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Identifying markers volatiles in Brazilian virgin oil by multiple headspace solid-phase microextraction, and chemometrics tools

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ABSTRACT

A protocol was optimized to determine the volatile profile from monovarietal virgin olive oil (VOO) by multiple headspace solid-phase microextraction (MHS-SPME) followed by gas chromatography-mass spectrometry (GC–MS) analysis. For this, a Plackett-Burman (PB) and central composite rotational designs (CCRD) were used to define the best condition of extraction. Moreover, fatty acids profile and principal component analysis (PCA) was used to identify markers among the cultivars. The amount of 0.1 g of sample was enough to express the volatile composition of the olive oils by MHS-SPME. Volatile compounds [nonanal, *(Z)*-3-Hexen-1-ol, *(Z)*-3-Hexenyl Acetate, Hexyl Acetate, 3-Methylbutyl Acetate, *(E)*-2-Hexen-1-ol, *(E)*-2-Hexenyl Acetate] and fatty acids [C17:1, C18, C18:1, C18:2] were those reported such as the markers in the varieties of olive oils. The PCA analysis allowed the classification of the most representative volatiles and fatty acids for each cultivar. Through two principal components was possible to obtain 81.9% of explanation of the variance of the compounds. The compounds were quantified using a validated method. The MHS-SPME combined with multivariate analysis showed a promising tool to identify markers and for the discrimination of olive oil varieties.

1. Introduction

Virgin olive oil (VOO) has a typical and characteristic aroma derived from volatile and non-volatile compounds. Approximately 280 compounds were identified in the volatile fraction of VOO (Lukić, Carlin, Horvat, & Vrhovsek, 2019; Paiva-Martins & Kiritsakis, 2017).

The formation of volatile compounds in VOO occurs enzymatically, depending on pH and temperature. The most significant fraction of these volatiles is attributed to compounds C6 and C5, which are produced from polyunsaturated fatty acids by the lipoxygenase (Niinemets, Kännaste, & Copolovici, 2013; ul Hassan, Zainal, & Ismail, 2015). Thus, their concentrations will be relative to the activity of each enzyme involved in the lipoxygenase pathway and the fatty acid profile of the olive oils, which is dependent on the olive cultivar and maturation. Several studies have reported the strong influence of cultivars (Crizel et al., 2020; Lukić et al., 2019; Mansouri et al., 2017; Matos et al., 2007; Talhaoui et al., 2016) and maturation (Baccouri et al., 2008; Bouchaala, Lazzez, Jabeur, Daoud, & Bouaziz, 2014; Dag et al., 2011; Magagna et al., 2016; Yu et al., 2021), on the volatile compounds from VOO.

On the other hand, irrigation, soil, environmental conditions, and agronomic practices such as harvesting, pruning and storage also impact the oil quality obtained. It was already reported in VOO produced in Greece (Eriotou, Karabagias, Maina, Koulougliotis, & Kopsahelis, 2021; Kosma et al., 2017), Morocco (Mansouri et al., 2017), Turkey (Üçün-cüoğlu & Sivri-Özay, 2020) and Croatia (Lukić et al., 2019).

The evaluation of volatile compounds in VOO is an incredibly

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challenging task due to the diversity of compounds identified.

The solid phase microextraction (SPME) technique has been widely used for the analysis of volatiles in food. However, SPME does not perform an exhaustive extraction, being necessary to calibrate using spiked blank samples, which compromises the quantification of the analytes (Tena & Carrillo, 2007). Moreover, the behavior of the analytes in real samples and standards solutions are different.

To overcome this drawback, multiple headspace (MHS) extractions were developed. The technique consists of in to do several extractions on the same sample to promote an exhaustive extraction. Accordingly, the analyte concentration decay exponentially, and the total peak area can be calculated as the sum of the areas of each extraction (Canellas, Vera, & Nerín, 2016).

The MHS considers the total amount of analyte obtained with the total area, regardless of its distribution coefficient between the fiber, sample, or headspace. Thus, the technique eliminates the effect of the headspace (HS) composition caused by the matrix effect when all analyte is extracted since it performs an exhaustive extraction. Finally, the analyte concentration can then be quantified by an external standard approach made in water, by submitting mixtures of selected compounds at different concentrations to MHS-SPME (Tena & Carrillo, 2007).

Several factors might affect the extraction process by MHS-SPME as the type of fiber, temperature and time of extraction, agitation, desorption time, among others. The multivariate statistical tools have been used to optimize the factors that affect the volatile extraction by MHS (Maruti, Durán-Guerrero, Barroso, & Castro, 2018; Oliveira et al., 2020; Serrano, Beltr án, & Hern ández, 2009).

Multivariate statistical tools have the advantage of evaluating the individual effect of these variables as well as the interaction between them, allowing to reach best analytical conditions using mathematical models (da Costa, Dal Bosco, & de Ramos, 2020; Magagna et al., 2016; Sales, Portolés, Johnsen, Danielsen, & Beltran, 2019).

The MHS technique is seldom utilized to investigated the volatile profile of VOO. Several works used HS-SPME and standards that assist in tentative determining the quantities of analytes in this matrix (Malheiro, Casal, Rodrigues, Renard, & Pereira, 2018; Peres et al., 2013; Zago, Squeo, Bertoncini, Difonzo, & Caponio, 2019).

Currently, Brazil has started to produce VOO. The culture of the olive tree has become viable in the country due to microclimates, such as mountainous areas and temperate subtropical climate. Arbequina, Arbosana, Grappolo, Koroneiki, Maria da Fé, and Picual are some of the cultivars used to obtain olive oil in the country (Ballus, Meinhart, de Souza Campos, & da Silva, 2014; Crizel et al., 2020; da Costa et al., 2020; Zago et al., 2019). Studies showed that, at the physical-chemical level, the oils produced in Brazil have a chemical composition similar to those produced in other countries (Borges, Ramalhosa, Seiquer, & Pereira, 2018). Regarding the volatile profile, poor information on Brazilian VOO was related or short investigated using MHS. Brazilian olive oils have received international awards for their outstanding sensory quality. However, there's little information on the olives cropped in Brazil. Considering the impact of the volatile fraction on the quality and commercialization of olive oils, it is fundamental to characterize the performance of these fruits growing in this new region outside the Mediterranean (Filoda, Chaves, Hoffmann, & Rombaldi, 2021).

Therefore, this study aims to evaluate the volatile profile of VOO obtained from cultivars growing in southeastern Brazil through MHS. To do this, an MHS-SPME protocol was optimized using multivariate statistical tools. Moreover, the fatty acid composition of the samples was evaluated, since plays a key role in the formation of volatile compounds in VOO by the lipoxygenase way.

2. Material and methods

2.1. Reagents and standards

n-Heptane was supplied by Sigma-Aldrich (St. Louis, MO, USA). Chloroform was purchased from Neon (Suzano, SP, Brazil). Diethyl ether and potassium iodate were purchased from Qhemis by Hexis Científica (Jundiaí, SP, Brazil). Phenolphthalein was purchased from Dinâmica (Indaiatuba, SP, Brazil). Methanol, ethanol 95%, isooctane, glacial acetic acid, potassium hydroxide, starch, and sodium thiosulphate were purchased from Synth (Diadema, SP, Brazil). Water was purified in a Milli-Q system (Millipore, USA). All chemicals were analytical reagent grade or higher purity.

The following standards were used for the volatile profile determination: Hexanal, 1-Heptanal, *(E)*-2-Hexenal, Nonanal, Dodecane, *(R)*-(+)-Limonene, *(Z)*-3-Hexenyl acetate, *(Z)*-3-Hexen-1-ol, Hexyl 3-Methylbutanoate, 3-Octanone, 1-Hexanol, *(E)*-2-Hexen-1-ol from Sigma-Aldrich (St. Louis, MO, USA); Hexyl Acetate was supplied from Acros Organics by Thermo Fisher Scientific (Geel, Belgium). All standards 97–99% pure.

Fatty acids methyl esters standard mixture (37 FAME Mix), SPME fiber DVB/CAR/PDMS (50/ 30 μm, 1 cm) and the *n*-alkanes used to determine the linear retention indices (LRI) were purchased from Supelco (Bellefonte, PA, USA).

2.2. VOO samples

The most economically important southeastern Brazilian olive cultivars (*Olea europaea* L.) were considered, such as Koroneiki, Arbosana, Arbequina, and Grappolo. Only healthy olive samples were hand-picked at the usual maturity level for each cultivar during the local customary harvest period (February/March). The olives were cultivated in Delfim Moreira (latitude: 22° 30′ 32″ S; longitude: 45° 16′ 48″ W; altitude: 1200 m above sea level) and Consolação (latitude: 22° 33′ 03″ S; longitude: 45° 55′ 15″ W; altitude: 1080 m above sea level) southeastern of Brazil, in two different crop years (2019 and 2020), with a tree spacing of 4 m within rows and 6 m between rows (417 plants/ha), without irrigation. In both locations, the rainfall is above 1300 mm and not more than 1850 mm per year, and the temperature range from −3 to 30 °C, with a temperature medium of 15–18 °C. The pruning was carried out when the harvest was completed (March/April), followed by a fertilizer with organic fertilizer and three times per year with ammonium sulfate, urea, and potassium chloride. The fertilizers were surceased three months before the picking.

About 20 trees per cultivar were marked and utilized during the study. Approximately 4 kg of olives were handpicked. The fruits have been picked aleatory in the base and the top of the trees to achieve a homogenous and representative harvest at a maturation index of 2–3, determined following the guidelines of Estación de Olivicultura y Elaiotecnia, Jaén, Spain (Hermoso et al., 1991). Both locations have tropical climates. All olive trees were approximately 6 years old. The ID name of samples are presented in Table 1.

After harvest, the olive oils were extracted following the EEC/1019/02 EU Regulation. Malaxation process was performed for 30 min at 25 °C. The oil samples were stored in Amber glass bottles of 100 mL completely filled, at low temperature, and analyzed within 12 months of

Table 1
Codification of VOO samples.

Cultivar	Crop year	Region	ID
Koroneiki	2019	Delfim Moreira	KN1
		Consolação	KN2
	2020	Delfim Moreira	KN3
		Consolação	KN4
Arbequina	2019	Delfim Moreira	AQ1
		Consolação	AQ2
	2020	Delfim Moreira	AQ3
		Consolação	AQ4
Arbosana	2019	Delfim Moreira	AS1
	2020	Delfim Moreira	AS2
Grappolo	2019	Consolação	GP1
	2020	Consolação	GP2

extraction.

