoid carryover of the fiber.

Identification of the volatile compounds in almond oil samples was performed in full scan mode (40–550 *m/z*), by the combination of NIST mass spectra and gas chromatographic retention times of standard compounds. When standards were not available, volatile compounds were tentatively identified using GC–MS spectra only. In this sense, the compounds having ≤90% similarity with the NIST library were not taken into consideration. Chromatographic responses of detected volatile compounds (area counts) were monitored.

Statistical Analysis

The results of the two-level factorial experimental design for optimization of HS-SPME conditions were analyzed using the statistical package "Statgraphics Plus for Windows Version 5.1". A standardized Pareto diagram was constructed at a 5% of significance, showing each estimated effect in decreasing order of magnitude. The length of each bar is proportional to the standardized effect, which corresponds to the estimated effect divided by its standard error. Also, the vertical line can be used to judge which effects are statistically significant at a 95% confidence level. In this sense, bars that extend beyond the line correspond to effects that are statistically significant.

After HS-SPME optimization, all samples (eight Marcona, eight Guara and eight Butte) were analyzed by using optimum extraction conditions found followed by GC–MS identification of volatile compounds. Experimental data were processed with the aid of the "SPSS statistical package Version 15.0". Cluster analysis was carried out by applying the Ward method for agglomeration with the square of Euclidean distance as the criterion of proximity. The presence of different categories within almond samples was investigated using stepwise linear discriminant analysis (LDA). This is a supervised pattern recognition technique used not only to recognize different classes in a set of data but also to obtain classification functions (i.e., vectors) that make it possible to predict to which group a sample belongs using the appropriate latent variables [23]. These latent variables are linear combinations of the initial selected variables that maximize the resolution among the groups. The method used for variable selection was to minimize the Wilks' lambda statistics (λ_w). This parameter is calculated as the sum of the squares of the distances between points belonging to the same category divided by the total sum of the squares. In order to construct LDA vectors, the means obtained for replicates of samples were included, reducing the internal dispersion of the categories. In order to validate the classification rule developed for this study, a test set was constructed on the basis of the leave-one-out algorithm [23].

Results and Discussion

Optimization of the HS-SPME Process

The effect of main variables affecting the HS-SPME process in Butte 4 almond oil using DVB/CAR/PDMS fiber was studied by performing the experiments shown in Table 1. Different volatile compounds were detected depending on the experiment performed. The influence of each experimental factor on the extraction of each volatile compound was evaluated by means of standardized Pareto diagrams. Table 2 shows the influence of each experimental factor and interactions on the response (peak areas) of nine main volatile compounds detected, 1,3-dimethylbenzene; nonane; octanal; nonanal; dodecane; nonanoic acid; 2-undecenal; dodecanal and tetradecanal.

For the nine volatile compounds presented in Table 2, no significant influence of the stirring speed (A) was observed. These results agreed with previous studies performed in fish oil emulsions [11]. On the other hand, other studies have reported an increase in total peak areas for samples under stirring mode compared with under non-stirring mode. This fact may be explained by fluid turbulence induced by the stirring mode in the liquid and gaseous phases. Consequently, this phenomenon leads to an increase in the partition coefficient and diffusion rate of the analyte from the headspace to the fiber [24].

The extraction time (B) showed itself to be significant with a positive influence on the chromatographic responses of nonanal, dodecane, nonanoic acid and 2-undecenal. As expected, the positive effect of the extraction temperature (C) was higher for less volatile compounds as dodecane

Table 2 Influence of factors and interactions on the response of nine main volatile compounds from Butte 4 almond oil

Volatile compound	Standardized Effects						
	A	B	C	AB	BC	AC	ABC
1,3-dimethylbenzene	ns	ns	ns	+	ns	ns	ns
Nonane	ns	ns	ns	ns	ns	ns	ns
Octanal	ns	ns	ns	+	−	−	ns
Nonanal	ns	+	ns	+	ns	ns	ns
Dodecane	ns	+	+	+	+	ns	+
Nonanoic acid	ns	+	+	ns	+	ns	ns
2-undecenal	ns	+	ns	ns	ns	ns	ns
Dodecanal	ns	ns	ns	ns	ns	ns	ns
Tetradecanal	ns	ns	ns	ns	ns	ns	ns

ns not significant, + positive effect, − negative effect

and nonanoic acid, probably due to an increase in their transfer to the headspace.

