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Recognition of volatile compounds as markers in geographical discrimination of Spanish extra virgin olive oils by chemometric analysis of non-specific chromatography volatile profiles

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ABSTRACT

Chromatographic profiles obtained by headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography (GC) were processed as continuous and non-specific signals through multivariate analysis techniques in order to select and identify the most discriminate volatile marker compounds related to the geographical origin of extra virgin olive oils. The blind analysis of the chromatographic profiles was carried out on several steps including preliminary mathematical treatments, explorative analysis, feature selection and classification. The results obtained through the application of stepwise linear discriminant analysis (SLDA) method revealed a perfect discrimination between the different Spanish geographical regions considered (La Rioja, Andalusia and Catalonia). The assignment success rate was 100% in both classification and prediction by using cross validation procedure. In addition, it must be noted that the proposed strategy was able to verify the geographical origin of the samples involving only a reduced number of discriminate retention times selected by the stepwise procedure. This fact emphasizes the quality of the accurate results obtained and encourages the feasibility of similar procedures in olive oil quality and traceability studies. Finally, volatile compounds corresponding to the predictors retained were identified by gas chromatography–mass spectrometry (GC–MS) for a chemical interpretation of their importance in quality virgin olive oils.

1. Introduction

Extra virgin olive oil is a valuable and prized foodstuff in Mediterranean countries. In order to guarantee and promote high quality extra virgin olive oils that show particular quality properties attributed to their geographical area of production, the European Union (EU) legislates and regulates the creation of protected designations of origin (PDO) [1,2]. Oils labelled with this designation acquire an "added-value" which results in a higher price in the market. This fact highlights the importance of ensuring the authentication of these products with the aim of providing real protection for the rights of consumers and reliable producers from fraudulent practices involving, for instance, mixing with cheaper oils in order to make a profit. Therefore, food safety and traceability are essential challenges that must be faced in quality control tasks in order to achieve an objective differentiation among the different olive oils to ensure the declared origin and the quality labelled. According to these goals, reliable methods based on the evaluation of quality factors and properties are required.

In this sense, flavour is a remarkable factor that plays an important role in the determination of extra virgin olive oils authenticity. Distinctive sensorial properties can be attributed to a complex mixture of more than one hundred volatile compounds [3,4] from different chemical classes, mainly aldehydes, alcohols, esters, hydrocarbons and ketones. In high quality virgin olive oils, C_6 and C_5 compounds enzymatically generated through the lipoxygenase (LOX) pathway represent the most important fraction of volatile profiles. [5–7].

The official procedure established by European legislation and the International Olive Oil Council (IOOC) [8–10] for the sensory analysis of olive oil's flavour involves notable problems related to poor repeatability and subjectivity [11]. Therefore, since the volatile profile of olive oils represents a fingerprint of the samples, 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 approaches for evaluating olive oil authenticity.

A number of previous studies have reported the effectiveness of different analytical procedures in the development of strategies focusing on olive oil characterisation purposes [12–17]. In this context, gas chromatography (GC) coupled with mass spectrome-

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try (MS) has been the most widely employed, involving different extraction and pre-concentration techniques of the volatile fraction in order to extract minor compounds and obtain representative profiles [18–20]. The results reported showed that solid phase microextraction (SPME) technique [21,22], had important advantages over other extraction procedures in the study of virgin olive oils. SPME is a sensitive, solvent-free, cost-efficient and fast method which allows the extraction and the concentration steps to be performed simultaneously. SPME in combination with GC–MS analysis has been successfully applied in olive oil characterisation and classification research to evaluate the effect of geographical origin, environmental conditions, olive varieties and detection of adulterations [23–31]. This analytical methodology has been traditionally associated with target analysis involving a first step of identification and quantification of specific markers existing in the volatile fraction. However, finding adequate and sufficiently specific markers is not always a quick and reliable procedure and therefore the quality assurance study may be tackled by considering the chromatographic profiles as continuous and non-specific signals, avoiding a priori identification of compounds. In this sense, realisable classification strategies involving foodstuff fingerprints have been developed in recent studies by applying different chemometrical tools mainly over spectroscopic and spectrometric matrices directly acquired on untreated food product samples [32–37]. In the same way, chromatographic fingerprints have been studied in fields such as the petroleum industry and herbal medicine [38–40]. On the basis of these results it is reasonable to assume that multivariate analysis of chromatographic profiles may be an efficient strategy to recognize the volatile compound markers associated with the geographical discrimination of virgin olive oils.

