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Characterization of aroma compounds in Portuguese extra virgin olive oils from Galega Vulgar and Cobrançosa cultivars using GC–O and GC × GC–ToFMS

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ABSTRACT

Aroma compounds of virgin olive oils extracted from two olive cultivars – Galega Vulgar and Cobrançosa – grown in Beira Baixa region in central Portugal were investigated. Gas chromatography–olfactometry (GC–O) was carried out to select the important odorants for subsequent comprehensive gas chromatography/time of flight mass spectrometry (GC × GC–ToFMS) analysis. By GC–O fifteen odorants were identified. For the quantification of volatile compounds, headspace solid phase microextraction (SPME) technique was optimized. Under optimized conditions, 22 volatile compounds were quantified in all samples. *Trans*-2-hexenal was the most abundant compound. A discriminant analysis (DA) was used to discriminate among olive oil samples obtained from olives of the two cultivars with different harvest time/ripening stages. Concerning the harvesting time and cultivar, nine volatiles showed to have discriminant power among samples, namely heptanal, *trans*-2-hexenal, 1-octen-1-ol, nonanal, 2,3-butanedione, ethyl-2-methylbutyrate, hexanal, *cis*-3-hexenylacetate and 3-methylbuthylacetate.

1. Introduction

The research on virgin olive oil (VOO) sensory characteristics is mostly based on trained panels to recognize and evaluate many attributes representing several descriptors. Moreover, the official method to perform organoleptic assessment of VOO is based in well-established methodology, with selected and well trained assessors (EC, 2008). However, this technique is not exempt of risks and sometimes the classification errors may lead to serious economic losses (Aparicio, Morales, & García-González, 2012). More precise and diagnostic chemical information may be expected with gas chromatography/mass spectrometry (GC–MS) analyses of aroma compounds of VOO (Frankel, 2010).

The identification of aroma compounds in VOO is a challenging task, as hundreds of compounds are quite often present in the volatile fraction, differing by orders of magnitude in their concentration (Boskou, 2006). Among these compounds, only a small fraction contributes to the aroma of olive oil (Angerosa, Servili, Selvaggini, Taticchi, Esposto and Montedoro, 2004). Only those volatiles able to interact with the receptor proteins in the human olfactory bulb are responsible for a certain aroma (Belitz, Grosh, & Shieberle, 2009). The knowledge that not all of the volatiles occurring in a food contribute to its aroma was the reason for changing the methodology of their analysis and the developments of gas chromatography–olfactometry (GC–O) methods. Furthermore, the results led to the conclusion that less than 5% of the volatiles identified in foods contributed to their aromas (Grosch, 2000).

The aroma of olive oil is mainly attributed to aldehydes, alcohols, esters, hydrocarbons, ketones and furans (Morales & Tsimidou, 2000). Virgin olive oils produced from fruits of good quality, where the lipoxygenase (LOX) pathway is the predominant source of compounds biogeneration, are usually described by perception of fruity sensations, freshly cut grass, green fruits such as apple, banana, or vegetables, such as artichoke or tomato, accompanied by more or less intense taste notes of bitterness and pungency (Angerosa, 2002; Aparicio & Luna, 2002; Cerretani, Salvador, Bendini, & Fregapane, 2008). From a quantitative point of view, C6 linear unsaturated and saturated aldehydes represent the most important fraction of volatile compounds of high quality VOO (Angerosa et al., 2004). An increase on the intensity of fruity *vs.* green notes was observed in virgin olive oils, after sedimentation and racking, showing that olive oil flavor profile is affected by the technological operations (Reboredo-Rodríguez, González-Barreiro, Cancho-Grande, & Simal-Gándara, 2013).

Sample preparation is an essential step in the analysis of aroma compounds in VOO, greatly influencing the precision and accuracy of the results and the time and cost of the analysis. Concentration of olive oil aroma compounds using sorptive methods became the most widely used group of methods in the past two decades, and

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since the last 10 years, solid phase microextraction became the most frequently used of sorbent-based method for the analysis of flavors and off-flavors in virgin olive oil (Cajka, Riddellova, Klimankova, Cerna, Pudil and Hajslova, 2010; Cavalli, Fernandez, Lizzani-Cuvelier, & Loiseau, 2003; Dierkes, Bongartz, Guth, & Hayen, 2011; Jiménez, Beltrán, & Aguilera, 2004; Kalua, Bedgood, Bishop, & Prenzler, 2006; Kanavouras, Kiritsakis, & Hernandez, 2005; Vaz-Freire, Silva, & Freitas, 2009; Vichi, Castellote, et al., 2003; Vichi, Pizzale, Conte, Buxaderas and López-Tamames, 2003). Therefore, gas chromatography–olfactometry (GC–O) is considered a good tool for screening sensory active compounds. The technique consists of isolation of the volatile fraction and concentration of the extract, and then an aliquot is separated by GC and the effluent is split into a flame ionization detector (FID) and a sniffing port (Morales, Rios, & Aparicio, 1997; Reiners & Grosch, 1998).

The identification and the quantification of the compounds causing flavor or off-flavor is considered one of the keys for VOO quality control and a good indicator of olive oil quality changes (Kalua et al., 2007). Moreover, olive cultivar as well as ripening stage, strongly influences the abundance of volatiles compounds and consequently this knowledge may be applied to produce VOO with particular sensory notes from chosen cultivars at certain ripening stages (Prenzler, Robards, & Bedgood, 2007). Also, VOO obtained under irrigation conditions can display higher whole aroma concentration than the rainfed ones (Baccouri et al., 2007). Different water stress levels in olive trees affected the amount of VOO volatile compounds, showing that *trans*-2-hexenal, hexan-1-ol, and *cis*-3-hexen-1-ol concentrations were higher in olive oils extracted from olives from irrigated groves (Gómez-Rico, Salvador, La Greca, & Fregapane, 2006). Hence, different amounts of total volatiles can be related to diverse agronomic conditions.