Positive significant interactions between stirring speed and extraction time were observed for 1,3-dimethylbenzene, octanal, nonanal and dodecane. On the other hand, the extraction process of volatile compounds was less influenced by the interaction between stirring speed and extraction temperature. In this case, a negative significant effect was observed on the chromatographic response of octanal being no significant for the rest of evaluated compounds. Positive significant interactions between extraction temperature and extraction time were observed for dodecane and nonanoic acid. In contrast, a negative significant effect was obtained in the chromatographic response of octanal. Finally, a positive significant interaction among stirring speed, extraction time and extraction temperature was observed for dodecane.

It is important to note that significant negative effects have been observed for BC and AC interactions of octanal. This fact can be attributed to the extraction temperature, a factor present in both BC and AC interactions. In HS extraction mode, an increase in the sample temperature increases the volatility of the analytes and the headspace sensitivity, but with SPME this effect is counteracted by simultaneously lowering the concentration in the fiber coating due to the fact that adsorption is an exothermic process [25].

When considering the total sum of peak areas of the nine volatile compounds studied as response, the standardized Pareto chart shown in Fig. 1 can be obtained. As it can be seen, only the extraction temperature was significant and with a positive effect. This means that overall HS-SPME extraction efficiency improves when the temperature increases from 35 to 60 °C. Stirring and extraction time factors and all the possible interactions (AB, BC, AC and ABC) were not found to be significant.

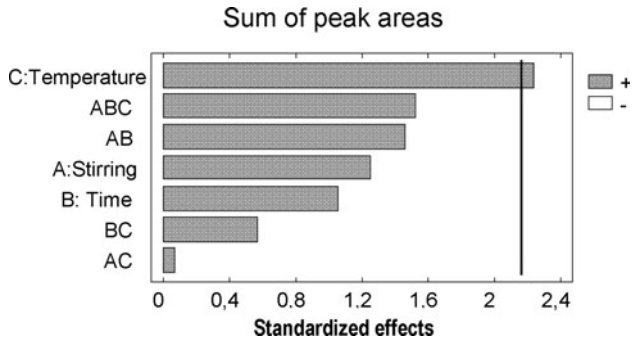

Fig. 1 Pareto chart for the standardized effects of the sum of peak areas for nine main detected compounds in Butte 4 almond oil: 1,3-dimethylbenzene, nonane, octanal, nonanal, dodecane, nonanoic acid, 2-undecenal, dodecanal, tetradecanal. The *vertical line* in the chart defines the 95% confidence level

On the basis of these results, an extraction temperature of 60 °C and extraction time of 60 min were selected as optimal conditions. This value of extraction time was selected as being significant for four of the nine studied compounds (nonanal, dodecane, nonanoic acid and 2-undecenal) and the presence of positive significant interactions between extraction time and other factors (as seen in Table 2). In spite of no significant positive effects being observed for stirring speed in none of the volatile compounds evaluated, 110 rpm was chosen as optimal stirring speed due to the positive significant interactions observed between this factor and the rest of variables for some of the studied compounds like dodecane.

Analysis of Almond Oils

The analysis of major volatile compounds in oil samples from different almond cultivars provides useful information which corroborated other reports [18] and added to the volatile fingerprint of almonds in the literature. The initial part of this work was the optimization of an HS-SPME–GC–MS method for extraction of volatile compounds in almond oils. The second part of the study was the analysis, under optimum extraction conditions found, of all almond oil samples (24) in order to determine the volatile profile of samples belonging to different almond cultivars (Marcona, Guara and Butte). Extractions were performed in duplicate using two different fibers to consider in this study fiber-to-fiber variation, which has been recognized as a possible problem in SPME analysis [26]. Acceptable results were obtained for repeatability, with relative standard deviations of replicates ranging from 5 to 12%, depending on the analyzed volatile compound. The purpose was to obtain discriminating parameters that could be useful in differentiating the almond cultivars to detect adulteration practices.