Standard quality parameters for olive oil (acidity value, peroxide index (IP), K_{232}, K_{270}, and ΔK) were carried out according to the European Community Regulation EEC/2568/91 and presented in Table 2.

2.3. Fatty acid composition

Fatty acid methyl esters (FAMEs) were extracted with *n*-Heptane after transesterification with methanolic potassium hydroxide solution, following EEC/2568/91 EU Regulation. The fatty acid profile was determined by gas chromatograph. Chromatographic separation was performed on an Agilent 7890A (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector (FID), a split/splitless injector, and a DB-23 column (60 m × 0.25 mm id., 0.25 μm film thickness) (Agilent J&W Scientific Inc., Folsom, CA, USA). Injector and detector temperatures were held at 250 °C and 280 °C, respectively. Nitrogen was used as carrier gas at constant flow rate 1.0 mL/min and flow rate of the gas in the flame ionization detector (FID) was held at 30:30:300 mL/min (H_2/N_2/synthetic air). The oven temperature was programmed at 50 °C during the first minute, increasing to 175 °C at a rate of 25 °C per min, follow to a final temperature of 223 °C at a rate of 6 °C per min and kept for 25 min. The split ratio was 1:100 and the injected volume was 1.0 μL. The compounds were identified using FAME mix. The results were calculated by internal normalization of the chromatographic peak and expressed in relative percentage of each fatty acid peak area. Samples were injected in triplicate (n = 3).

2.4. Volatile characterization

2.4.1. Experimental design

Previously to perform the experimental designs, the amounts of sample were defined based on the exponential decay for all targeted analytes. This step is crucial for MHS analysis since small masses might have a significant loss from the first extraction or even be below the detection limits of the equipment whereas copious quantities do not allow exponential decay and quantitation. Hence, 0.1 g, 0.2 g, and 0.5 g of VOO mix containing the same proportion of all oils evaluated was subjected to MHS.

Next, a multivariate experimental statistical design was applied to determine an optimal condition to extract the volatile profile from VOO by MHS-SPME. Firstly, a Plackett-Burman design (PB) was carried out to select variables that could affect the extraction ($\alpha < 0.05$) of volatile from VOO. The parameters investigated were incubation time (X_1 = min), extraction temperature (X_2 = °C), extraction time (X_3 = min), agitation (X_4 = rpm) and desorption time (X_5 = min). The total volatile area was used as a response. Variables with significant effect on the answer were evaluated using a central composite rotational design (CCRD) to determine the best conditions for extraction. A 2^2 factorial design with four axial points and five repetitions in the central point was used. The experimental domains used for PB and CCRD were showed in Table 3. The statistical significance of the models was evaluated by ANOVA ($\alpha = 0.05$), considering the coefficient of determination (R^2) and the lack of fit.

Table 2
Quality parameters.

ID	Free acidity (%)	Peroxide value (mEq.O_2/kg)	K_{232}	K_{268}	ΔK
KN1	0.29 ± 0.00	6.24 ± 0.59	1.54 ± 0.03	0.16 ± 0.02	−0.01 ± 0.00
KN2	0.34 ± 0.00	4.97 ± 0.01	1.18 ± 0.05	0.10 ± 0.01	−0.01 ± 0.00
KN3	0.19 ± 0.00	7.06 ± 0.59	1.58 ± 0.21	0.18 ± 0.00	−0.01 ± 0.00
KN4	0.19 ± 0.00	7.07 ± 0.60	1.58 ± 0.07	0.17 ± 0.01	−0.01 ± 0.00
AQ1	0.19 ± 0.00	14.15 ± 1.16	1.47 ± 0.07	0.10 ± 0.01	−0.01 ± 0.00
AQ2	0.24 ± 0.00	6.65 ± 0.00	1.62 ± 0.14	0.19 ± 0.01	−0.01 ± 0.00
AQ3	0.18 ± 0.01	7.49 ± 0.00	1.76 ± 0.09	0.12 ± 0.00	−0.01 ± 0.00
AQ4	0.20 ± 0.01	7.49 ± 0.01	1.63 ± 0.09	0.10 ± 0.00	−0.01 ± 0.00
AS1	0.19 ± 0.00	9.56 ± 0.58	1.64 ± 0.05	0.16 ± 0.01	−0.01 ± 0.00
AS2	0.19 ± 0.00	6.66 ± 0.01	1.87 ± 0.05	0.19 ± 0.01	−0.01 ± 0.00
GP1	0.38 ± 0.00	5.40 ± 0.57	1.33 ± 0.18	0.18 ± 0.01	−0.01 ± 0.00
GP2	0.24 ± 0.03	10.40 ± 0.60	1.46 ± 0.05	0.19 ± 0.01	−0.01 ± 0.00

2.4.2. Instrumental analysis

The determination of volatile compounds was carried our using an Agilent 8890 gas chromatograph equipped with a PAL RSI 85 autosampler and coupled to an Agilent 7010B triple quadrupole mass spectrometer with a high-efficiency electron ionization (EI) source (Agilent Technologies, Palo Alto, CA, USA). A polar VF-WAXms column (60 m × 0.25 mm × 0.50 μm, Agilent J&W Scientific Inc., Folsom, CA, USA) was used. The injector was set at 250 °C and the automatic injections were made in splitless mode. Carrier gas was helium (White Martins, SP, Brazil) at the constant flow rate of 1 mL/min, at a linear velocity of 25.6 cm/s. The oven temperatures were the following: 40 °C hold for 1 min; 4 °C/min until 240 °C and hold for 1 min. The transfer line and the MS ion source were set at 250 and 230 °C, respectively. Electron impact mass spectra were recorded at 70 eV ionization energy. All mass spectra were acquired by electron ionization in the *m/z* 35–400 range at 25 ms of scan time. The quadrupole temperature was set at 150 °C. Agilent MassHunter Workstation (Version 10.0, Agilent Technologies) was used for all acquisition control and data processing.

The volatile identification was obtained using authentic standards and by comparing their mass spectral data with the information from the National Institute of Standards and Technology mass spectral library (NIST17) and the Flavour and Fragrance Natural and Synthetic Compounds (FFNSC3) database library. Furthermore, linear retention index (LRI) was calculated using *n*-alkanes standards. Only a minimum similarity match of 80% was considered and the experimental LRI within a ± 30 units range compared with the reported retention index of all identified volatiles.

2.4.3. Quantitative analysis of volatile compounds by MHS-SPME procedure

The VOO mass previously determined was weighted in a 20 mL headspace vial and submitted to MHS-SPME. Four extractions of the sample were performed using the procedure developed using experimental designs. The total area (A_T) of each volatile compound was determined using the peak areas obtained from the first extraction trough the equation (1).

$$A_T = \sum_{i=1}^{i\to\infty} A_i = \frac{A_1}{1-e^{-q}} = \frac{A_1}{1-\beta} \quad (1)$$

where A_T is the total peak estimated area, A_1 the peak area obtained in the first extraction, and the exponent q is a constant describes the exponential decline associated with the β.

The term β is a constant value obtained from the slope of the linear regression of the logarithms of the individual peak areas as a function of the number of extractions, according to the equation (2).

$$\ln A_i = \ln A_1 + (i-1)\cdot \ln\beta \quad (2)$$

where A_i is the relative peak area obtained in the *i*th extraction. This formula represents a linear equation of the $y = ax + b$ type, where $\ln A_1$ is the intercept on the y axis, and $\ln \beta$ is the slope.

Table 3
Plackett-Burman (PB) design for screening the variables and central composite rotational design (CCRD) followed in the experiments to optimize the extraction of VOO volatile.

PB				DCCR					
Variable	−1	0	1	Variable	-α	−1	0	1	α
incubation (X_1 - min)	3.84	8.00	12.16	**extraction (X_3 - min)**	20.86	25	35	45	49.14
extraction (X_2 - °C)	35	45	55	**desorption (X_5 - min)**	2.17	3.00	5.00	7.00	7.83
extraction (X_3 - min)	18.11	30.00	41.89						
agitation (X_4 - rpm)	300	400	500						
desorption (X_5 - min)	1.41	2.75	4.09						

2.4.4. Quantification of volatile compounds

To calculate the concentration of each volatile compound in VOO external calibration curves was performed using seven concentrations of analytical standards (from 0.1 mg/kg to 3.1 mg/kg) which were analyzed in the same conditions. When an authentic standard was not available, the analyte was estimated using a similar compound. Analytical curves were plotted using total area vs concentration. The concentrations in samples were obtained using the area total obtained from the 1st extraction through the term β and equation (1).

The concentrations were expressed as μg/100 g of VOO. Each VOO sample was analyzed in triplicate.

2.5. Statistical analysis

2.5.1. Analysis of variance

Analysis of variance (ANOVA) with Type III sums of squares was performed using the GLM (General Linear Model procedure) of the IBM SPSS (version 25.0, IBM Corporation, New York, USA). All dependent variables were analyzed using a one-way ANOVA with or without Welch correction, depending on if the requirement of the homogeneity of variances was fulfilled or not. The main factor studied was the prevalence of fatty acids and volatile compounds by the varieties of VOO. Means were compared using Tukeý́s or Dunnett T3 test also depending on if equal variances could be assumed or not. All statistical tests were performed at a 5% significance level.

2.5.2. Principal components analysis

A stepwise linear discriminant analysis (s-LDA) was used to extract the best discriminant variable to separate VOO varieties (12 variables overall). Volatile compounds and fatty acid profiles were used to find differences among the cultivars. The analysis was performed to find as many classification variables as possible. For discrimination of the groups, Wilk's lambda method was used, which probability values of F to enter set at 0.05 and to remove at 0.10. The prediction capacity of the discriminant model was studied by cross-validation. Three discriminant functions were constructed to the four-class, considering the cultivars studied. With this procedure, it was possible to achieve an optimal separation into the class, searching for a maximization and minimization of the variance between-class and within-class, respectively. The s-LDA was performed by using the software IBM SPSS (version 25.0, IBM Corporation, New York, USA).

The variables selected were used to do a principal components analysis (PCA). PCA was performed in R version 3.6.3 (R Development Core Team (2020), 2020) with RStudio interface version 1.4.1717 (RStudio Team. (2020), 2020), using the 'factoextra' package (Kassambara & Mundt, 2017) and the plots were generated using 'ggplot2' package (Wickham, 2016).