The aim of the present study was to analyze flavor compounds in two of the most important extra virgin olive oils (EVOO) from Portugal: one produced from the cultivar Galega Vulgar and the other from cultivar Cobrançosa, in early stages of ripening. Our goal was to select the main compounds responsible for major odor notes in these olive oils using GC–O, and then to quantify the detected odorants, as well as other compounds known from the literature to have influence on olive oil flavor using a dedicated method based on solid phase microextraction and comprehensive gas chromatography/mass spectrometry. Up to now, there is no detailed information available on the identification of the main odorants in Portuguese EVOO by GC–O, although it is known that they are influenced by the time of harvesting (ripening stage) of the fruits, affecting consumers' sensory evaluation of these oils. Concerning this, a correct evaluation of the effects of early stages of ripening on VOO aroma is a very important aspect, especially for Galega cultivar, the most important Portuguese olive cultivar. This cultivar is strongly attacked by pests and diseases, so if the harvesting period could start earlier, this would be very important from a sustainable agriculture point of view, minimizing pesticides use.

2. Material and methods

2.1. Olive samples, chemicals and reagents

Olive samples cvs. Galega Vulgar and Cobrançosa (8 samples of each cultivar), were harvested in 2011 in two olive groves in Beira Baixa region (center of Portugal). Samples were organized into four groups, by cultivar and by two harvest times: first harvest time corresponds to Galega Vulgar and Cobrançosa olives picked in October; second harvest time corresponds to Galega Vulgar and Cobrançosa olives picked in November.

Their ripening indices (RI) were determined following the guidelines of Estación de Olivicultura y Elaiotecnia, Jaén, Spain (Hermoso, Uceda, Frias, & Beltran, 1997). Only healthy fruits were selected for olive oil extraction. Average water content in the crude pastes was 55 and 54% in 'Galega' and 'Cobrançosa', respectively. Olive oils were extracted using an Abencor system (MC2 Ingenieria Sistemas, Seville, Spain), equipped with a hammer mill (3000 rpm), a thermobeater (50 rpm) and a centrifuge (3000 rpm). Oil extraction was performed by thermobeating at 28–30 °C, for 30 min. European Union chemical quality criteria for olive oil (acidity value, peroxide index (IP) and UV light absorption (K_{232} and K_{270})) was carried out following the analytical methods described in EEC/2568/91 EU Regulation. Fatty acid composition was performed by GC/FID in accordance with EEC/2568/91 EU Regulation. Samples for volatile compound analysis were stored at −20 °C until analysis. Standards of volatile compounds used for quantitation were purchased at Sigma Aldrich (Poznań, Poland) with the highest available GC standard grade. Dichloromethane was purchased from Sigma Aldrich in a Chromasolv purity (99.9%). SPME fibers — Carboxen/Polydimethylsiloxane (CAR/PDMS); Polydimethylsiloxane (PDMS); Polydimethylsiloxane/Divinylbenzene (PDMS/DVB) and 2 cm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) were purchased from Sigma Aldrich and preconditioned according to manufacturer's recommendation.

2.2. GC–O analysis

Gas chromatography–olfactometry analyses were performed on a HP 5890 II (Hewlett Packard, Santa Clara, CA) gas chromatograph equipped with an olfactory port. To obtain samples for GC–O analyses, 90 g of oil were dissolved in 250 mL of dichlorometane. Volatile compounds were isolated using solvent assisted flavor evaporation (SAFE) apparatus (Engel, Bahr, & Schieberle, 1999). Rotary vane pump was used in SAFE apparatus to reduce the pressure to values lower than 30 kPa to ensure vacuum transfer of volatiles. SAFE apparatus was cooled using liquid nitrogen. Extract containing volatile compounds was concentrated using Kuderna-Danish concentrator to a volume of approximately 0.5 mL. Concentrated extract was analyzed on two columns of different polarities: non polar DB-5 column (30 m × 0.32 mm × 0.5 μm, Agilent Technologies, USA) and a polar Supelcowax-10 (30 m × 0.25 mm × 0.5 μm, Supelco, Bellefonte, PA), both with Y type glass splitters allowing simultaneous detection of compounds by flame ionization detector and sniffing. The oven program for GC–O analysis was the following: 40 °C for 1 min, then 6 °C/min to 180 °C, followed by 20 °C/min to 260 °C (3 min), for DB-5 column and 40 °C (0 min), then 5 °C/min to 170 °C, followed by 25 °C/min to 250 °C (4 min) for Supelcowax-10 column. A volume of 2.5 μL of extract was injected into the column in a splitless mode with purge valve closed for 2 min. Retention indices were calculated for each compound using homologous series of C_7–C_{24} n-alkanes.

2.3. Development of SPME extraction method

For the development of the extraction method using solid phase microextraction (SPME), a mixture of 1-pentene-3-one, 2-penten-1-ol, hexanal and 2-hexenal in refined rapeseed oil was used, so the selected volatile compounds represent different chemical classes. In a first step, the fiber providing the highest peak responses was chosen at a temperature of 40 °C. Then, different extraction temperatures were compared (40 °C, 50 °C and 60 °C), and different extraction times (5, 10, 15, 20, 30 and 60 min). Peak areas for analyzed compounds added to refined rapeseed oil in concentrations of 0.1, 0.5, 1 and 10 mg/L were compared for each fiber to evaluate linearity of compounds adsorption onto the fiber's surface. The evaluation was performed on a HP 6890 gas chromatograph equipped with FID detector and the same polar column as in GC–O experiment. Peak areas were compared in SPME method development procedure.

2.4. SPME–GC × GC–ToF-MS analysis

Quantitative analysis was performed using SPME and gas chromatograph coupled to time of flight mass spectrometer. Volatile compounds were identified and quantified using GC × GC–ToF-MS system (Pegasus

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Table 1
Fatty acid composition (%) and quality criteria (acidity, peroxide index and UV absorbances) of Galega Vulgar and Cobrançosa virgin olive oils. In each row superscript indexes indicate differences based on Tukey test. No indexes indicate no differences between samples.