The same main volatile compounds were detected in all almond oil samples after analysis by HS-SPME–GC–MS as expected, since differences among cultivars are lower than those from different nuts. As a result, a total of 22 compounds were identified, which are listed in Table 3. Fig. 2 shows the full-scan chromatogram of Butte 4 almond oil sample obtained at optimized conditions.

12 compounds were selected for method evaluation since all of them were present in all samples and have been usually identified in edible oils in previous studies [17]. The presence of straight chain alkanals was detected for all oils (octanal, nonanal, decanal, dodecanal and tetradecanal). In general terms, it can be said that volatile content was higher for Spanish almond cultivars (Marcona and Guara) in comparison with Butte samples, as can be seen in Fig. 3.

Octanal and nonanal are oleic acid derivatives and it is important to point out that their content is clearly lower in

Table 3 Major volatile compounds identified for almond cultivars oils (Marcona, Guara and Butte) under optimum extraction conditions (60 °C, 60 min, 110 rpm)

Peak No.	Volatile compound[a]	Retention time (min)
1	3-Methyl-3-pentanol	3.1
2	Octane	4.1
3	1,3-Dimethylbenzene	6.2
4	Nonane	7.8
5	5-Hexen-2-ol	8.4
6	2-Methylpentane	9.8
7	Decane	14.3
8	Octanal	14.5
9	Undecane	19.5
10	Nonanal	19.6
11	Dodecane	23.7
12	Decanal	23.8
13	2-Decenal	25.8
14	Nonanoic Acid	26.3
15	Tridecane	27.3
16	Undecanal	27.5
17	2-Undecenal	29.4
18	Tetradecane	30.7
19	Dodecanal	30.9
20	Tetradecanal	37.1
21	Pentadecanal[b]	42.5
22	*n*-Hexadecanoic acid	46.1

[a] Volatile compounds were identified with a combination of mass spectra and retention time of standard compounds

[b] Volatile compound was tentatively identified with mass spectra only

samples from Butte cultivar compared with Spanish almond cultivar ones (Guara and Marcona), as it can be observed in Fig. 3. This fact is consistent with previous studies indicating that the Butte cultivar has the lowest oleic and stearic acids concentrations and the highest linoleic acid content [10].

Multivariate Analysis

In order to obtain the association between samples on the basis of nearness criteria among objects, a cluster analysis to the 12 volatile compounds, previously selected, was performed. As a result, the dendrogram shown in Fig. 4 arises, where two groups are differentiated at a dissimilarity level between 15 and 25. A sharp differentiation between the first group, consisting of Spanish cultivars, and the second one, the American almond samples, can be observed. In conclusion, the dendrogram confirms the fundamental association between Spanish cultivars as different from the American almond cultivar, Butte.

Based on this information, a discriminant analysis was applied to individual values of volatile compounds according to a stepwise method with the following three groups consisting of a single cultivar each: Marcona (group 1), Guara (group 2) and Butte (group 3). Two discriminant functions were obtained using the variable selection rule for minimizing Wilks′ lambda. The variance explained by the two discriminant functions was 92.2 and 7.8%, respectively.

Projections of cultivars scores on the two determined discriminant functions are shown in Fig. 5. The variables octanal, nonanal, tetradecanal, nonanoic acid, decane and