3. Results and discussion

The determination of volatiles from VOO requires reliable methods that express the real volatiles content of the samples. Assessing the volatile profile also allows establishing target analytes descriptive of the characteristics of the sample groups (Aparicio-Ruiz, García-González, Morales, Lobo-Prieto, & Romero, 2018; da Costa et al., 2020; Quintanilla-Casas et al., 2020). To do this, the optimization of the MHS-SPME was conducted and described below.

3.1. Screening of HS-SPME in the extraction

DVB/CAR/PDMS fiber was used since it is the most suitable coating for extracting volatiles from olive oils (Peres et al., 2013). The sample amount of 0.1 g was used for screening under HS-SPME conditions since this mass allows a detailed profile of volatiles in VOOs (Stilo et al., 2019). As described in section 2.4.1, the application of experimental design made it possible to evaluate the effects of analysis parameters and interactions between them.

The parameters optimized were evaluated to avoid any matrix modification. In this way, the extraction temperatures were kept between 35 and 55 °C. Higher temperatures might form artifacts of lipid oxidation (Mascrez & Purcaro, 2020). The total area of extracted analytes was used as an answer for data evaluation. Fig. 1A shows the Pareto chart, at a significance level of 5%, obtained from the PB design.

All the variables studied showed a positive effect in the extraction of volatile compounds from VOO; however, only extraction time and desorption time were significant ($\alpha < 0.05$). Therefore, these factors were selected for the CCRD. Variables that showed no effect were standardized, using 5 min of incubation time, 45 °C of extraction temperature, and stirring at 400 rpm.

Saturated design, such as PB, should be evaluated in light of the curvature test to avoid masking the statistically significant factors and indicate the experiment's optimal ranges and the proposed process's robustness (Ferreira, Caires, & da Borges, 2017; Oliveira, Monsalve, Nerin, Padula, & Godoy, 2020; Ren et al., 2008). In this step, the positive curvature was significant, indicating that the mean of the responses from the fractional design is higher than the mean of the central point. Because of this and the positive effects of the factors studied, the experimental domain applied to CCRD (Table 3) was expanded.

The CCRD design was performed using extraction and desorption. The model showed an R^2 of 0.929, which indicates a good prediction against the experimental data. According to the response surface (Fig. 1B), the optimized conditions using 0.1 g of sample were 45 min of extraction time and 7 min of desorption time. These conditions were used to optimize the MHS-SPME method.

3.2. MHS-SPME optimization

The MHS method allows quantifying with greater precision the

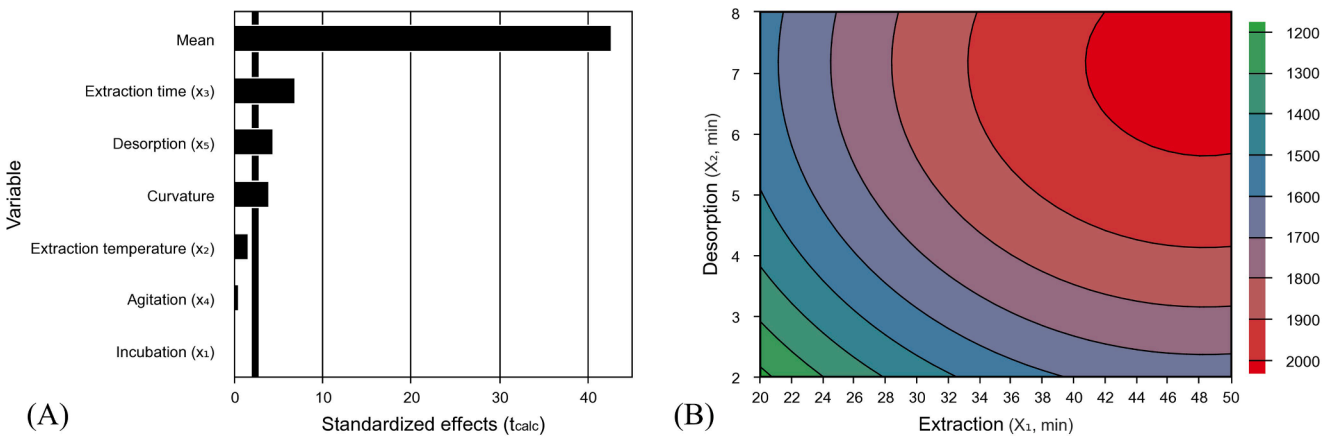

Fig. 1. Pareto chart of Plackett-Burman design ($p < 0.05$) (A) and response surface of the extraction time as a function of the desorption time (B).

volatile compounds, eliminating the matrix effect through exhaustive extractions. However, the amount of sample to provide exponential decay of the analytes should be carefully determined. For evaluation of the decay condition, three amounts of olive oil were studied (Fig. 2).

At each extraction performed, the aim is to reduce the total volatile analyte area by at least 5% (Serrano et al., 2009). It is possible to observe that, the amount of sample of 0.1 g (Fig. 2A) was enough for a significant decay between the extractions. The results obtained made it possible to obtain β values between 0.54 and 0.90 (Table 4). With an exceptionally low mass, the volatilization of analytes may be a narrow as some of them may be below the LODs. On the other hand, excessive amounts of the sample may show similar areas in the headspace after successive extractions, making it impossible to predict the β value. Thus, the technique is feasible, since MHS-SPME obtains β values within the range of 0.4–0.95. Values below 0.4 indicate that the application of MHS is not necessary as the analyte is exhaustively isolated in the first extraction. If the value is above 0.95, the application is affected due to the high concentration of the analyte in the HS even after several extractions (Tena & Carrillo, 2007). In our study, the increase in sample amount indicated low area variability, showing that the volatiles extracted by

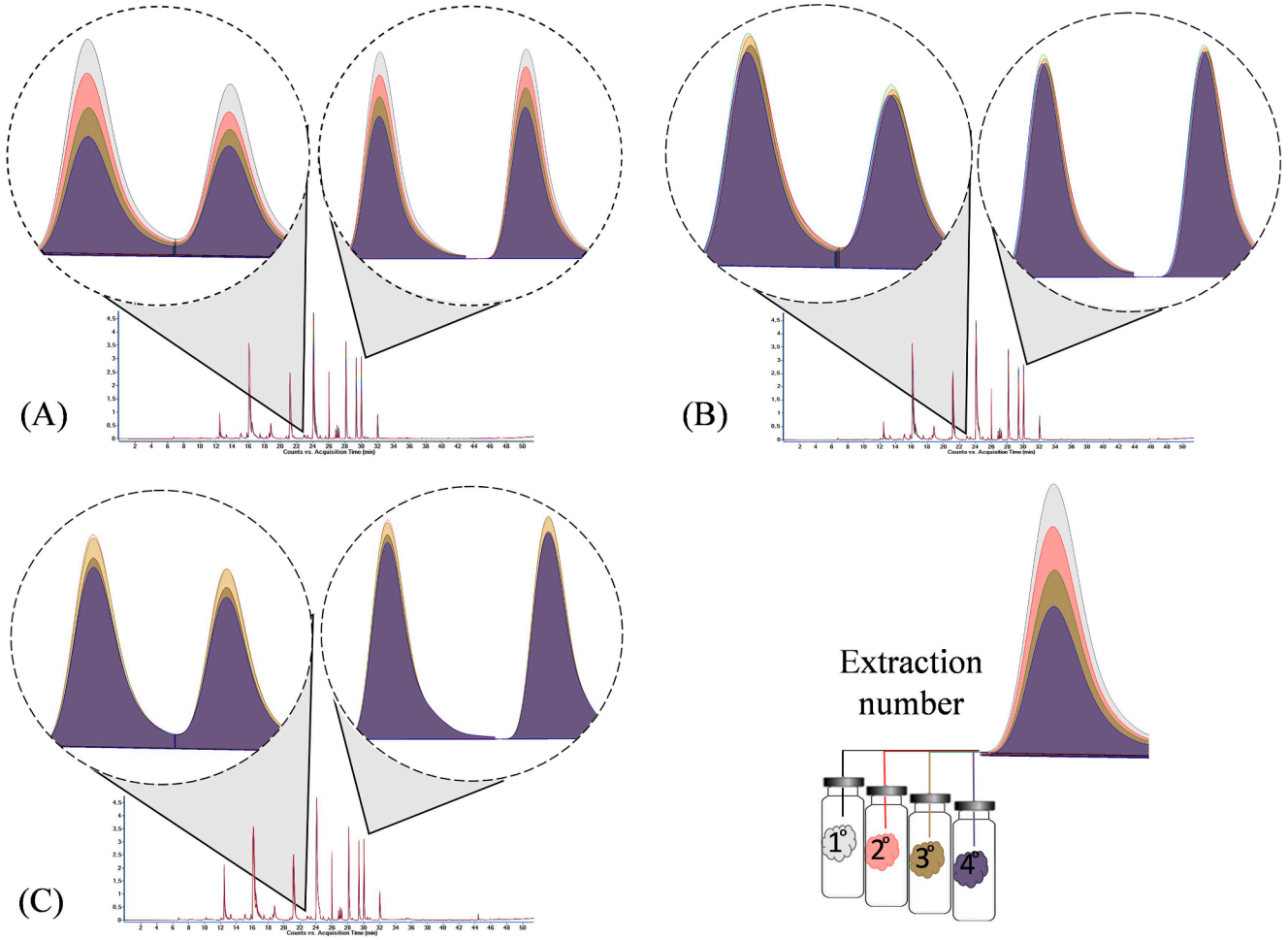

Fig. 2. Decay of the peak area with four consecutive extractions in different amounts of sample: 0.1 g (A), 0.2 g (B), 0.5 g (C).

Table 4
Obtained β value by the technique of MHS-SPME to the samples and calibration curve.