The aim of the present study lies in identifying a series of volatile compounds able to discriminate extra virgin olive oils in accordance with geographical origin through the development of a strategy based on the blind analysis of the volatile fraction by means of combining the non-specific volatile profiles with multivariate analysis, in order to work directly with the whole fingerprint. The methodology proposed was based on the combined use of headspace (HS) SPME/GC–MS with the subsequent analysis of the chemical fingerprints using a SLDA method [41,42] and a PCA data compression strategy to classify olive oils from different geographic origins. Finally, the identification of the volatile compounds selected for the geographical differentiation of the categories to be studied was carried out.

Therefore, the main novelty of the present work concerns the study of the potential of using non-specific volatile profiles to identify the volatile marker compounds related to the geographical discrimination of EVOOs through the development of a discrimination strategy based on combining the non-specific volatiles profiles with multivariate analysis. This procedure could be of great interest in olive oil authentication research because it involves the application of a large potential analytical technique in volatile component analysis (SPME/GC–MS) and presents a distinctive feature with respect to other previous studies carried out in this field because it avoids the need to carry out an a priori peak identification allowing direct work on the whole fingerprint. The proposed procedure was applied to the discrimination of extra virgin olive oils produced and manufactured in La Rioja, a region located in the north of Spain and distinguished by the certificated POD "Aceite de La Rioja" since 2004, from other protected extra virgin olive oils produced in southern and eastern regions of Spain according to their origin.

To our knowledge the identification of features related to the geographical origin of extra virgin olive oils through the blind analysis of chromatographic profiles has not been previously reported.

2. Material and methods

2.1. Extra virgin olive oil samples

The data set comprised a total of forty extra virgin olive oil samples from several Spanish regions, all of them labelled with the PDO quality trademark and collected in the same harvest. Nine of the samples corresponded to extra virgin olive oils from PDO "Aceite de La Rioja", twenty-three were produced and manufactured in several PDOs from Andalusia, and eight samples came from PDO Siurana and PDO Les Garrigues (Catalonia). The samples were divided into three categories (Andalusia, La Rioja and Catalonia) according to their geographical origin.

To avoid oxidation and rancidity processes, oil samples were stored at room temperature, in amber glass bottles and in dark conditions until the analysis was carried out. All the samples were analysed in triplicate.

2.2. HS-SPME

The extraction of the volatile compounds was carried out by HS-SPME. The conditions associated with this extraction procedure were selected taking into account the results reported in previous research involving the analysis of the virgin olive oil volatile fraction [4,43]. In accordance with these results, a divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm) fibre purchased from Supelco (Bellefonte, PA, USA) was selected. Before use, the fibre was conditioned in accordance with the manufacturer's specifications. HS-SPME analyses were performed by placing a 3 g sample of extra virgin olive oil into a 20 ml headspace vial. Then the vial was hermetically sealed with a PTFE septum and preincubated at 40 °C for 5 min under magnetic stirring at 250 rpm. Then, the extraction was carried out by exposing the fibre to the headspace for 60 min. When the sampling was completed the SPME device was removed from the vial and immediately inserted into the injection port of a GC/MS system for thermal desorption at 260 °C for 5 min.

2.3. Apparatus and chromatographic conditions

Analyses were performed with a Varian 3800 gas chromatograph (Varian Chromatography Systems, Walnut Creek, CA, USA) equipped with a CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland) and connected to an ion-trap mass spectrometer (Varian Saturn 2200). Separation of compounds was carried out using a CP-WAX 52-CB column (30 m × 0.25 mm I.D., 0.25 μm film thickness) purchased from Varian. Helium was used as carrier gas with a flow rate of 1 ml min^{-1}. Injection was performed in splitless mode for 2 min. Oven temperature was initially held at 40 °C for 10 min, and subsequently programmed to raise to 200 °C at a rate of 3 °C/min. The manifold, GC–MS interface and ion trap temperatures were kept constantly at 60, 280 and 200 °C, respectively. Mass spectra were performed in electron impact mode (EI) at 70 eV in the 35–350 m/z range. The identification of the compounds was achieved by comparing the mass spectra of the unknown peaks with the information available in the National Institute of Standards and Technology (NIST) library.