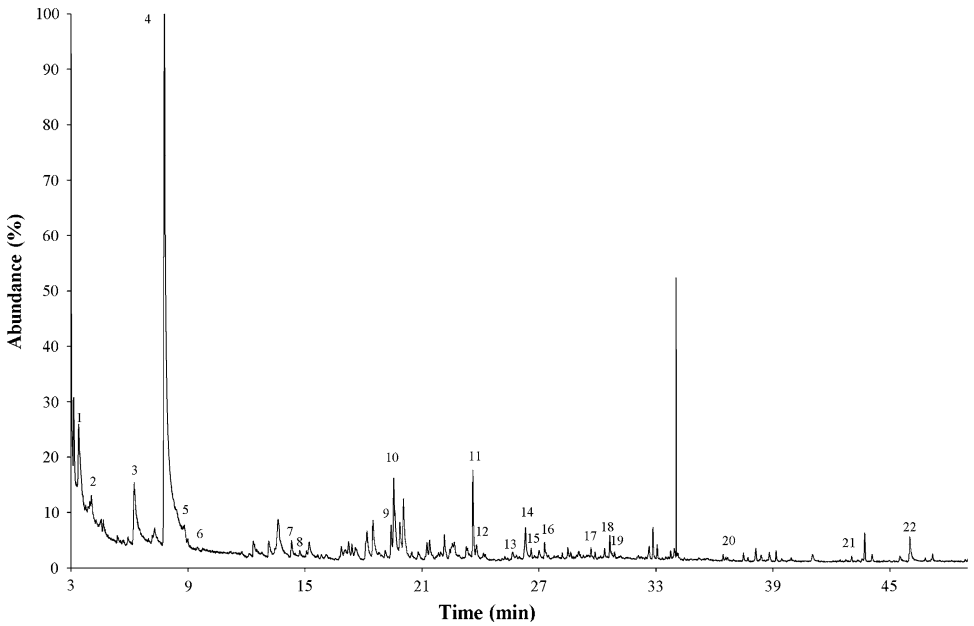

Fig. 2 HS-SPME–GC–MS chromatogram for volatile compounds obtained from Butte 4 almond oil under optimum extraction conditions (60 °C, 60 min, 110 rpm). Numbered peaks are listed in Table 3

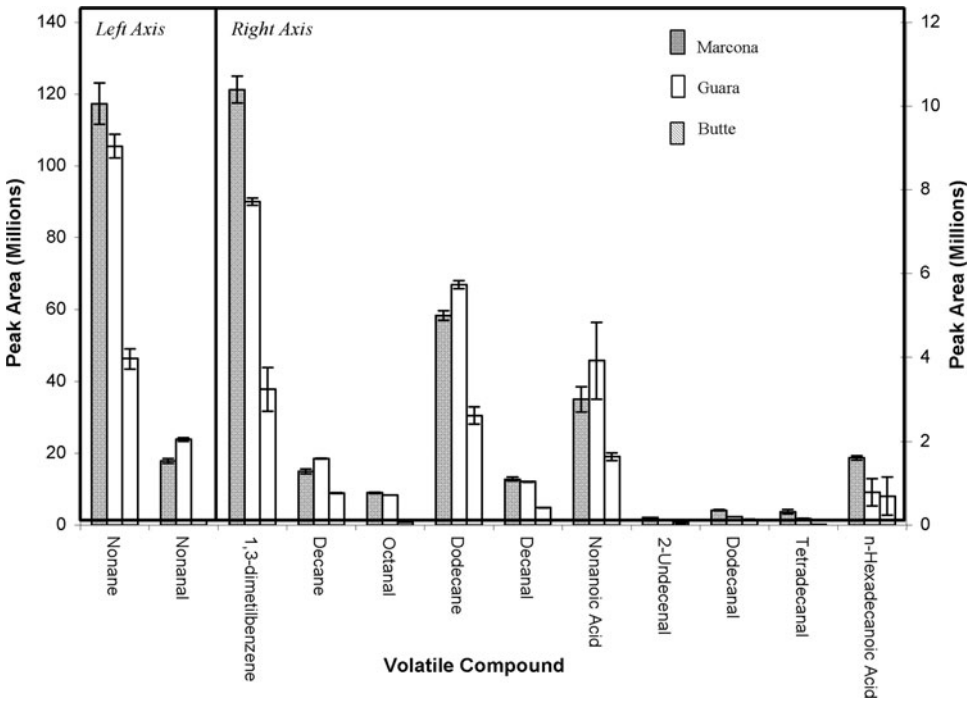

Fig. 3 Comparison of peak areas obtained for 12 volatile compounds found in almond oils from different almond cultivars: Marcona, Guara and Butte (60 °C, 60 min, 110 rpm)