Compound	β	Compound	β
Acetic Acid	0.72	*(E)*-2-Heptenal	0.75
Hexyl 3-Methylbutanoate	0.76	Nonanal	0.87
2-Methyl-2-Propanol	0.81	*(E.E)*-2.4-Hexadienal	0.75
1-Methoxy-2-Propanol	0.78	*(E.Z)*-2.4-Hexadienal	0.87
1-Penten-3-ol	0.69	*(E.E)*-2.4-Heptadienal	0.75
3-Methylbutanol	0.81	Benzaldehyde	0.56
1-Pentanol	0.71	Ethyl Acetate	0.59
3-methyl-3-Buten-1-ol	0.71	3-Methylbutyl Acetate	0.85
4-Penten-1-ol	0.82	Hexyl Acetate	0.77
(E)-2-Penten-1-ol	0.59	*(Z)*-3-Hexenyl Acetate	0.76
(Z)-2-Penten-1-ol	0.64	*(E)*-2-Hexenyl Acetate	0.79
1-Hexanol	0.84	*(Z.Z)*-3-Hexenyl 2-Methyl-2-Butenoate	0.70
(Z)-3-Hexen-1-ol	0.77	Methyl 3-Hydroxybutanoate	0.76
(E)-2-Hexen-1-ol	0.80	Toluene	0.56
Benzyl Alcohol	0.85	Cycloheptatriene	0.77
Phenylethyl Alcohol	0.79	3-Ethyl-1.5-Octadiene	0.78
2-Methylbutanal	0.90	*p*-Cymene	0.88
3-Methylbutanal	0.90	*(E)*-4.8-Dimethylnona-1.3.7-Triene	0.84
Pentanal	0.71	Penten-3-one	0.54
Hexanal	0.70	Acetoin	0.74
(E)-2-Pentenal	0.58	1.4-Cyclohex-2-Enedione	0.77
(Z)-3-Hexenal	0.76	D-Limonene	0.72
1-Heptanal	0.75	*(E)*-β-Ocimene	0.88
(E)-2-Hexenal	0.73		

the SPME fiber is insignificant compared to the total amount. Moreover, no analyte was extracted with a single extraction using the method proposed.

The method developed has been validated. The validation focused on the linearity, precision, the limit of detection (LOD), and quantification (LOQ), presented in Table 5. LOD and LOQ were calculated by three and ten times, respectively, in a relation between the standard deviation of the regression and the slope of the calibration curves (Uhrovčík, 2014). The precision, expressed as the relative standard deviation (RSD%), varied from 0.45 to 13.75%.

3.3. Volatile profile quantitation of VOO by MHS-SPME

The MHS technique leads to a significant quantification of analytes, contributing to the precise concentration of volatiles in the real sample since the approach pulls out the matrix effect. As an alternative, to quantify the volatile analytes of the VOOs, our optimized MHS-SPME method was applied to the samples. Through Eq. (1), the total area of the analytes was determined. The volatile concentrations were determined using an external calibration curve since AT is known, as shown in Table 6.

Concentrations of analytes above the maximum values of the calibration curve can be assumed. Serrano et al. (2009) showed in their study that extrapolating the maximum levels of the calibration curve does not attribute relevant errors in the analysis once the calculated areas used are theoretical.

This extrapolation is necessary because the excessive increase in the concentration of standards causes a loss of linearity at high concentrations. Thus, the feasibility of using an external calibration curve to calculate parameter b was performed. The AT of each compound was calculated following the MHS-SPME theory, from the analyte area in a single extraction, and the calculated β parameter.

Forty-seven volatile compounds were identified in the cultivars evaluated. *(E)*-2-Hexenal, *(E,Z)*-2,4-Hexadienal, *(E)*-4,8-Dimethylnona-1,3,7-Triene were in highest concentration in the samples evaluated. The volatile composition of the studied olive oils was qualitatively similar to those nationally and internationally (da Costa et al., 2020; Peres et al., 2013; Yu et al., 2021; Zago et al., 2019).

Borges et al. (2018) studied the volatile fraction of the olive oil cultivar Arbequina produced in Brazil and Spain, and their results indicate a similar profile. Despite Brazil has unfavorable regions for the cultivation of the fruit and its by-products due to the abiotic conditions required by the olive tree (Kiritsakis & Shahidi, 2017), the quality of the VOOs produced was relevant.

Higher concentrations of *(E)*-2-Hexenal were found in the cultivars studied, with Arbequina being the cultivar with the highest and lowest significant concentration ($p < 0.001$), with values of 685.97 μg/100 g (AQ3) and 35.57 μg/100 g (AQ1), respectively. Compounds formed in the lipoxygenase (LOX) pathway, such as Hexanal, 1-Hexanol, *(Z)*-3-Hexen-1-ol, *(Z)*-3-Hexenal, *(Z)*-3-Hexenyl Acetate, and *(E,Z)*-2,4-Hexadienal were detected in high concentration in the cultivars evaluated. This information is in line with that presented in other studies, which show significant concentrations of these analytes in olive oil (da Costa et al., 2020; Lukić et al., 2019; Malheiro et al., 2018; Stilo et al., 2019; Yu et al., 2021). These compounds are part of GLV's (green leaf volatiles), formed through the oxidation of linoleic acid and the action of endogenous enzymes such as lipoxygenase, hydroperoxide lyase, alcohol dehydrogenase, and alcohol acetyltransferase, thus producing aldehydes, alcohols, and esters (ul Hassan et al., 2015). Moreover, show a similarity that can be compared to green leaves, grasses, apple-like, herbaceous, fruity, citrus, which attribute positive sensory characteristics to the VOOs (Angerosa et al., 2004).

(E)-4,8-Dimethylnona-1,3,7-Triene was detected in all cultivars evaluated with concentration varying from 35.59 to 51.80 μg/100 g. This compound was recently reported as one marker in in Brazilian VOO (Stilo et al., 2023).

Cultivars Koroneiki and Grappolo, in different crops, showed high concentrations of Nonanal. A significant difference ($p < 0.001$) for the analyte was found, with the KN2 sample being the one with the highest concentration (35.20 μg/100 g). In the Arbosana cultivar, only AS1

Table 5
Validation parameters for the MHS-SPME developed method.

Volatile compound	Linear range (mg/kg)	Regression curve	R^2	LOD (mg/kg)	LOQ (mg/kg)
Hexyl 3-Methylbutanoate	0.03–3.1	$y = 7.21 \bullet 10^4X - 1.39 \bullet 10^7$	0.974	0.34	1.12
1-Hexanol	0.01–2.1	$y = 1.10 \bullet 10^5X - 2.11 \bullet 10^6$	0.996	0.04	0.13
(Z)-3-Hexen-1-ol	0.01–3.1	$y = 2.65 \bullet 10^5X - 9.57 \bullet 10^6$	0.994	0.13	0.44
(E)-2-Hexen-1-ol	0.02–3.1	$y = 8.04 \bullet 10^4X - 2.96 \bullet 10^6$	0.995	0.09	0.28
Hexanal	0.03–3.1	$y = 1.98 \bullet 10^5X - 1.98 \bullet 10^7$	0.986	0.21	0.69
1-Heptanal	0.03–3.1	$y = 1.21 \bullet 10^5X - 2.82 \bullet 10^7$	0.965	0.28	0.92
(E)-2-Hexenal	0.03–3.1	$y = 8.65 \bullet 10^4X - 2.10 \bullet 10^7$	0.960	0.23	0.76
Nonanal	0.01–3.1	$y = 4.77 \bullet 10^4X - 1.05 \bullet 10^7$	0.945	0.19	0.65
Hexyl Acetate	0.02–3.1	$y = 1.45 \bullet 10^5X - 2.20 \bullet 10^7$	0.979	0.19	0.65
(Z)-3-Hexenyl Acetate	0.01–3.1	$y = 1.35 \bullet 10^5X - 1.93 \bullet 10^7$	0.983	0.19	0.65
D-Limonene	0.02–3.1	$y = 4.68 \bullet 10^5X - 1.06 \bullet 10^8$	0.951	0.34	1.12
3-Octanone	0.02–3.1	$y = 7.04 \bullet 10^4X - 4.55 \bullet 10^6$	0.995	0.10	0.33
Dodecane	0.01–3.1	$y = 1.14 \bullet 10^5X - 2.74 \bullet 10^7$	0.934	0.27	0.90

Table 6
Volatile profile (μg/100 g of oil) of olive oils from cvs. Arbequina, Arbosana, Grappolo and Koroneiki, by MHS-SPME ($n = 3$; mean with the value of standard deviation represented in brackets).