	Galega Vulgar		Cobrançosa	
	October	November	October	November
C16:0	16.32 ^{b}	15.97 ^{b}	15.30 ^{b}	14.24^{a}
C16:1	2.16 ^{b}	2.29 ^{b}	1.22 ^{a}	1.07^{a}
C17:0	0.12	0.11	0.13	0.14
C17:1	0.33 ^{b}	0.31 ^{ab}	0.23 ^{a}	0.24 ^{a}
C18:0	1.74 ^{a}	1.77 ^{a}	2.96 ^{b}	3.27 ^{b}
C18:1	73.81 ^{b}	73.72 ^{b}	70.53 ^{a}	70.63 ^{a}
C18:2	3.85 ^{a}	4.32 ^{a}	7.84 ^{b}	8.90 ^{b}
C18:3	0.78	0.77	0.70	0.64
C20:0	0.38 ^{a}	0.35 ^{a}	0.50 ^{b}	0.49 ^{b}
C20:1	0.28 ^{b}	0.25 ^{b}	0.21 ^{a}	0.20 ^{a}
C22:0	0.10	0.11	0.11	0.12
Acidity (% oleic acid)	0.27 ^{b}	0.19 ^{a}	0.33 ^{b}	0.33 ^{b}
Peroxide index (meq O_2 kg^{-1})	5.49 ^{a}	4.45 ^{a}	7.41 ^{b}	7.37 ^{b}
K_{270}	0.137 ^{a}	0.120 ^{a}	0.209 ^{b}	0.190 ^{b}
K_{232}	1.221 ^{a}	1.233 ^{a}	1.476 ^{b}	1.379 ^{b}

4D LECO, St. Joseph, MI). The GC was equipped with a DB-5 column (25 m × 0.2 mm × 0.33 μm, Agilent Technologies, Santa Clara, CA) and Supelcowax 10 (1.2 m × 0.1 mm × 0.1 μm, Supelco Bellefonte, PA) as a second column. For two dimensional analysis modulation time was optimized and set at 5 s, mass spectra were collected at a rate 150 scans/s. Main oven temperature was 40 °C (1 min), then increased 5 °C/min to 180 °C and 20 °C/min to 240 °C. Secondary oven was run at temperatures 5 °C higher than in the first one. Transfer line was 250 °C. Injection port temperature was 220 °C (in case of liquid injections to identify compounds in SAFE extracts) and 260 °C (in case of samples run using SPME).

2.5. Statistical analysis

A discriminant analysis (DA) was performed using the software Statistica, version 6, from Statsoft, Tulsa, USA. DA was used on a 16 × 12 matrix, containing all the 16 samples characterized by their volatile compounds (12) identified by CG–O, except ethyl isobutyrate, 2,4-heptadienal and *trans*-2-nonenal, as their concentrations were rather low and constant throughout the samples. DA was performed to determine which of these compounds discriminate among the 4 groups of olive oils obtained from olives harvested in the same period (Galega harvested in October and November and Cobraçosa harvested in October and November). These groups were *a priori* defined (Burgard & Kuznicki, 1990). In DA, the basic underlying idea is to see whether groups differ with regard to the mean of a variable and then use that variable to predict group membership. The procedure is identical to the one-way analysis of variance or to the multivariate analysis of variance if several variables are used (Bofinger, 1975; Burgard & Kuznicki, 1990).

The discrimination model was built by forward stepwise analysis using the following options: tolerance of 0.010; F to enter equal to 1.00 and F to remove equal to 0.00. The classification functions obtained to characterize each group of samples can be used in future to determine to which group, each unknown sample most likely belongs. The classification matrix shows the number of samples used in the DA and of known group origin, that were correctly classified and those that were misclassified.

3. Results and discussion

3.1. Olive oils characterization

Fatty acids profiles of analyzed olive oil samples differed between olive cultivars: palmitic (C16:0), palmitoleic (C16:1) and oleic acid (C18:1) contents in Galega oils were higher than in Cobrançosa oils (Table 1). In what concerns polyunsaturated fatty acids (PUFA), mean linoleic acids (C18:2) weight percentage was 4.1% in Galega and 8.4% in Cobrançosa olive oils. Together with oleic acid content, they are the main differences between the two oils (>4%). The amounts of stearic (C18:0) and linolenic acids (C18:3) were higher for Cobrançosa oils. The differences in remaining fatty acids were of a lesser importance. According to the quality criteria defined by the European Union, acidity, peroxide value and UV absorbances, all the samples used in this study can be labeled as "Extra Virgin Olive Oil". However, Cobrançosa olive oils presented higher peroxide and UV absorbances values, which may be ascribed to their higher content of PUFA, which are rather prone to oxidation.

3.2. GC–O analyses of Galega and Cobrançosa olive oils

Screening volatile compounds by SPME–GC × GC ToF-MS resulted in over 300 chromatographic peaks for each cultivar. When the main peaks were considered (100–120) these compounds could be classified into several classes – aldehydes, ketones, esters and alcohols – to name the dominant ones. Fig. 1 shows area percent of peaks of compounds representing these classes in both cultivars. The high amount of data