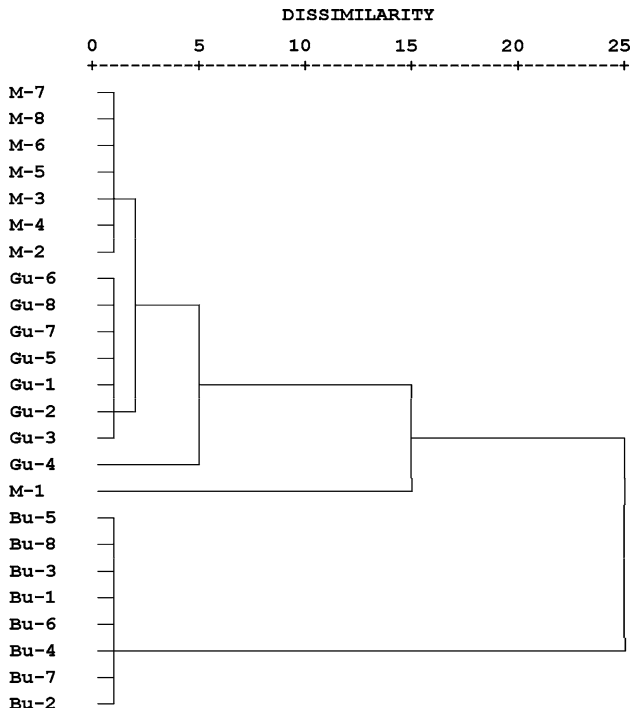

Fig. 4 Dendrogram obtained from cluster analysis of 12 volatile compounds: nonane, nonanal, 1,3-dimethylbenzene, decane, octanal, dodecane, decanal, nonanoic acid, 2-undecenal, dodecanal, tetradecanal and *n*-hexadecanoic acid. *Gu* Guara, *M* Marcona, *Bu* Butte

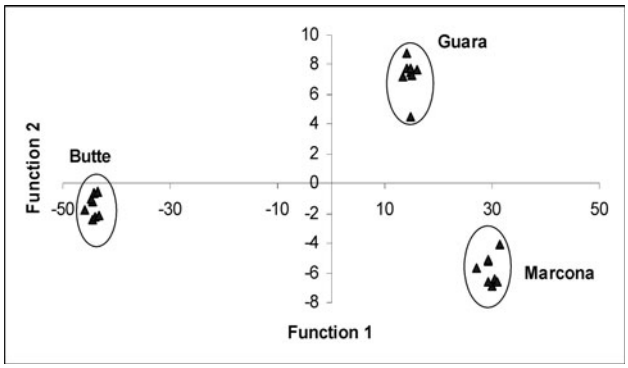

Fig. 5 Projections of cultivar scores on the space determined by the two discriminant functions

nonane were included in the analysis. On the other hand, the variables 1,3-dimethylbenzene, dodecane, decanal, 2-undecenal, dodecanal and *n*-hexadecanoic acid were not included in the calculations because their tolerance level was below the established minimum. The first discriminant function was predominantly influenced by nonanal, tetradecanal and nonanoic acid content. By using the calculated discriminant functions, samples were correctly classified in 100% of the cases, as it can be seen in Fig. 5. Also, it can be observed that samples from the Guara cultivar showed higher dispersion compared with those of Marcona and Butte cultivars. Finally, by applying the leave-one-out methodology, the generality of the LDA model was proved by correctly classifying external data.

Conclusions

HS-SPME–GC–MS has proved to be a valuable method for the extraction and detection of a large range of volatile

compounds in almond oils. It has shown itself to be a rapid, repeatable and sensitive technique. DVB/CAR/PDMS was employed as the extracting fiber and a 2^3 factorial design demonstrated that temperature and extraction time were the parameters having significant influences on the efficiency of the extraction process. A total of 22 volatile compounds were detected in almond oils the straight chain alkanal (octanal, nonanal, decanal, dodecanal, tetradecanal) being the main chemical group present. From the three cultivars studied, American Butte showed a lower nonanal content, which could be attributed to its lowest oleic acid content in comparison with Spanish almond cultivars. Finally, the use of multivariate techniques applied to the obtained volatile compounds profile has been successful for the classification and discrimination of different almond cultivars. In this sense, LDA gave a better performance as all the studied samples were correctly classified, in contrast with cluster analysis which allowed the classification of Spanish samples as a consistent group different from the American almond cultivar. These results constituent an important base for avoiding adulteration practices in food products.

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