Compound	LRIlit	LRIexp	AQ1	AQ2	AQ3	AQ4	AS1	AS2	GP1	GP2	KN1	KN2	KN3	KN4	*p*-value
Acetic Acid	1447	1476	25.91a,b (2.32)	25.87a,b (1.51)	22.53a (1.12)	23.15a (2.21)	29.72a,b (3.26)	22.93a (0.16)	32.47b, c (3.57)	22.46a (1.33)	39.41c,d (4.80)	41.17d (6.27)	23.35a (0.61)	25.13a,b (1.79)	0.006*
Hexyl 3-Methylbutanoate	1475	1500	24.04a,b (0.26)	24.09b (0.10)	23.85a,b (0.10)	23.80a,b (0.07)	24.06a,b (0.20)	23.81a,b (0.10)	23.93a, b (0.04)	23.73a (0.07)	24.04a,b (0.06)	23.98a,b (0.07)	23.84a,b (0.07)	24.05a,b (0.05)	0.006**
2-Methyl-2-Propanol	871	896	1.45b (0.06)	1.59b,c (0.10)	1.82c (0.12)	n.d.	1.19a (0.07)	n.d.	1.61b,c (0.15)	1.57b,c (0.05)	1.56b,c (0.05)	1.57b,c (0.09)	1.40a,b (0.02)	1.41a,b (0.13)	<0.001**
1-Methoxy-2-Propanol	1146	1139	11.26b-d (1.47)	1.31a (0.09)	5.07a-d (2.06)	6.57a-d (1.49)	7.89a-d (1.89)	8.31c-d (0.97)	1.63a,b (0.23)	4.61a-c (1.28)	4.65c,d (0.33)	1.85a,b (0.32)	15.29a-d (4.30)	12.13d (1.47)	<0.001*
1-Penten-3-ol	1161	1163	2.54d,i (0.03)	2.04a-c,e-h (0.08)	1.99e-h (0.04)	2.06b,c,f,h (0.02)	3.98j (0.15)	1.96a,e,g (0.02)	1.70a,b (0.05)	3.56a-j (0.30)	3.15i (0.09)	2.39c,d (0.05)	1.71a,b,e,f (0.08)	3.44c,d,g-j (0.23)	<0.001*
3-Methylbutanol	1209	1211	29.19 g (1.06)	7.19e (0.17)	5.74a-d (0.25)	4.70d (0.02)	8.31e (0.35)	4.36c (0.03)	5.58a-d (0.24)	5.21a,b,d (0.10)	5.07b (0.04)	5.61a (0.06)	3.91f (0.03)	5.08a-d (0.16)	<0.001*
1-Pentanol	1255	1254	7.63e (0.18)	3.82a (0.04)	6.75a-f (0.52)	5.57f (0.17)	5.28c,f (0.07)	5.50f (0.15)	3.77a (0.03)	4.61d (0.09)	4.21a-d (0.23)	4.14b (0.06)	3.76a,b (0.01)	4.21a,b,d (0.12)	<0.001*
3-Methyl-3-Buten-1-ol	1232	1259	3.82a-d,f-h (0.06)	3.89a,b,f,g (0.07)	n.d.	n.d.	4.03f (0.05)	n.d.	3.76a-d (0.05)	3.56c,d,h (0.02)	4.33f-h (0.12)	5.04e (0.10)	3.55a,c (0.01)	3.63b,d,g,h (0.01)	<0.001*
4-Penten-1-ol	1297	1307	3.56b (0.02)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.67a (0.01)	3.78a,b (0.24)	n.d.	3.54b (0.01)	0.001*
(E)-2-Penten-1-ol	1318	1319	n.d.	n.d.	n.d.	n.d.	3.60b (0.01)	n.d.	3.49a (0.01)	n.d.	n.d.	n.d.	n.d.	n.d.	<0.001**
(Z)-2-Penten-1-ol	1325	1327	4.08b (0.03)	3.83a (0.03)	4.08b (0.02)	3.82a (0.03)	4.70d (0.09)	4.18b (0.05)	3.75a (0.04)	5.55f (0.06)	4.85e (0.06)	4.47c (0.02)	3.77a (0.02)	4.53c (0.06)	<0.001**
1-Hexanol	1359	1358	59.20e (2.46)	37.93a-c (3.02)	14.99f (0.83)	23.87b (0.34)	29.98a,b (1.31)	24.10b (0.60)	25.12a, b (1.13)	15.41f (0.10)	26.88a (0.41)	29.30a (0.88)	6.87d (0.39)	36.84c (1.25)	<0.001*
(Z)-3-Hexen-1-ol	1384	1395	14.65a,b (0.60)	24.04d (1.29)	17.17b,f (0.69)	20.11d,f (0.55)	45.52c,g (1.37)	57.34 h (1.45)	13.82a, b (0.62)	38.64 g (0.19)	79.15e (2.12)	44.46c (0.09)	13.94a (0.50)	73.55e (1.75)	<0.001*
(E)-2-Hexen-1-ol	1403	1413	36.96f (0.65)	5.87a,e (0.23)	25.82g (0.98)	21.60g (0.60)	12.94h (0.02)	40.48f (0.75)	4.60a,b (0.06)	5.46e (0.03)	4.60b,c (0.10)	4.19c,d (0.04)	4.07d (0.03)	5.51e (0.06)	<0.001*
Benzyl Alcohol	1905	1913	6.58a (0.12)	5.53a (0.33)	2.94c (0.14)	2.41c (0.09)	8.66a-d (1.22)	2.77c (0.04)	5.57a (0.21)	1.59d (0.09)	3.82b (0.14)	6.02a-c (0.59)	1.73d (0.08)	2.32c (0.10)	<0.001*
Phenylethyl Alcohol	1922	1951	5.97a-e (0.55)	6.17a,c (0.36)	3.74b (0.01)	3.75b,d,e (0.04)	8.12a-e (1.45)	3.90a,d (0.02)	4.55a-d (0.13)	3.60e (0.02)	5.85a,e (0.31)	8.65a-e (1.14)	3.69b,e (0.03)	4.27c (0.03)	<0.001*
2-Methylbutanal	917	923	15.78h (0.19)	13.09a-g (0.33)	13.95f,g (0.24)	12.46c,e,g (0.08)	17.35h (0.35)	12.25d,e (0.11)	12.68a-e (0.14)	n.d.	12.95a,b,f (0.04)	13.07a,c,f,g (0.14)	11.85d (0.03)	12.18b,d,e (0.17)	<0.001*
3-Methylbutanal	920	927	12.72a-d (0.18)	12.27a,b (0.05)	13.59e (0.13)	12.68a,c (0.11)	12.32a,b (0.04)	12.28a-d (0.17)	12.55a-e (0.29)	n.d.	12.61a,c (0.08)	12.51a-d (0.08)	12.04b,d (0.12)	12.68c,d (0.03)	<0.001*
Pentanal	979	993	13.57a-f (0.23)	12.39a,c (0.24)	13.26c-f (0.07)	12.91a-f (0.08)	15.69g (0.30)	12.50a,c,d,f (0.20)	13.28a-f (0.31)	18.59h (0.10)	14.88b,e,g (0.48)	12.59a,b,d (0.02)	14.00b,d-f (0.30)	14.05b,d-f (0.31)	<0.001*
Hexanal	1088	1099	19.70c (0.43)	25.11a (0.72)	40.53d,f (1.70)	38.26d,f (0.69)	50.66d-f (2.37)	42.00f (0.93)	21.20a-c (0.89)	23.89a (0.39)	49.53e (0.97)	36.22d (0.37)	16.23b (0.21)	42.77d-f (1.81)	<0.001*
(E)-2-Pentenal	1143	1152	29.85e (0.02)	30.40a,c,d (0.07)	30.26a-d (0.05)	30.24c (0.03)	30.53d (0.02)	30.08a,b (0.02)	30.07a, b (0.01)	30.35c (0.01)	30.40c,d (0.03)	30.32c (0.02)	30.05b (0.02)	30.37c (0.03)	<0.001*

(*continued on next page*)

Table 6 (*continued*)

Compound	LRIlit	LRIexp	AQ1	AQ2	AQ3	AQ4	AS1	AS2	GP1	GP2	KN1	KN2	KN3	KN4	*p*-value
(Z)-3-Hexenal	1146	1173	29.67a (0.01)	29.96b (0.04)	31.21c (0.16)	30.17a,b (0.11)	32.32a-c,e (0.79)	35.06e (0.43)	29.67a (0.01)	85.22f (2.29)	31.06a-c (0.31)	29.91a,b (0.06)	43.30d (0.34)	79.68f (2.27)	<0.001*
1-Heptanal	1188	1211	28.62b,c,e (0.04)	28.71b,d (0.01)	28.63b,c,e (0.05)	28.68b,c (0.02)	28.67b,c,e (0.04)	28.80a,e (0.01)	28.80a-e (0.04)	28.79a-e (0.09)	28.63c (0.01)	28.95a (0.03)	28.81a-e (0.06)	29.05a,d (0.06)	<0.001*
(E)-2-Hexenal	1216	1224	37.57e (0.61)	75.18b (2.85)	685.97f (19.48)	485.92h (9.60)	380.26g (4.83)	502.05h (13.70)	55.68a (1.73)	256.06i (1.80)	150.93c (5.88)	90.09b (3.01)	118.56d (3.13)	205.73c,d,i (15.47)	<0.001*
(E)-2-Heptenal	1361	1353	29.79a (0.01)	29.85b,c (0.02)	29.89b-d (0.02)	29.84a,b (0.01)	30.04f (0.02)	29.90c,d (0.01)	29.94d,e (0.02)	29.87b,c (0.01)	29.99e (0.02)	n.d.	n.d.	30.10g (0.03)	<0.001**
Nonanal	1397	1416	n.d.	n.d.	n.d.	n.d.	32.94a (0.22)	n.d.	33.16a (0.21)	33.48a (0.08)	34.63b,c (0.27)	35.20c (0.25)	33.18a (0.08)	34.46b (0.29)	<0.001**
(E,E)-2,4-Hexadienal	1428	1428	29.98c (0.01)	30.36b (0.02)	39.52e (0.70)	34.10d (0.13)	30.15b,c (0.05)	36.41e (0.15)	29.72a (0.02)	53.57f (0.54)	30.04c (0.02)	29.80a (0.01)	34.82d (0.26)	49.94f (0.87)	<0.001*
(E,Z)-2,4-Hexadienal	1441	1436	31.05a,b (0.48)	32.46b (0.41)	75.92d (3.38)	49.42c (1.39)	31.71a,b (0.44)	59.22d (0.27)	30.36a (0.30)	130.86e (3.28)	31.57a,b (0.68)	n.d.	53.16c (0.29)	112.45f (3.79)	<0.001*
(E,E)-2,4-Heptadienal	1487	1497	31.01a,b,d-f (0.16)	30.16a-f (0.64)	30.19a,d-f (0.03)	30.23d-f (0.06)	32.84c (0.33)	30.36e,f (0.05)	31.05a (0.13)	30.11d,e (0.03)	32.23a-f (0.33)	31.75b,c (0.10)	29.99d (0.06)	30.55a,f (0.09)	<0.001*
Benzaldehyde	1558	1571	29.95a,b (0.03)	30.94c (0.08)	30.17d (0.02)	29.96a,b (0.02)	30.60c (0.05)	30.10a,b,d (0.04)	29.94a,b (0.04)	29.91a,b (0.05)	30.03a (0.02)	29.96a (0.01)	29.86b (0.01)	29.98a,b (0.02)	<0.001*
Ethyl Acetate	902	893	22.01a,c (0.02)	21.94c,d (0.02)	21.98a-d (0.04)	n.d.	22.64e (0.08)	n.d.	22.08a,b (0.03)	21.94c,d (0.03)	22.01a,c (0.01)	22.10a (0.02)	n.d.	21.88b,d (0.01)	<0.001*
3-Methylbutyl Acetate	1126	1134	22.30b (0.03)	22.30b (0.03)	22.07a (0.03)	22.01a (0.01)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<0.001**
Hexyl Acetate	1276	1286	23.21f (0.05)	23.80c,g (0.11)	23.41c,f (0.02)	24.04e,g (0.06)	23.12f (0.09)	23.39c,f (0.03)	22.55a,b (0.06)	22.24b (0.01)	27.67d (0.11)	30.69a,c-h (1.22)	25.62e (0.28)	33.19h (0.05)	<0.001*
(Z)-3-Hexenyl Acetate	1328	1333	23.17f (0.13)	29.63c,d (0.88)	24.70c (0.13)	27.90d (0.23)	31.82d (0.70)	37.89g (0.58)	21.03a (0.10)	21.83h (0.08)	72.40b (1.11)	78.56b (1.37)	58.64e (2.50)	91.48b (4.01)	<0.001*
(E)-2-Hexenyl Acetate	1339	1348	20.52b (0.02)	n.d.	20.48a,b (0.03)	20.44a (0.03)	n.d.	20.49a,b (0.02)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.032**
(Z,Z)-3-Hexenyl 2-Methyl-2-Butenoate	–	1372	20.44a-d (0.01)	20.44a,c,e (0.02)	20.50a-f (0.02)	20.53e-g (0.01)	20.53e,f (0.02)	20.75g,h (0.04)	20.46a-d (0.01)	20.54e-g (0.01)	20.73b,d,f-h (0.06)	20.49a,b,e,f (0.01)	20.42c,d (0.01)	20.92h (0.04)	<0.001*
Methyl 3-Hydroxybutanoate	1475	1503	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	23.53a (0.02)	n.d.	25.62c (0.34)	24.25a,b (0.26)	n.d.	23.44b (0.02)	0.002*
Toluene	1046	1061	41.64e,g (0.20)	41.93e (0.44)	36.26a,b,g (1.02)	38.04b (0.20)	41.52c,e (0.54)	37.67a,b (0.10)	39.14a-c (0.48)	47.96f (0.17)	46.74f (0.39)	256.60d (19.24)	36.92a (0.22)	39.75a-c,e,g (0.75)	<0.001*
Cycloheptatriene	1089	1067	35.45b (0.08)	35.97a-d (0.10)	35.35b (0.13)	35.26b (0.07)	35.54a,b,d,e (0.22)	35.34b,e (0.04)	35.54a,b (0.11)	36.58d,e (0.19)	36.43a,b,d,e (0.27)	51.61c (0.40)	35.41b,e (0.05)	35.57a,b (0.18)	<0.001*
3-Ethyl-1,5-Octadiene	1080	1093	35.99b (0.11)	n.d.	n.d.	n.d.	35.28a (0.04)	n.d.	35.89b (0.15)	n.d.	35.38a (0.08)	n.d.	n.d.	35.39a (0.07)	<0.001**
p-Cymene	1284	1298	33.63a (0.54)	33.52a (0.26)	33.41a (0.17)	33.70a (0.65)	33.47a (0.80)	34.21a (0.49)	33.94a (1.26)	33.61a (0.54)	33.45a (0.84)	33.32a (0.01)	34.02a (0.12)	34.53a (0.79)	0.459**
(E)-4,8-Dimethylnona-1,3,7-Triene	1312	1322	35.59f (0.09)	37.25b,e (0.15)	36.33e (0.04)	40.39a (0.25)	36.84b,e,f (0.31)	36.83b,e (0.11)	40.70a (0.09)	51.80c (0.86)	46.14c (0.13)	45.96a-d (1.43)	43.59d (0.37)	79.70g (0.42)	<0.001*

