

Research Article

Improvement of sensorial and volatile profiles of olive oil by addition of olive leaves

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The changes caused by the addition of olive leaves (0, 5, and 10%) during the extraction of olive oil and malaxation time (20, 30, and 30 min) in the volatile profile and sensory attributes of olive oil from cv. Cobrançosa were studied. To investigate such transformations, a central composite designs from the Response Surface Methodology (RSM) was used, retrieving 13 runs combining leaf percentages and malaxation times. Each run was extracted in triplicate (39 olive oils overall).

Sensory attributes were improved to leaves addition, mainly green and fruitiness attributes in olfactory and gustatory-olfactory sensations, but high malaxation times (>30 min) reduced pungent and bitter notes. Leaves addition increased the amounts of total volatiles, particularly the GLV's (green leaves volatiles) (*E*)-2-hexenal, (*Z*)-3-hexenal, and (*Z*)-3-hexenyl acetate, directly correlated with the improved sensory attributes. RSM models showed positive linear effect with leaves addition, but a negative effect with malaxation time. These results suggest the use of olive leaves as effective odorants for the olive mill industry, while enabling the reduction of malaxation times and by-product amounts.

Practical applications: The results obtained clearly open new lines of research to use olive leaves, a sub-product of olive oil extraction, in a valuable way. Olive leaves can be used as natural sources of odorants for olive oils. Furthermore, their use during the extraction of olive oils from overmature olives may also lead to an improvement of the volatile fraction and provide enhanced sensory properties to the consumers, thus conferring an added value to these oils. Another important practical application is the extraction process. In our work, we advise to optimize both the percentage of leaves and the malaxation time as much as possible, as they facilitate both sensory and volatile fractions of the extracted olive oils.

Keywords: olive leaves / malaxation / olive oil / sensorial / volatiles / Response Surface Methodology

Received: April 12, 2017 / Revised: June 25, 2017 / Accepted: June 27, 2017

DOI: 10.1002/ejlt.201700177

1 Introduction

Olive leaves represent an important by-product of the olive oil extraction industry, immediately after olive mill wastewaters. With a steady increase in production worldwide,

particularly in the last decade [1], the olive oil industry is seeking for effective alternatives to these industrial residues. According to Bouaziz et al. [2], about 3 to 10% of the total mass that enters the olive mills corresponds to olive leaves that are collected together with olives. In general, these leaves are discarded, or partially used for composting and animal feed. While no effective valorization is given to this sub-product, their primary fate is destruction, representing an extra financial effort for this industry. However, several strategies are being attempted to valorize olive leaves within the food sector. One of those strategies includes their use (as

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fresh leaves or as an extract) to improve the quality, composition, stability, and properties of olive oil and other vegetable oils [3, 4]. Indeed, the use of fresh olive leaves during extraction has improved the chemical composition of olive oils extracted from overmature olives [5] by increasing pigments (lutein, β -carotene, chlorophyll a, and pheophytin a) and vitamin E contents. This leads to an increase in their oxidative resistance and, consequently, shelf life. Other authors have also reported an increase in the number of antioxidants and antioxidant properties [6], as this effect is mainly attributed to the extraction of phenolic compounds from the olive leaves, mainly oleuropein [7, 8] and flavonoids [9]. Nevertheless, other chemical classes also contribute to the overall quality of olive oils and need to be studied under the addition of olive leaves. The sensory properties and volatile fraction are two aspects that need to be taken into account, as they act directly and indirectly, respectively, in the commercial classification of olive oils. In particular, the use of olive leaves to improve olive oil characteristics can cause sensory defects that are not permitted in the extra-virgin olive oil category, in accordance with European regulations [10]. Furthermore, another important aspect that influences the sensory and volatile profiles of olive oils is malaxation, mainly the dualistic feature time-temperature used during this extraction step [11, 12]. Excessive malaxation times and temperatures lead to the reduction of the formation of volatile compounds connoted with pleasant attributes, like green, fresh-cut grass formed during the lipoxygenase pathway, while raising the potential formation of off-flavors and the development and promotion of molecular mechanisms, namely the conversion of amino acids [13].

Therefore, in the present work, we intend to investigate if the addition of fresh olive leaves, combined with different malaxation times, could improve the sensory properties and volatile composition of olive oils from cv. Cobrançosa, a widespread olive cultivar in Portugal. To achieve such a goal, three mass percentages of olive leaves (0, 5, and 10% of fresh weight) and three malaxation times (20, 30, and 40 min at 25°C) were studied using a central composite design to retrieve the number of runs and factors combinations. A trained panel also assessed the sensory profile, and the volatile composition was determined by headspace solid-phase microextraction (HS-SPME) gas-chromatography with mass spectrometry (GC/MS).