2.4. Data analysis

The chromatographic fingerprint for each sample was composed for as many variables as retention times recorded during the data acquisition time. The range from 2 to 35 min turned out to be the most informative region and therefore was retained for further pre-processing and classification steps. Moreover, before applying the multivariate chemometric approach to data analysis,

a signal pre-processing step was required in order to obtain a uniform representation of the signals in which retention times were synchronized [44]. The strategy to eliminate the systematic shift observed in the profiles involved a target peak alignment. [45].

In order to eliminate the effect of irreproducibility which is closely related not only with the loss of sensitivity in the fibre over time but also to mass spectrometer instruments, the data matrix was submitted to a row profile pre-treatment before calculating the mean of the replicates. This scaling method consists of dividing each value of the original variables by the row sum:

$$y_{iv} = \frac{x_{iv}}{\sum_{v=1}^{v} x_{iv}}$$

Therefore, the results of each individual variable are expressed as a percentage of the total sum of signals [16,46].

One decisive parameter that has to be set prior to the application of any supervised classification method is the number of categories to be considered and the particular requirements that a sample must fulfil in order to be assigned to a certain class. Therefore, in the present geographical classification approach three categories (Andalusia, La Rioja and Catalonia) were considered.

It must be noticed that the application of LDA as pattern recognition technique is subject restriction concerning the ratio between the number of samples and the number of variables, in such a way that the number of variables should not exceed the number of samples. Since the present work involves a profile study, the number of variables is much larger than the number of objects and therefore, two different strategies were considered to achieve a reduction in the dimensionality of the data and to remove noisy and non-informative variables before the application of LDA. In the first attempt, principal component analysis (PCA) was performed in order to select the number of significant principal components. The resulting PCA matrix score was the input for LDA. In a second attempt SLDA was applied as a classification method to select the most suitable variables in order to optimize the discrimination. In this strategy, LDA classification method was developed by applying a forward stepwise variable selection algorithm using a Wilks' Lambda as a selection criterion and an *F*-statistic factor to determine the significance of the changes in Lambda when the influence of a new variable is evaluated (*F* value to enter = 1; *F* value to remove = 0.5). Therefore, only the most discriminant variables involved in sample differentiation were selected. LDA is probably the most frequently applied pattern recognition technique [47–50]. LDA is based on a search for directions (discriminant functions) which achieve maximum separation among categories by maximizing the between-class variance and minimizing the within-class variance. Each of these discriminant functions represents a new variable called the canonical variable which turns out to be a linear combination of the original predictors. In general, when there are *c* categories, only $(c-1)$ independent discriminant functions can be computed. The principal advantage of the proposed strategy is the ability to perform a feature selection. Regarding this fact, only those variables which helped to improve classification performance were used whereas variables without discriminant information were discarded.

In order to determine model stability the models achieved should be validated by cross-validation procedure through a test set of samples not used to construct the model. However, the number of samples available in the present work was limited and therefore was not sufficient to support the creation of an external test set. Nevertheless, it was possible to apply cross validation procedure several times with a different number of cancellation groups including leave-one-out (LOO) procedure. This methodology allows significant measurement of the predictive ability [51].

The alignment was implemented in MATLAB® version R2010a [52]. Pre-treatments and SLDA were carried out using V-Parvus software [53].

3. Results and discussion

3.1. Exploratory analysis

Since the proposed strategy is based on blind multivariate analysis of chromatographic profiles, areas of chromatographic peaks were not integrated; nor was a priori assignment of the peaks to its corresponding volatile compound carried out. The aligned and row profile scaled data set comprising 722 variables and 40 samples constituted the starting point for the pattern recognition analysis. The number of categories was defined according to the aim of discriminate protected extra virgin olive oils from different Spanish regions. The three categories considered were: La Rioja, Andalusia and Catalonia.