(*continued on next page*)

Table 6 (*continued*)

Compound	LRIlit	LRIexp	AQ1	AQ2	AQ3	AQ4	AS1	AS2	GP1	GP2	KN1	KN2	KN3	KN4	*p*-value
Penten-3-One	1020	1035	8.28a (0.01)	8.33a (0.02)	9.15e (0.01)	8.88d (0.03)	8.54b (0.04)	8.50b (0.03)	8.45a,b (0.05)	11.15f (0.13)	8.77c,d (0.04)	8.68a-d (0.06)	8.50b,c (0.01)	10.16g (0.11)	<0.001*
Acetoin	1286	1314	8.90a-c (0.08)	8.88a-c (0.13)	8.71b,c (0.04)	8.93a-c (0.08)	9.33a-d (0.15)	8.82a-c (0.12)	9.92a-d (0.37)	9.78d (0.11)	9.27a,d (0.01)	9.05a-c (0.11)	8.44b (0.01)	9.09a,c (0.08)	<0.001*
1,4-Cyclohex-2-Enedione	–	1789	n.d.	n.d.	n.d.	n.d.	n.d.	8.59a (0.05)	n.d.	8.76b (0.02)	n.d.	n.d.	n.d.	9.03c (0.05)	0.002*
D-Limonene	1200	1224	33.06d,g (0.01)	37.53b (0.34)	33.49f-h (0.07)	33.23c-h (0.04)	33.24c,e,f, h (0.02)	33.31e,h (0.01)	32.92a (0.01)	34.79i (0.08)	33.08a,c,d, e (0.04)	32.93a (0.01)	33.24c,f (0.01)	34.81i (0.04)	<0.001*
(*E*)-β-Ocimene	1250	1268	33.18d (0.01)	33.66b (0.07)	34.06e (0.04)	38.88a (0.22)	33.67b (0.06)	36.46f (0.19)	39.98a (0.38)	46.26g (0.73)	33.10c (0.01)	n.d.	33.06c (0.02)	33.29b (0.01)	<0.001*

n.d. – not detected.

[a-j]In the same line, for each volatile compound, mean values with different letters differ significantly ($p < 0.05$); *$p < 0.05$, by means of Levene's test. P values are those from one-way Welch ANOVA analysis. Means were compared by Dunnett T3's test, since equal variances could not be assumed; **$p > 0.05$, by means of Levene's test. P values are those from one-way ANOVA analysis. Means were compared by Tukey's test, since equal variances could be assumed.

presented nonanal contents, with a concentration of 32.94 μg/100 g. This compound has a significant increase during the oxidative process (Morales & Przybylski, 2013). Malheiro et al. (2018) showed that, in the process of obtaining olive oil, there is a significant increase in Nonanal, especially during centrifugation and clarification, wherever oxygen is incorporated into the oil.

In general, aldehydes, alcohols, hydrocarbons, esters, ketones, terpenes, and acids were quantified in the VOO samples. Considering the total area of the compounds identified, aldehydes were the most representative class among all, with contents ranging from 32.2% to 68.6% of the total identified compounds, followed by hydrocarbons (from 9.1% to 32.8%), alcohols (from 5.8% to 19.2%) and esters (from 6.9% to 14.2%). Other classes had contents lower than 8% in relation to the total of identified compounds. A circos plot (Fig. 3), created using the circlize package (Gu, Gu, Eils, Schlesner, & Brors, 2014) in the R software, show those representative classes. These results are similar with those presented in other works with olive oil, showing high levels of aldehydes for extra virgin olive oils (Aparicio-Ruiz et al., 2018; Ouni, Flamini, & Zarrouk, 2016; Üçüncüoğlu & Sivri-Özay, 2020; Zago et al., 2019). Aldehydes are the most important volatile fraction in the determination of high quality virgin oils (Kalua et al., 2007).

Significant variations of aldehydes and alcohols within the same cultivars in different regions may reflect the planting density of olive trees, which may influence the enzymatic activity of alcohol dehydrogenase (ADH), as well as variations in esters due to the influence of alcohol acetyltransferase (AAT) (Angerosa et al., 2004). The main responsible for the formation of this class of volatiles are fatty acids, mostly polyunsaturated ones, such as linoleic and linolenic, contributing to the formation of the main aldehydes that also contribute to the alcohols class, reducing compounds such as *(E)*-2-Hexenal to *(E)*-2-Hexen-1-ol and Hexenal to 1-Hexanol (da Silva, Costa Freitas, Cabrita, & Garci, 2012; Kalua et al., 2007; ul Hassan et al., 2015). Therefore, the fatty acid composition is relevant for the formation of volatile compounds in olive oil.

3.4. Fatty acids profile of VOO

The Fatty acids profiles and their amounts in the analysed olive oils are shown in Table 7. All results obtained for fatty acids showed significant differences ($p \leq 0.001$) between cultivars. Higher concentrations of palmitic acid (C16:0) and palmitoleic acid (C16:1) are observed for Arbequina and Arbosana samples. Grappolo is the cultivar with the lowest concentrations. This condition is contrary to the analysis of oleic acid (C18:1), since the two samples, GP1 and GP2, have the highest concentrations of this fatty acid, with values of 80.16% and 80.80%, respectively. Values ranging from 6.00% to 8.94% linoleic acid (C18:2) were obtained for Arbequina. Cultivar Koroneiki showed the highest concentrations of stearic acid (C18:0). Linolenic acid values (C18:3) were variable among cultivars. When evaluating the composition of polyunsaturated fatty acids, which have a strong relationship with the lipoxygenase enzymatic pathway, samples of Arbequina (AQ1 and AQ4) stood out. The GP2 sample, on the other hand, has the lowest average percentages among the samples, followed by KN3 and KN1.

It is evident that the fatty acid composition is more related to the cultivar compared to the geographical origin, as already presented by some works (Ballus et al., 2014; Dabbou et al., 2010; Polari, Mori, & Wang, 2021). Thus, intrinsically the composition of volatiles must be evaluated in conjunction with fatty acids, to concisely elucidate their formation.

3.5. Principal components analysis

Principal component analysis was done to identify trends and similarities among the samples. For this purpose, an s-LDA was applied to all volatile compounds and quantified fatty acids, to classify target compounds that, even with geographic differences, were present in the

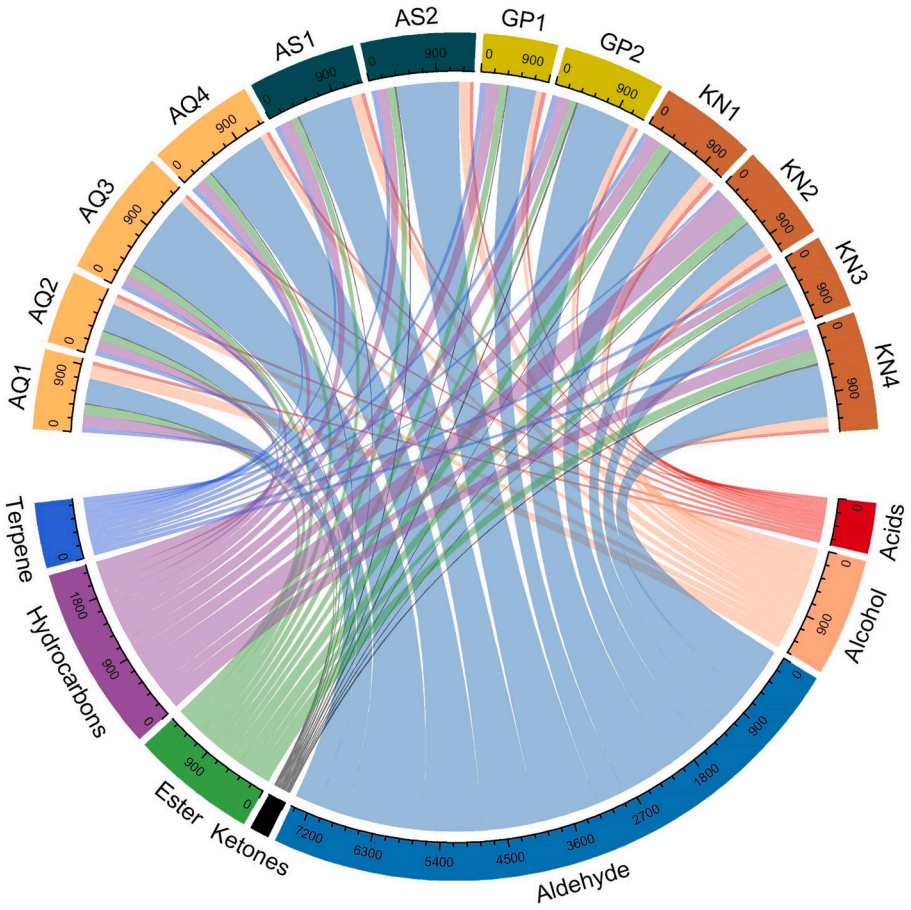

Fig. 3. Classes of volatile compounds in studied samples by Circos plot.

cultivar. Such purpose is desired since with the MHS-SPME technique we extract as much information as possible from the cultivars, eliminating any associated matrix factor, which allows a better interpretation of each cultivar.