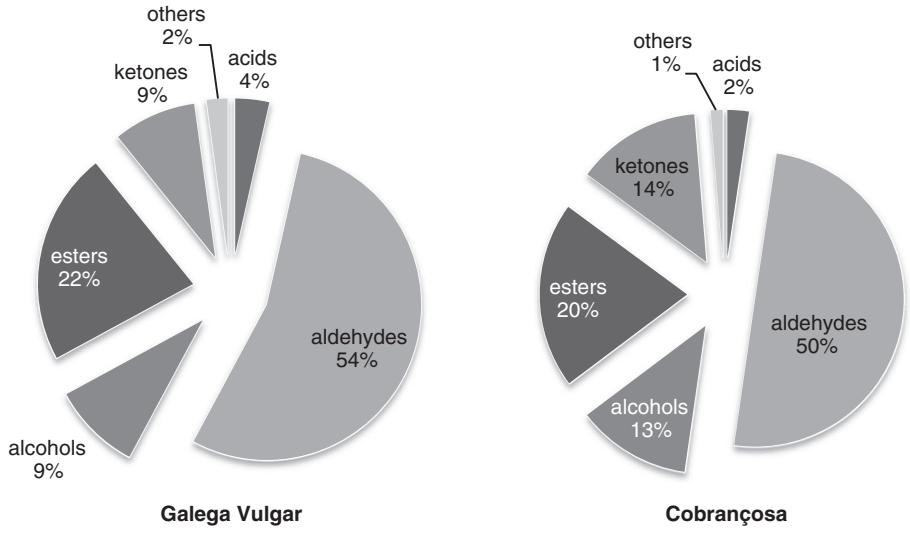

Fig. 1. Main classes of volatile compounds in Galega Vulgar and Cobrançosa virgin olive oils evaluated by SPME–GC × GC ToFMS.

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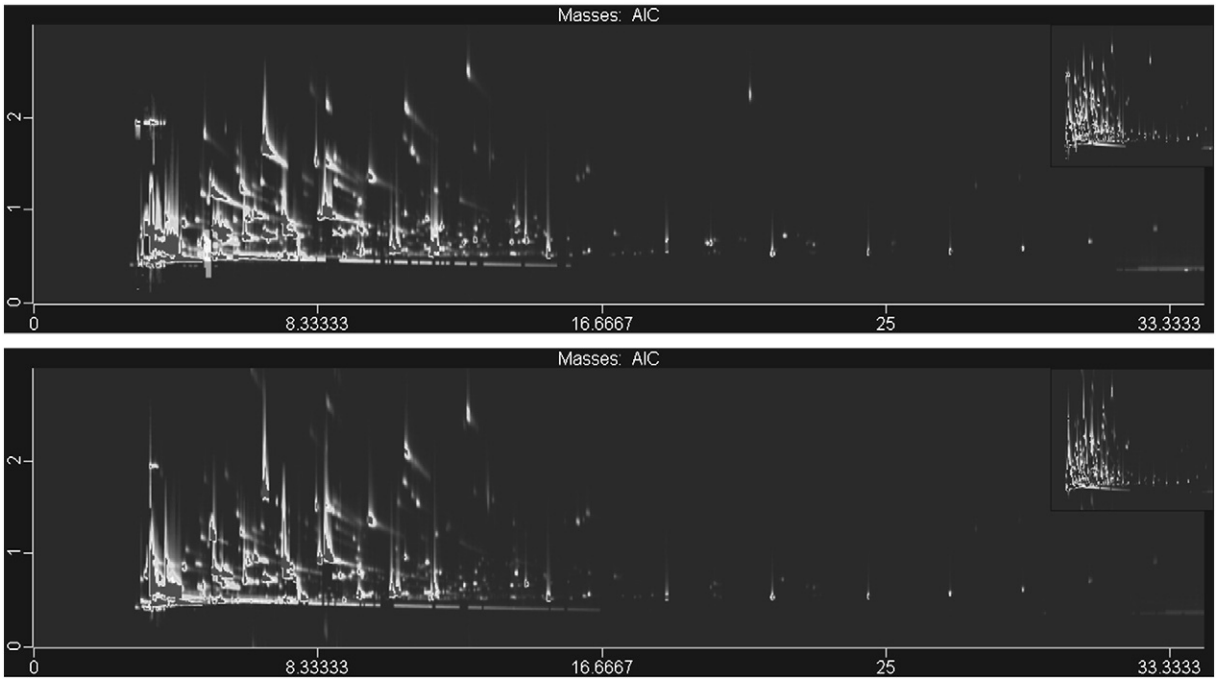

Fig. 2. Example of a contour plot for Galega (top) and Cobrançosa (bottom) VOO obtained by CG × GC ToFMS.

generated by GC × GC ToF-MS (Fig. 2) shows that the evaluation of odorants in VOO should be sensory oriented. To select volatile compounds for quantitative analyses, gas chromatography–olfactometry was performed for the olive oils from both varieties. It must be referred that the influence on sensory detection of each volatile compound depends on its concentration and odor threshold, which determine the odor activity value (OAV) (Belitz et al., 2009).

Fifteen main odor fractions were detected in analyzed oils. The main odor notes and the respective odor threshold (OT) are listed in Table 2. They ranged from buttery, different fruity notes, fatty and fatty-soapy, as well as several green notes of different character. Compounds responsible for these odor impressions were identified using gas chromatography/mass spectrometry and confirmed by injecting authentic standards of the investigated compounds. The odorants more easily identified were those with descriptors "green", also prominent when the olive samples were sensory characterized. Consumers dislike high intensities of bitter and pungent, whereas they like almost all aroma descriptors qualified with the adjective "green" (Morales, Angerosa, & Aparicio, 1999). However, "green" is not a single characteristic, but can have several different manifestations (Hongsoongnern & Chambers, 2008). Hexanal, *trans*-3-hexenal, *cis*-3-hexenal, *trans*-2-hexenal, hexyl acetate, *cis*-3-hexenyl acetate, hexan-1-ol, *trans*-3-hexen-1-ol, *cis*-3-hexen-1-ol, and *trans*-2-hexen-1-ol are examples of compounds that give a green-type description covering a wide range from mild green to intense cut grass, in accordance with the results obtained using pure compounds (Hatanaka, 1996). In Spanish olive oils, five compounds (hexanal, *trans*-3-hexenal, *cis*-3-hexenal, *trans*-2-hexenal and hexan-1-ol) had OAVs higher than 1, clearly contributing to the green aroma of olive oil. The other five volatile compounds having green or fruity odors evaluated by GC–O, but with OAVs lower than 1, do not clearly contribute to the green aroma of olive oil by themselves; however, their presence is important for its final overall aroma (Aparicio & Morales, 1998). The odorants with higher OAV are frequently essential for the aroma. However, there are exceptions where odorants with high OAVs are suppressed in the aroma and, conversely, compounds with lower OAVs are important contributors to the final aroma (Grosch, 2001). Another important feature is that the presence of high intensity of green smell enhances the bitterness perception (Caporale, Policastro, & Monteleone, 2004).