In a first attempt, principal component analysis (PCA) was performed as a preliminary step in multivariate analysis in order to extract and display the main information existing in multivariate data. The results obtained when PCA was performed on centred matrix were slightly better than those obtained from autoscaled matrix, thus only the centred data results were considered and graphically analyzed by the scores plots obtained when the objects were projected into the principals' components. This figure showed a partial overlapping between the three geographical regions. However, it could be pointed out that the first principal component evidenced a trend towards discrimination amongst Andalusia and Catalonia samples, and olive oils from La Rioja appeared partially separated from Andalusia olive oils on the second principal component (Fig. 1).

3.2. PCA data compression

Since LDA could not be performed directly on the data matrix because of the small ratio between the number of samples and the number of variables, the classification model was developed on the first principal components in order to reduce the dimensionality of the data. For the present work the first ten principal components, corresponding to a 91.5% of total explained variance were retained (Table 1), and the predictive abilities of the model were validated

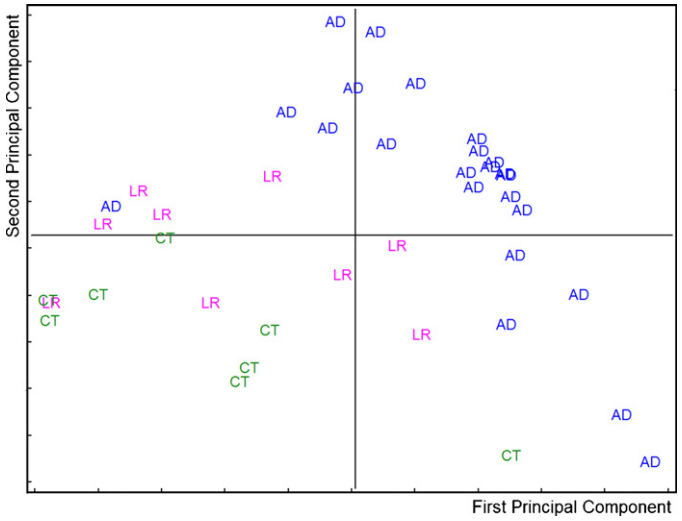

Fig. 1. Score plot on the two first principal components of the centred data. The four geographical categories considered were labelled as LR (La Rioja), AD (Andalusia) and CT (Catalonia).

Table 1
Percentages of individual and cumulative variance explained by the ten first principal components.

Component	Individual explained variance (%)	Cumulative variance (%)
1	34.38909	34.389
2	19.38792	53.777
3	11.31755	65.095
4	7.67206	72.767
5	6.20519	78.972
6	3.96593	82.938
7	3.24114	86.179
8	2.03767	88.217
9	1.81407	90.031
10	1.43749	91.468

by cross validation through 5 and 7 cancelation groups and LOO procedure. The results reported in Table 2 showed the unsuitability of this strategy for achieving an accurate classification due to low percentages of correct prediction for extra virgin olive oils from La Rioja.

The poor ability of the data to discriminate between the three defined categories could be closely related to the fact that PCA selects a direction that retains maximal structure among the data in a lower dimension and LDA selects the direction that achieves maximum separation among the given classes.

3.3. SLDA

SLDA was performed on the chromatographic profiles in order to extract only the variables with the highest discriminant ability between the geographical regions studied. This strategy involved a substantial reduction of the dimensionality of the data in such a way that only six variables were retained by the stepwise procedure and used as input in LDA classification. The successful results obtained after performing LDA on the reduced data set showed unequivocal discrimination between the three geographical regions considered, so that all the samples were correctly classified and predicted. The prediction ability of the SLDA model developed was evaluated by cross-validation (Table 3).