From the fatty acid profile, a discriminant model with three significant discriminant functions was obtained, explaining 100% of the variance, where the first two functions explain 98% of the variance of the experimental data (Fig. 4A). In this case, the first discriminant shows eigenvalue obtained of 44.19, and the canonical correlation 0.989. In the second discriminant function the canonical correlation presents a value of 0.965 and eigenvalue of 13.35. A lowest eigenvalue (1.16), and discriminant function (0.733) is obtained in the third discriminant function, once accounting for 2% of variance, and 100% of cumulative variance. For volatiles (Fig. 4B), the three significant discriminant functions explained 100% of the variance, however, the first two functions have already reached this observed variance. The first discriminant function of volatile compounds shows a higher eigenvalue and a canonical correlation of 1.000, accounting 99.9% of variance. The high eigenvalue and canonical correlation obtained in both analyses show how well the classification differentiates the groups. More information about the eigenvalues, correlations, discriminant functions, and classification functions is supported in the supplemental material.

Out of the 14 variables/fatty acids identified, only four were classified as discriminating cultivars, namely C17:1, C18:0, C18:1, and C18:2. For the volatiles, of the 47 variables/volatiles identified, seven were classified as cultivar discriminants, namely *(E)*-2-Hexen-1-ol, *(Z)*-3-Hexen-1-ol, *(E)*-2-Hexenyl acetate, *(Z)*-3-Hexenyl Acetate, 3-Methylbutyl Acetate, Hexyl Acetate, and Nonanal. Table 8 shows the satisfactory classification performance of s-LDA by 'leave-one-out' cross-validation for volatile and fatty acids profile.

Linear discriminant analysis (LDA) reduces dimensionality and extracts important features from a data matrix. This dimensioning can lead to a singular matrix, as it occurs in a lower dimension in an attempt to separate the vectors into classes. When the data dimension is too large for the classification vectors, the reduced matrix may become impossible or generate an incorrect classification. This is a disadvantage when working with small sample groups (Sharma & Paliwal, 2015).

The s-LDA technique is an alternative to this problem. This technique uses forward and backward regression models, selecting the appropriate features in a non-exhaustive way. F-testing removes insignificant terms and inserts the most correlated features, resulting in a dispersion matrix fully exploited in its information spaces, which contain some helpful discriminant for classification. Thus, it is to be expected a small sample size doesn't compromise the model (Huberty, 1984; Krusienski, Sellers, McFarland, Vaughan, & Wolpaw, 2008).

From the variables of fatty and volatile acids selected by s-LDA, principal component analysis (PCA) was applied to these data. Fig. 5 shows the PCA obtained. Therefore, it was possible to identify which variables (volatiles and fatty acids) contribute to the difference between the monovarietal VOO studied.

PCA 1 and 2 were able to explain 81.9% of the selected data for cultivars. The first dimension shows a high eigenvalue (7.07) and accounting 64.3% of the variance. In PCA 2, a low eigenvalue of 1.73 is presented, and the variance is 17.6%. The eigenvalue, loadings, and scores of PCA are shown in the supplemental material. Arbequina is the cultivar strongly classified with C17:1 and C18:2, in addition to having mostly volatile compounds 3-Methylbutyl acetate, *(E)*-2-Hexen-1-ol, and *(E)*-2-Hexenyl Acetate as markers. Authors reported a strong relationship of *(E)*-2-Hexen-1-ol with the Arbequina cultivar compared to other cultivars (da Costa et al., 2020; Hassine et al., 2015; Yu et al.,

Table 7
Fatty acids profile of olive oils from cvs. Arbequina, Arbosana, Grappolo and Koroneiki ($n = 3$; mean with the value of standard deviation represented in brackets).

Sample	AQ1	AQ2	AQ3	AQ4	AS1	AS2	GP1	GP2	KN1	KN2	KN3	KN4	*p*-value
C14	0.02a-e	0.02a-e	0.02a-e	0.02a,b	0.01c-e	0.02a,c	0.01b,d,e	0.01d	0.01a-e	0.02a-e	0.02a-e	0.02a-c,e	0.001*
	(0.01)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	
C16	15.38a	13.02b	13.20c	16.14a	13.24c	12.60d	9.34e	8.23f	11.97g,h	12.09g	10.69i	11.60h	<0.001*
	(0.02)	(0.03)	(0.02)	(0.17)	(0.01)	(0.03)	(0.03)	(0.04)	(0.01)	(0.02)	(0.03)	(0.05)	
C16:1	2.39a	1.46b	1.60c	2.28d	1.59c	1.51e	0.49f	0.43g	1.20h	0.86i	0.72j	0.80k	<0.001*
	(0.01)	(0.00)	(0.00)	(0.01)	(0.01)	(0.00)	(0.01)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	
C17	0.21a-c	0.17a	0.18a,b	0.23c	0.23b,c	0.18a	0.06d,e	0.07d,f	0.06e	0.06d,e	0.07d	0.08f	<0.001*
	(0.01)	(0.00)	(0.00)	(0.00)	(0.01)	(0.01)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	
C17:1	0.28a	0.24b,c	0.23b,d	0.25c,e	0.32a,d,e	0.25c,e	0.06f	0.08g,h	0.06f,g	0.06f	0.08g,h	0.08h	<0.001*
	(0.00)	(0.01)	(0.00)	(0.00)	(0.01)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	
C18	1.46a,b	1.67c	1.49a	1.50a	1.63c,d	1.49a	1.54b	1.59d	1.83e	1.97f	1.87g	1.94e-g	<0.001*
	(0.02)	(0.01)	(0.00)	(0.00)	(0.01)	(0.00)	(0.00)	(0.01)	(0.00)	(0.00)	(0.00)	(0.02)	
C18:1	64.97a	71.82b	70.09c	65.73a	72.21b	71.59b	80.16d	80.80d	76.39e	76.26e	76.90f	74.64b,c,e,f	<0.001*
	(0.04)	(0.02)	(0.05)	(0.12)	(0.14)	(0.05)	(0.02)	(0.47)	(0.03)	(0.05)	(0.04)	(0.66)	
C18:2	8.94a	6.00b	6.33c	8.77d	4.95e	5.56f	4.61g	3.13h	4.01i	4.21j	3.72k	4.48g,j	<0.001*
	(0.02)	(0.02)	(0.00)	(0.03)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)	(0.00)	(0.02)	(0.04)	
C18:3	0.85d	0.95e,f	0.74f	0.73d,e	1.02e,f	0.83f	0.77a	0.77b	0.76c	0.73f	0.85g	0.93h	<0.001*
	(0.00)	(0.00)	(0.00)	(0.00)	(0.01)	(0.00)	(0.01)	(0.00)	(0.00)	(0.00)	(0.00)	(0.01)	
C20	0.33a,b	0.35a-d	0.36c,d	0.34a-d	0.35a-d	0.36c	0.25e	0.27f	0.31a	0.36b-d	0.39d,g	0.41g	<0.001**
	(0.00)	(0.01)	(0.00)	(0.01)	(0.01)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	
C20:1	0.34a,b	0.38c,d	0.36a,c	0.32b	0.38c-e	0.38a-f	0.42f	0.40e	0.28g	0.33b	0.36a-g	0.39d,e	<0.001*
	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.01)	(0.00)	(0.00)	(0.00)	(0.00)	(0.01)	(0.00)	
C21	0.02a-d	0.02a-d	0.02a-d	0.03a-d	0.02a-d	0.02a,b	0.01a-d	0.02a-d	0.01c,d	0.01a-d	0.02a,c	0.02b,d	<0.001*
	(0.00)	(0.00)	(0.00)	(0.01)	(0.01)	(0.00)	(0.01)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	
C22	0.11a,b	0.14c,d	0.13c,e	0.11a,b	0.13a,c,d	0.14d	0.07f	0.08b,e,f	0.09b	0.11a-e	0.14c,d	0.15d	<0.001*
	(0.00)	(0.00)	(0.00)	(0.00)	(0.01)	(0.00)	(0.00)	(0.01)	(0.00)	(0.01)	(0.00)	(0.00)	
C24	0.05a-c	0.06c-f	0.08f,g	0.07d-g	0.06c-e	0.08g	0.04a,b	0.05b-d	0.03a	0.05b-d	0.07d-g	0.08e-g	<0.001**
	(0.01)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.01)	(0.00)	(0.00)	(0.01)	(0.00)	(0.00)	
SFA	17.58a	15.45b	15.49b	18.45a	15.68b	14.91c	11.33d	10.35e	14.32f	14.68c	13.28g	14.31c,f	<0.001*
	(0.04)	(0.03)	(0.02)	(0.17)	(0.04)	(0.03)	(0.01)	(0.04)	(0.02)	(0.01)	(0.02)	(0.08)	
MUFA	67.99a	73.89b,c	72.34d	68.74a	74.50b	73.78c	81.13e,f	81.75e,g	77.93g,h	77.52i	78.10g,h	75.95b-d,f,g,i	<0.001*
	(0.03)	(0.02)	(0.04)	(0.13)	(0.12)	(0.05)	(0.02)	(0.47)	(0.03)	(0.05)	(0.02)	(0.65)	
PUFA	9.79a	6.95b	7.10b	9.52c	5.97d	6.41e	5.38f	3.93g	4.77h	4.94i	4.59j	5.43f	<0.001*
	(0.02)	(0.02)	(0.01)	(0.03)	(0.01)	(0.02)	(0.01)	(0.02)	(0.01)	(0.00)	(0.02)	(0.05)	

C14:0, myristic acid; C16:0, palmitic acid; C16:1, palmitoleic acid; C17:0, margaric acid; C17:1, margoleic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, linolenic acid; C20:0, arachidic acid; C20:1, eicosanoic acid; C22:0, behenic acid; C24:0, lignoceric acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

[a-k]In the same line, for each volatile compound, mean values with different letters differ significantly ($p < 0.05$); *$p < 0.05$, by means of Levene's test. P values are those from one-way Welch ANOVA analysis. Means were compared by Dunnett T3′s test, since equal variances could not be assumed; **$p > 0.05$, by means of Levene's test. P values are those from one-way ANOVA analysis. Means were compared by Tukey's test, since equal variances could be assumed.