The green odorants identified were mainly produced from polyunsaturated fatty acids by the activity of each enzyme from the LOX pathway (Olias, Perez, Rios, & Sanz, 1993). The perception known as "green" odor notes are regarded as freshness and liveliness, which are characteristics of good quality virgin olive oils by consumers (Angerosa, 2002).

Apart from the condition of the fruit at harvest, as the presence of pests and diseases, differences in post-harvest handling of the fruit, produce olive oils with different flavors and off-flavors (Angerosa et al., 2004; Morales, Luna, & Aparicio, 2005; Vichi et al., 2009a, 2009b). Storage of the fruit after harvest and of the olive oil before reaching the consumer changes the volatile composition of olive oil. The highest sensory significance, evaluated by OAV, correspond to 1-octen-3-ol for mustiness-humidity, ethyl butanoate, propanoic and butanoic acids for fusty sensory defect, acetic acid, 3-methyl butanol and ethyl acetate for winey-vinegary and several saturated and unsaturated aldehydes and acids for rancid sensory defect (Morales et al., 2005). The presence of several volatile phenols was also ascribed to

Table 2
Main odoriferous fractions of Galega Vulgar and Cobrançosa virgin olive oils detected by GC–O method (RI—retention index; OT—odor threshold, Reiners & Grosch, 1998; Morales et al., 2005).

	Odor	Compound	RI (SPB-5)	OT (μg/kg)
1	Buttery	2,3-Butanedione	580	9.2
2	Pungent, green	1-Penten-3-one	680	0.73
3	Fruity	Ethyl isobutyrate	757	1.2
4	Fruity	Methyl-2-methylbutyrate	778	0.25
5	Green (fresh cut grass)	Hexanal	803	80
6	Green	*Trans*-2-hexenal	850	420
7	Citrus	Ethyl-2-methylbutyrate	855	0.72
8	Flowery	Cis-3-hexen-1-ol	858	1100
9	Fatty soapy	Heptanal	903	500
10	Fruity	3-Methylbutyl acetate	939	5
11	Mushroom	1-Octene-3-ol	978	1
12	Banana	*Cis*-3-hexyl acetate	1009	200
13	Fatty	2,4-Heptadienal	1011	3620
14	Soapy	Nonanal	1104	150
15	Fatty	*Trans*-2-nonenal	1162	900

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bad storage conditions of olives (Vichi, Romero, Tous, Tamames, & Buxaderas, 2008; Vichi et al., 2009a, 2009b). The absence of the C6 aldehydes, alcohols and esters from the LOX pathway and the presence of many aldehydes from chemical oxidation, including hexanal from both chemical and enzymatic reactions, characterize the off-flavor of olive oil (Morales & Przybylski, 2000). In what concerns hexanal, when in lower amounts, it is described as green, while in higher amounts, it is described unpleasantly sebaceous. Dierkes et al. (2011) refer that only concentrations higher than 900 μg/kg have a negative impact on olive oil quality. Nonanal or the ratio hexanal/nonanal was proposed as an appropriate way to measure olive oil oxidation (Angerosa, Mostallino, Basti, & Vito, 2000; Morales et al.,