Since each canonical variable represents a direction (discriminant function) with maximum separation among categories, the plots obtained by projecting the samples on the canonical variables are highly regarded as powerful visualization tools. Thus, the reliability of the classification results obtained from the proposed strategy were graphically confirmed by the bi-plots obtained when the extra virgin olive oil samples were projected onto the space defined by the first two LDA canonical variables (Fig. 2). This figure revealed a clear separation between the three categories. It also can be argued that protected extra virgin olive oils from Catalonia were largely separated from those produced in Andalusia by the first canonical variables, while protected extra virgin olive oils from La Rioja were mainly separated from the other two categories by the second canonical variable.

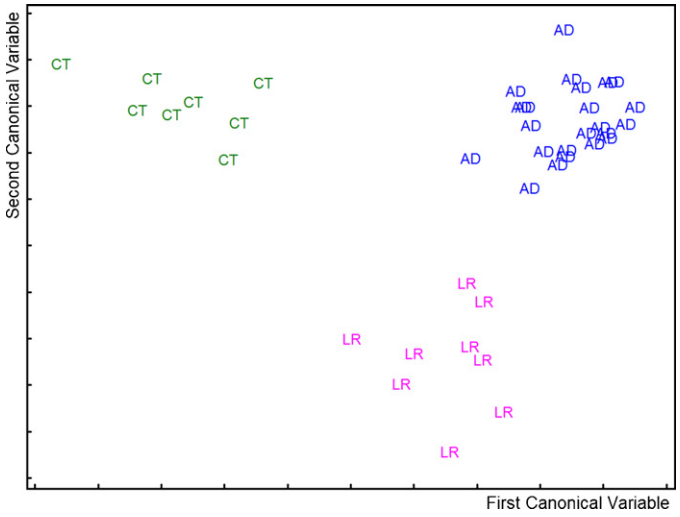

Fig. 2. Score plot on canonical variables of extra virgin olive oil samples after performing SLDA. Samples were shown by their category symbol: LR (La Rioja), AD (Andalusia) and CT (Catalonia).

It must be pointed that only six informative predictors from the large set of original variables were enough to discriminate successfully between the three categories. Therefore, the highlight of the proposed SLDA strategy was the ability to achieve a high compression of the dimensionality, which resulted in the development of low complexity models able to discriminate extra virgin olive oils on the basis on their geographical origin.

3.4. SLDA discriminant variables: identification of volatile compounds markers

The quality of the SLDA classification model developed on non-specific chromatographic profiles was evaluated from a statistical point of view, without taking into account any chemical considerations, to obtain the subset of discriminant variables. Therefore a final step based on the identification of the volatile compound selected by the proposed feature selection algorithm was carried out to gain further knowledge about the results obtained from a chemical standpoint.

Table 4 summarised the volatile markers identified in accordance with the six retention times selected by SLDA for the geographical discrimination of extra virgin olive oils PDO "Aceite de La Rioja" from protected extra virgin olives oils from Andalusia and Catalonia. The identification of the compounds was carried out using the information available in the mass spectra library with the only exception of the peak corresponding to the retention time 9.15 min. Taking into account the fragment ions detected for this peak, the corresponding compound was identified as a pentene dimer in accordance with the mass spectra information reported in a previous characterization study [54].

Table 2
Percentages of correctly classified samples corresponding to the LDA model developed on the PCA score matrix.

	5 CV		7 CV		LOO	
	Classification (%)	Prediction (%)	Classification (%)	Prediction (%)	Classification (%)	Prediction (%)
Category rates (R_c)						
Andalusia	93.91 (108/115)	78.26 (18/23)	95.03 (153/161)	86.96(20/23)	95.54 (879/920)	82.61 (19/23)
La Rioja	93.33 (42/45)	55.56 (5/9)	93.65 (59/63)	55.56 (5/9)	98.33 (354/360)	55.56 (5/9)
Catalonia	100.0 (40/40)	87.50 (7/8)	98.21 (55/56)	75.00 (6/8)	99.69 (319/320)	87.50 (7/8)
Total rate (TR)	95.00	75.00	95.36	77.50	97.00	77.50

The number of correct/total classifications (predictions) appears in brackets. Each object is classified a number of times according to the number of cross-validation groups (5, 7 and 40 (LOO)) and predicted once.

Table 3
Percentages of correctly classified samples corresponding to the SLDA classifications developed.