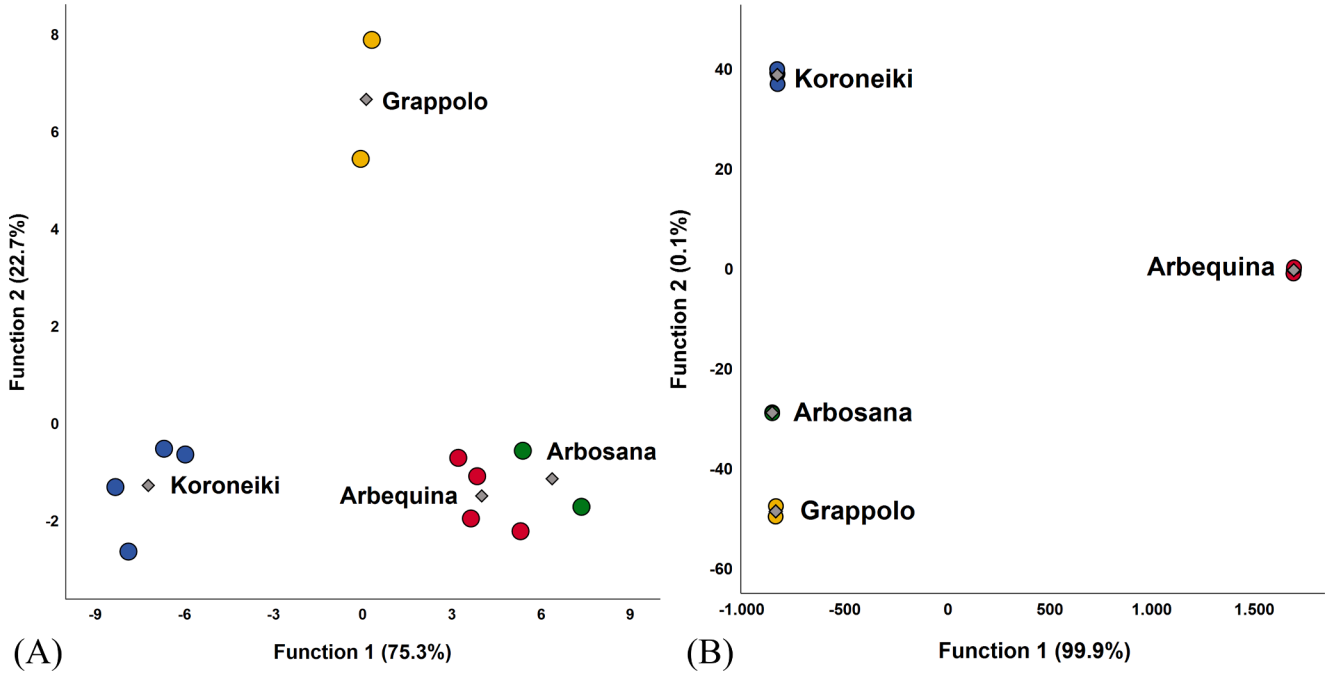

Fig. 4. Linear discriminant analysis of fatty acids (A) and volatile profile (B) of Arbequina, Arbosana, Grappolo, and Koroneiki cultivars.

Table 8
Prediction obtained for each virgin olive oil variety using data from the four fatty acids (in italic) and seven volatile compounds (in bold) selected according to the criteria described in the methodology. Reports are shown in percentage and number of samples correctly assigned (in brackets) by the model to the different cultivars.

Cultivar	Koroneiki	Arbequina	Grappolo	Arbosana	Total	Classified
Koroneiki	*100% (4)*	*0*	*0*	*0*	*4*	*100% (4)*
	100% (4)	**0**	**0**	**0**	**4**	**100% (4)**
Arbequina	*0*	*100% (4)*	*0*	*0*	*4*	*100% (4)*
	0	**100% (4)**	**0**	**0**	**4**	**100% (4)**
Grappolo	*0*	*0*	*100% (2)*	*0*	*2*	*100% (2)*
	0	**0**	**100%** *(2)*	**0**	**2**	**100%** *(2)*
Arbosana	*0*	*0*	*0*	*100% (2)*	*2*	*100% (2)*
	0	**0**	**0**	**100%** *(2)*	**2**	**100%** *(2)*

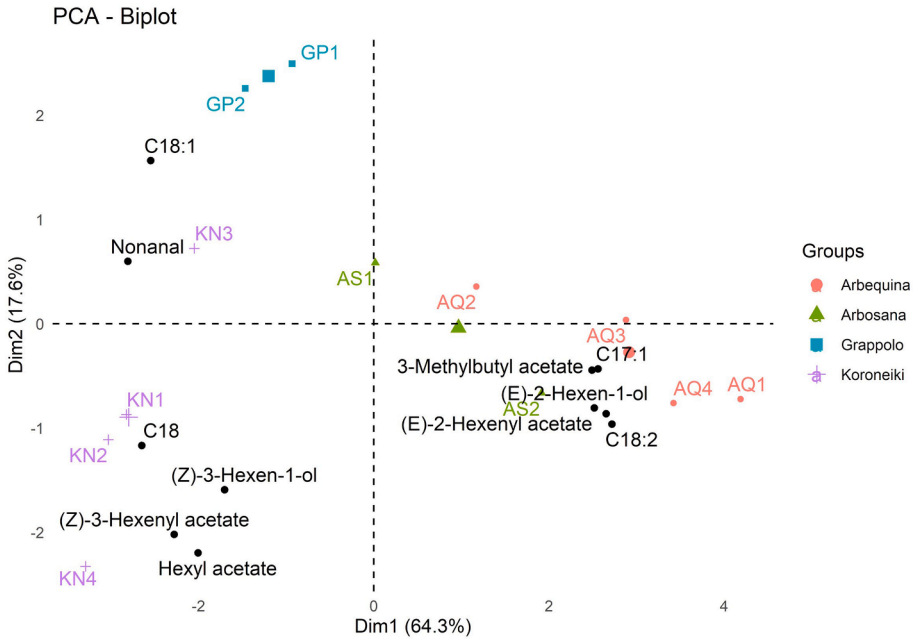

Fig. 5. Principal component analysis (PCA) on using fatty acids and volatile compounds from VOO.

2021), as well as more representative values of C18:2 in this cultivar. With the accurate quantification of these analytes through the MHS-SPME, we see *(E)*-2-Hexenyl Acetate as the most representative of Arbequina. This fact is associated with the conversion of *(E)*-2-Hexen-1-ol through alcohol acetyltransferase (AAT). Sánchez-Ortiz, Pérez, and Sanz (2013) observed in his work a higher concentration of C6 compounds associated with the high activity of hydroperoxide lyase in this cultivar. This is in line with the result obtained in our study.

Arbosana samples were grouped in the same region of Arbequina. Polari et al. (2021) presented data that support this information, since the authors found higher concentrations of *(E)*-2-Hexenal in Arbequina and Arbosana cultivars, compared to Koroneiki. It is through this aldehyde that the classifying analytes of Arbequina and Arbosana are obtained, namely *(E)*-2-Hexen-1-ol and *(E)*-2-Hexenyl Acetate.

Cv. Koroneiki has a strong association with stearic acid (C18:0). The result obtained is in line with the one presented by Yu et al. (2021), which showed this fatty acid as more significant compared to other cultivars studied, in addition to showing that the aldehyde *(Z)*-3-Hexenal is more abundant in this cultivar. This fact can be associated with the weight of *(Z)*-3-Hexen-1-ol and *(Z)*-3-Hexenyl Acetate for the classification of the samples, since these analytes are the main ones formed from *(Z)*-3-Hexenal through alcohol dehydrogenase (ADH) and alcohol acetyltransferase (AAT) (Sánchez-Ortiz et al., 2013; ul Hassan et al., 2015). Our results showed the cultivar Grappolo classified by higher concentrations of oleic acid (C18:1), against the one presented by (Ballus et al., 2014), which showed in its work higher concentrations of this fatty acid compared to cv. Arbequina.

4. Conclusions

A protocol for quantification of volatile compounds from VOO by MHS-SPME was optimized using a Plackett-Burman design followed by a central composite rotational design. The method was validated, and 47 volatile compounds responsible for the aroma of olive oil were identified and quantified. The technique showed as a promising alternative for characterization of the volatile profile of VOO. Also, when combined with multivariate technics, it can be used as a discriminative tool and incorporated as a routine analysis. By s-LDA was possible to select the most relevant volatiles and fatty acids for classification. Our results showed markers strongly related to cultivars of olive, such as C18:1 and nonanal to GP; hexyl acetate and C18 for KN; *(E)*-2-Hexenyl Acetate, *(E)*-2-Hexen-1-ol, C17:1 and C18:2 for AQ and AS. By presenting a precise and robust quantification, the technique allows decision-making about the best aromatic profile related to the cultivar. We found that the quality of Brazilian oil was like European oils.

Finally, it is suggested to study new cultivars using the protocol developed to comprehensively investigate other factors and clarify the classification behavior through other multivariate methods.

CRediT authorship contribution statement

Adriano Freitas Lima: Software, Validation, Formal analysis,

Investigation, Writing – original draft, Visualization. **Wellington da Silva Oliveira:** Software, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Aline de Oliveira Garcia:** Software, Methodology, Validation, Writing – original draft, Writing – review & editing, Funding acquisition. **Eduardo Vicente:** Investigation, Resources, Writing – review & editing. **Helena Teixeira Godoy:** Writing – review & editing, Resources, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.112697.

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