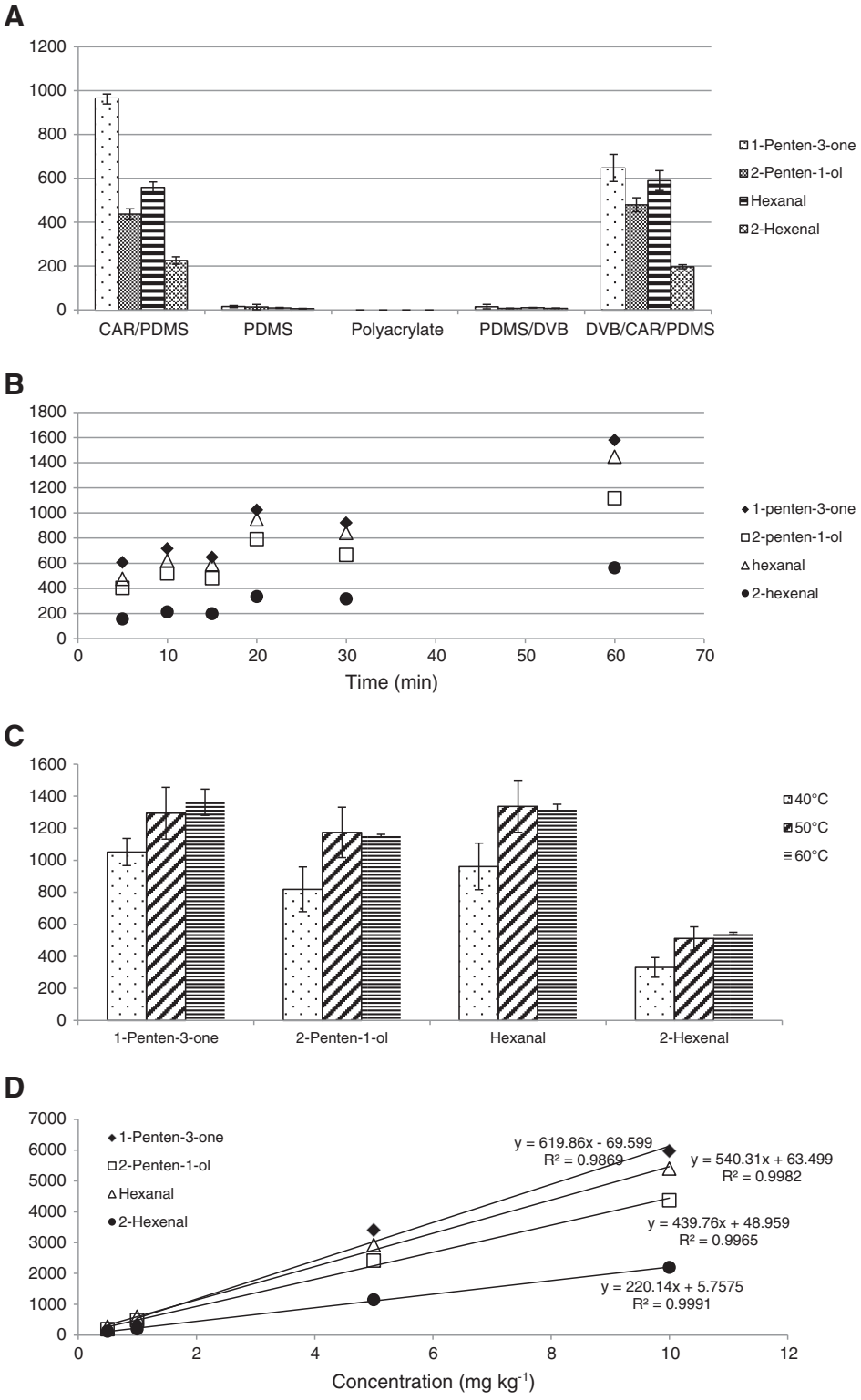

Fig. 3. Main parameters determined for SPME extraction (A — efficiency of extraction using different fibers and, for DVB/CAR/PDMS, B — extraction time, C — extraction temperature, D — linearity).

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1997). Hexanal (fatty), 2-heptanal (oxidized, tallow), nonanal (fatty, waxy, painty) and decanal (penetrating, waxy) are examples of volatile compounds present in oxidized olive oils (Morales & Przybylski, 2000). As expected, compounds from final oxidation steps were not detected as olive oil extraction was performed under controlled conditions, as well as storage conditions of olive oils till the moment of analysis.

3.3. Optimization of SPME extraction parameters

Fig. 3 shows the summary of the main parameters determined for SPME extraction. In the first stage, efficiency of fibers in extraction of volatiles was determined. Two of the examined fibers – CAR/PDMS and DVB/CAR/PDMS – acted in a similar way extracting vast amounts of volatiles in a 20 minute extraction at 40 °C. The amounts of extracted compounds for these two fibers were comparable except for 1-penten-3-one. It indicates that the main constituent of the fiber that adsorbs oil volatiles is Carboxen. The fiber containing DVB does not present important higher peak areas. The other fibers extracted minute amounts of volatiles when compared to the CAR/PDMS and DVB/CAR/PDMS fibers. The polymer based fibers are characterized by a high ability to adsorb volatiles when compared to fibers in which absorption takes place. As in polymer based fibers adsorption dominates, the question is whether such fiber provides sufficient linearity to quantify volatile compounds. Therefore, adsorption of tested compounds in a concentration range from 0.5 to 10 mg/L was evaluated. For both most efficient fibers, the linearity in the examined range was very good. The graph presented in Fig. 3 presents data for DVB/CAR/PDMS fiber. Similarly, for CAR/DVB fiber the R^2 values were all above 0.98. Therefore, both fibers have almost the same affinity and usability in extraction of oil volatiles. Increasing temperature of extraction favors the migration of compounds into the headspace, which is reflected in the temperatures tested — the highest amounts of extracted compounds were obtained for 60 °C. In all cases, samples were preheated for 5 min at a given temperature followed by fiber exposure to the sample headspace. Volatiles were extracted for different time from 5 up to 60 min. Despite the highest peak areas obtained for 60 minute sampling, 20 minute extraction was chosen as a compromise between extraction efficiency and analysis time. As summarized, SPME was used for the extraction of volatile compounds from oils by various authors (Jeleń, 2006). They noticed the high extraction efficiency of DVB/CAR/PDMS, PDMS/DVB and CAR/DVB fibers. Vichi, Pizzale, et al. (2003) found out the highest affinity of DVB/CAR/PDMS fiber for 6-carbon alcohols and that, for many compounds, the polymer based fibers did not attain equilibrium within 40 min. The influence of matrix/volatiles composition on the adsorption of particular compounds was investigated by Contini and Esti (2006). They noticed the loss of linearity for compounds present in high concentrations when PDMS/DVB fiber was used, where fiber saturation and compounds displacement can be responsible for this phenomenon. As checked out in the present study for both recommended fibers high linearity was achieved in a range of 0.5–10 mg/L. Thus optimized parameters were used for the extraction of volatiles from analyzed olive oil samples.

3.4. Comparison of aroma compounds in Galega and Cobrançosa olive oils

For olive oils comparison, 15 aroma compounds were chosen based on GC–O evaluation of main odor fractions in these olive oils. Additionally, seven compounds were selected based on literature data on olive oil aroma compounds: decanal (penetrating, sweet waxy odor; Kalua et al., 2007), *cis*-2-pentene-1-ol (banana; Morales et al., 1997), *trans*-hexen-1-ol (green, grassy; Kalua et al., 2007), hexyl acetate (green fruity, sweet; Kalua et al., 2007) and 2-pentylfurane (butter, green beans; Belitz et al., 2009). Table 3 shows 22 aroma compounds quantified using headspace SPME–GC × GC–ToF-MS for both investigated monovarietal olive oils in the four harvest time/ripening stages. The amounts of compounds varied considerably and the most abundant one was *trans*-2-hexenal. The prevailing ones were also hexanal, nonanal, *cis*-3-hexen-1-ol and 2,3-butanedione. During fruit ripening, *trans*-2-hexenal showed an increase for both cultivars in the early stages of ripening studied. This is not in accordance with the results of Gómez-Rico et al. (2006) for Cornicabra (a Spanish cultivar), as they concluded that, for both rainfed and irrigated olive groves, *trans*-2-hexenal and hexanal showed a decrease along fruit ripening. However, research in VOO from other cultivars showed the same pattern observed in the VOO of our study in early stages of ripeness (Angerosa, 2002). As expected, the group of C6 aldehydes was the most abundant, which explains the high intensity of orthonasal perception of these oils

Table 3
Aroma compounds (mg/kg) in Galega Vulgar and Cobrançosa olive oils determined by SPME–GC × GC–ToF-MS. In each row superscript indexes indicate differences based on Tukey test. No indexes indicate no differences between samples.