	5 CV		7 CV		LOO	
	Classification (%)	Prediction (%)	Classification (%)	Prediction (%)	Classification (%)	Prediction (%)
Category rates (R_c)						
Andalusia	100 (115/115)	100 (23/23)	100 (161/161)	100 (23/23)	100 (920/920)	100 (23/23)
La Rioja	100 (45/45)	100 (9/9)	100 (63/63)	100 (9/9)	100 (360/360)	100 (9/9)
Catalonia	100 (40/40)	100 (8/8)	100 (56/56)	100 (8/8)	100 (320/320)	100 (8/8)
Total rate (TR)	100	100	100	100	100	100

The number of correct/total classifications (predictions) appears in brackets. Each object is classified a number of times according with the number of cross-validation groups (5, 7 and 40 (LOO)) and predicted once.

Table 4
Identification of the six volatile compounds corresponding to the variables selected by SLDA.

Order of selection	Retention time (min)	Main m/z ions observed in MS spectra	Identification
1	16.44	39,41,55	E-2-hexenal
2	3.57	41,44,43	3-methylbutanal
3	16.16	41,39,55,70	3-methyl-butan-1-ol
4	9.15	41,39,43	Pentene dimer
5	25.50	67,39,41	Z-3-hexen-1-ol
6	24.18	56,41,39,55	Hexan-1-ol

The pathways involved in the production of volatile compounds in virgin olive oils play an important role in olive oil quality in such a way that the dominance of one over the other may lead to an unpleasant flavour increase. Hexan-1-ol, Z-3-hexen-1-ol, E-2-hexenal and pentene dimer are enzymatically produced from polyunsatured fatty acids through different branches of LOX pathway: Hexan-1-ol has linoleic acid (LA) as precursor, Z-3-hexen-1-ol and E-2-hexenal derive from the linolenic (LnA), whereas pentene dimers are generated by the dimerization of 1, 3-pentene radicals. The concentration of the C_6 compounds generated through LOX cascade is strongly associated with the cultivar origin, variety and processing conditions. On the other hand, 3-methylbutanal and 3-methyl-butan-1-ol are volatile compounds generated by the conversion of aminoacids [5–7]. In this regards, the LOX pathway is the most important process for the generation of volatile compounds in olive oils obtained from properly processed healthy and ripe fruits; the fact that most of the discriminat compounds were generated through this biochemical cascade may be rationalised by the high quality of the studied olive oils.

The contribution of the volatile compounds to the flavour has been investigated in previous studies in order to establish relationships between the volatile compounds and sensory attributes associated with both positive and defective perceptions [6,55,56,15]. E-2-hexenal and Z-3-hexen-1-ol were associated with green sensory perceptions. Moreover, E-2-hexenal was correlated with bitter and almond attributes, and Z-3-hexen-1-ol contributes to banana notes. Pentene dimer and hexan-1-ol were involved in the contribution of tomato odour notes as well as in leaf and walnut husk, and fruity sensations respectively. Finally the presence of 3-methyl-butan-1-ol should be briefly discussed. 3-Methyl-butan-1-ol is present in low concentrations in quality olive oils and accounted for spicy and malt notes. However, in high concentrations it contributes to mustiness-humidity and winey-vinegary sensory-defects. The low amount of 3-methyl-butan-1-ol found in the present study highlights the quality of the olive oils selected for the present study.

4. Conclusions

In the present study, a classification methodology based on the blind analysis of non-specific chromatographic profiles was proposed in order to select and identify the most discriminate volatile markers associated with the geographical origin of extra virgin olive oils. The results reported showed that the combination of HS-SPME/GC analysis with SLDA method resulted in a satisfactory discrimination between the studied categories involving only six input variables. The subsequent identification of the six volatile compounds selected as discriminant features provided a chemical interpretation of the results obtained. Although the results obtained are fairly promising, encouraging similar chemometric approaches to be considered in future quality olive oil assurance studies, it must be taken into account that the actual applicability of the classification methodology proposed would require further research, extending the study to a broader number of samples, in such a way that the constraints on the robustness of the classification model can be solved. Moreover it could be interesting to examine different origins and harvesting years.

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