	Compounds	Galega		Cobrançosa	
		October	November	October	November
1	Hexanal	0.47 ± 0.20	0.89 ± 0.16	0.66 ± 0.32	0.69 ± 0.32
2	Heptanal	0.14 ± 0.03 ^{b}	0.21 ± 0.04 ^{c}	0.06 ± 0.005 ^{a}	0.04 ± 0.02 ^{a}
3	Nonanal	0.46 ± 0.10 ^{ab}	0.72 ± 0.10 ^{b}	0.37 ± 0.13 ^{a}	0.22 ± 0.07 ^{a}
4	Decanal	0.02 ± 0.0008 ^{ab}	0.02 ± 0.001 ^{b}	0.01 ± 0.002 ^{a}	0.01 ± 0.003 ^{a}
5	*Trans*-2-hexenal	2.30 ± 0.52 ^{a}	6.99 ± 1.57 ^{b}	1.85 ± 0.60 ^{a}	3.28 ± 1.42 ^{a}
6	*Trans*-2-nonenal	0.03 ± 0.001	0.03 ± 0.001	0.03 ± 0.003	0.03 ± 0.002
7	*Trans, trans*-2,4-heptadienal	0.009 ± 0.001	0.01 ± 0.0007	0.01 ± 0.007	0.01 ± 0.001
8	*Trans, trans*-2,4-decadienal	nd	nd	nd	nd
9	*Cis*-2-penten-1-ol	0.32 ± 0.05 ^{ab}	0.25 ± 0.05 ^{a}	0.47 ± 0.08 ^{b}	0.46 ± 0.14 ^{b}
10	*Cis*-3-hexen-1-ol	0.31 ± 0.38	0.54 ± 0.43	0.14 ± 0.03	0.17 ± 0.07
11	*Trans*-2-hexen-1-ol	0.02 ± 0.007 ^{a}	0.05 ± 0.02 ^{b}	0.006 ± 0.004 ^{a}	0.009 ± 0.009 ^{a}
12	1-Octene-3-ol	0.10 ± 0.03	0.10 ± 0.04	0.14 ± 0.04	0.13 ± 0.03
13	2,3-Butanedione	0.54 ± 0.10	0.51 ± 0.08	0.60 ± 0.17	0.48 ± 0.20
14	6-Methyl-5-hepten-2-one	0.35 ± 0.07	0.31 ± 0.06	0.33 ± 0.08	0.27 ± 0.07
15	1-Penten-3-one	0.43 ± 0.22	0.20 ± 0.009	0.54 ± 0.19	0.54 ± 0.20
16	*Cis*-3-hexenyl acetate	0.02 ± 0.02	0.03 ± 0.03	0.01 ± 0.01	0.02 ± 0.01
17	Hexyl acetate	0.001 ± 0.0007	0.002 ± 0.001	0.0008 ± 0.005	0.002 ± 0.001
18	Ethyl isobutyrate	0.004 ± 0.0007	0.06 ± 0.09	0.002 ± 0.002	0.002 ± 0.002
19	Methyl-2-methylbutyrate	0.06 ± 0.03	0.06 ± 0.02	0.03 ± 0.009	0.05 ± 0.004
20	Ethyl-2-methylbutyrate	0.01 ± 0.004	0.07 ± 0.09	0.006 ± 0.0009	0.007 ± 0.0006
21	3-Methylbutyl acetate	0.27 ± 0.10	0.29 ± 0.13	0.21 ± 0.06	0.21 ± 0.06
22	2-Pentylfuran	0.003 ± 0.002 ^{a}	0.005 ± 0.0004^{ab}	0.004 ± 0.002 ^{ab}	0.007 ± 0.003 ^{b}
	Ripeness index	2.3 ± 0.5 ^{a}	3.9 ± 0.5 ^{b}	1.9 ± 0.7 ^{a}	3.6 ± 0.6 ^{b}

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Table 4
Coefficients of the linear classification functions, derived by stepwise discriminant analysis, describing each group of olive oils from the same period of harvesting.

	Galega Vulgar		Cobrançosa	
	October	November	October	November
	(GaOct)	(GaNov)	(CobOct)	(CobNov)
Heptanal	15075.48	24115.7	8931.70	9126.39
Trans-2-hexenal	160.28	291.6	91.43	97.41
1-Octen-1-ol	4284.20	6776.2	2854.88	2968.67
Nonanal	−14.58	502.2	−147.04	−165.53
2,3-Butadione	−1069.21	−2213.8	−478.34	−502.23
Ethyl-2-methylbutyrate	−5663.01	−9238.1	−3090.18	−3075.84
Hexanal	857.07	1427.1	503.92	512.86
Cis-3-hexenylacetate	−8436.52	−14,816.9	−4512.18	−4601.62
3-Methylbutyl acetate	709.81	1160.2	328.47	316.71
Constant	−1398.34	−3793.0	−532.49	−556.54

(Cerretani et al., 2008). Volatile compounds in VOO do not come from the fruit itself, they are formed during processing, namely during crushing and thermobeating, which influence the presence or absence of specific odorant compounds. For instance, 1-penten-3-ol, octane, hexanal and *trans*-2-hexenal significantly discriminate thermobeating temperatures, and 2-penten-1-ol discriminate the time of thermobeating (Kalua et al., 2006). Moreover, Salas (2004) suggest that the conditions that promote hydroperoxide lyase (HPL) and inhibit alcohol dehydrogenases (ADH) and alcohol acyltransferase (AAT) activities can be applied to increase the green aroma. Similarly, the conditions that promote AAT activity can be applied to enhance the fruity aroma.

Prenzler et al. (2007) refer that aroma volatiles are not present in significant amounts in fresh olive oils and that during the thermobeating step, in presence of very high concentrations of phenolic compounds, flavor development might be hindered. This could be expected in this study, since total phenols from both monovarietal olive oils were very high (data not shown). It should be noted that these conclusions can be quite different if the thermobeating step is performed in the absence of O_2 (Servili, Selvaggini, Taticchi, Esposto, & Montedoro, 2003).

In order to investigate which aroma compounds could discriminate among the two different periods of harvesting/ripening stages for both cultivars, a discriminant analysis was performed.

Concerning the harvesting time, 9 volatiles showed to have discriminant power among samples, namely heptanal, *trans*-2-hexen-1-al, 1-octen-1-ol, nonanal, 2, 3-butanedione, ethyl-2-methylbutyrate, hexanal, *cis*-3-hexenylacetate and 3-methylbuthylacetate. Table 4 presents the coefficients of the linear classification functions, derived by a stepwise discriminant analysis, describing each harvesting time for each cultivar. These functions can be used to determine to which group each case most likely belongs. In these functions, only the compounds with discriminant power were retained.

When the observed classifications were compared to those predicted by these classification functions, 93.8% of the samples were correctly classified in terms of harvesting period and cultivar (Table 5). For the harvesting period, only the samples from Galega oils were correctly separated by the groups *a priori* defined, while only 87.5% of the samples from Cobrançosa oils were correctly classified (Table 5). This misclassification may be due to similar volatile profiles of olive oils from Cobrançosa fruits collected in October and in November. Tura, Failla, Bassi, Attilio, and Serraiocco (2013) confirmed that the ripening stage influence both *trans*-2-hexenal and 1-penten-3-one, as well as Garcia, Magalhães, Fregapane, Salvador, and Paiva-Martins (2012) for the last compound. Prenzler et al. (2007) without pre-selection of volatile compounds, found that *trans*-2-hexenal, hexanol, 1-penten-3-ol and *cis*-2-penten-1-ol, contribute towards the discrimination of maturity stages.

Table 5
Classification Matrix — the diagonal shows the number of samples correctly classified.

		Predicted classifications			
		Galega		Cobrançosa	
		October	November	October	November
Observed classifications	% correct	GaOct	GaNov	CobOct	CobNov
GaOct	100	4	0	0	0
GaNov	100	0	4	0	0
CobOct	75	0	0	3	1
CobNov	100	0	0	0	4
Total	93.75	4	4	3	5

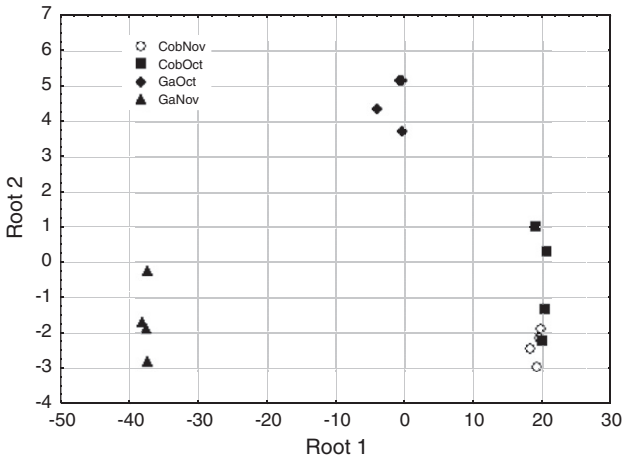

Fig. 4. Score plots of EVOO samples on the planes defined by the canonical roots 1 and 2 samples, after performing DA.

Fig. 4 shows the projections of the olive oil samples on the plane defined by the canonical roots 1 and 2, for the four groups of harvesting periods and cultivar *a priori* defined. In this plot, the clustering of the samples into the groups previously defined is well illustrated.

4. Conclusion

In Galega and Cobrançosa olive oils, fifteen odorants were identified by GC–O. In fact, the majority of the volatile compounds identified by GC × GC–ToF-MS system could not be sensory detected, probably due to their low concentrations and/or high odor threshold. So, relatively few odorants can explain the aromatic profiles of each monovarietal olive oil.

Galega and Cobrançosa olive oils in early stages of ripening showed a very similar aroma profile by GC–O. For the quantification of volatile compounds, headspace solid phase microextraction (SPME) technique was optimized. Under optimized conditions, volatile compounds were quantified in all samples. *Trans*-2-hexenal was the most abundant compound ranging from 2.3 to 7.0 mg/kg in average, for Galega oils, and from 1.9 to 3.3 mg/kg for Cobrançosa oils.

By discriminant analysis, nine volatiles showed to have discriminant power among samples, from different cultivars and harvest times, namely heptanal, *trans*-2-hexenal, 1-octen-1-ol, nonanal, 2,3-butanedione, ethyl-2-methylbutyrate, hexanal, *cis*-3-hexenylacetate and 3-methylbuthylacetate.

As well as improving the health properties of EVOO, the challenge for the producers should be the enhancement of the sensory quality of fresh olive oils. The present study confirms the importance of the ripening stage in the amount of volatile compounds. Therefore, the decision of the harvesting date will allow the production of high quality VOO with different sensory notes.

Moreover, changes in the crushing and thermobeating steps or the blending of different VOO can be used to improve and promote the

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generation of other volatile compounds to produce EVOO with more complexity and harmony.

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