2 Material and methods

2.1 Samples collection

Olives and olive leaves from cv. Cobrançosa were collected in a traditional olive grove located in Suçães (Mirandela 41°29'28.9"N 7°15'26.0"W), Trás-os-Montes (Northeast Portugal), in November 2015. About 50 kg of olives at a

maturation index 3–4 (according to Hermoso *et al.* [14]) were collected, together with approximately 3 kg of leaves. The olives were immediately transported to the laboratory and visually inspected to discard all olives and leaves with symptoms of attacks of pests and incidence of diseases. Once inspected, healthy olives were separated in 39 sub-samples of at least 1 kg for extraction.

2.2 Experimental design

In order to study the impact of olive leaves addition and malaxation time on the sensory and volatile profiles of olive oils from cv. Cobrançosa, one of the most representative of the Portuguese olive cultivars, Response Surface Methodology (RSM) by Minitab[®] software was used to retrieve the number of events and combinations to be studied. A one block face-centered ($\alpha = 1$) central composite design (CCD) was constructed. The two independent factors studied were the quantity of olive leaves added (X_1 : 0–10% w/w) and the malaxation time (X_2 : 20–40 min). The amounts of olive leaves were selected based on a previous study carried out in the same cultivar [5]. The malaxation times were within those commonly used, but selected in order to avoid prolonged exposure of olive paste to air, reducing the oxidation of olive oil. The response variables were the main volatile components identified and quantified, as detailed below, namely, (*E*)-2-hexenal, (*Z*)-3-hexenal, and total volatiles, in the olive oils obtained. Each one of the variables to be optimized was coded at three levels: -1 , 0 , $+1$, as presented in Table 1.

In total, 13 runs were carried out with five replications in the central point (5%–30 min. – Runs 1, 5, 6, 12, and 13). In each run, three independent olive oils were extracted, with a total of 39 independent olive oil extractions. To reduce systematic errors, the order of the olive oil extractions was randomly established. The experiments performed in the central point allowed the estimation of the influence of the experimental error, whereas the other experiments allowed the calculation of the regression coefficients of the model. The experimental data from the CCD was fitted to a second-order polynomial model, presented in Eq. (1):

$$Y = \beta_0 + \sum_{i=1}^2 \beta_i X_i + \sum_{i=1}^2 \beta_{ii} X_i^2 + \beta_{12} X_1 X_2 \quad (1)$$

Table 1. Independent variables and their coded and uncoded values

Coded value	Olive leaf (% w/w)	Malaxation time (min)
-1	0	20
0	5	30
1	10	40

where Y is the predicted response; β_0 is the constant (intercept); β_i is the linear coefficient, β_{ii} is the quadratic coefficient and β_{12} is the interaction coefficient. X_1 and X_2 are coded independent variables (leaf percentage and malaxation time, respectively).

The adequacy of the models was determined by evaluating the lack of fit, the coefficient of determination (R^2), and the adjusted- R^2 obtained from the analysis of variance (ANOVA) generated by the software. The statistical significance of the model and model variables was determined at the 5% probability level ($\alpha=0.05$).

2.3 Olive oils extraction

The olive oils were extracted within 24 h after olives and leaves were harvested, in a pilot extraction plant using an Abencor analyzer (Comercial Abengoa S.A., Seville, Spain) with three main units: A mill, a thermobeater where malaxation takes place at a controlled temperature, and a centrifuge. Olive samples were enriched with freshly chopped leaves at the defined amounts (0%, 5%, and 10% w/w) and then milled together. The olive paste obtained was homogenized, and about 700 g were transferred to the thermobeater unit for malaxation at pre-determined times (20, 30, and 40 min) in a thermostatic water bath at 25°C. In the final 5 min of each malaxation, 100 mL of water at 35°C was added to aid in the olive oil separation. The mixture was centrifuged and decanted, and the olive oil was collected and stored in 100 mL dark bottles.

2.4 Sensory analysis

The sensory analysis was performed according to the methodology described in the standards of International Olive Council (IOC), namely COI/T.20/Doc. No. 15/Rev. 8 [15], and by using the profile sheet provided in the COI/T.30/Doc. No. 17 [16]. A team of four trained panel members assessed the 39 olive oil samples. The number of trained panelists was decided according to the amount of olive oil available, conjugating also with the chemical parameters sample needs. Each trained panelist evaluated olfactory, gustatory-retronasal, and olfactory-gustatory sensations, in that order, all accounting for a total of 100 points. The following attributes were evaluated in the olfactory sensations (maximum of 35 points): olive fruitiness (0–7); other fruits (0–3); green (grass/leaves) (0–2); other positive sensations (0–3); and harmony (0–20). For the gustatory-retronasal sensations, the following parameters were assessed (maximum of 45 points): olive fruitiness (0–10); sweet (0–4); bitter (0–3); pungent (0–3); green (grass/leaves) (0–2); other positive sensations (0–3); and harmony (0–20). For the olfactory-gustatory sensations (maximum of 20 points), two parameters were assessed: Complexity (0–10) and persistence (0–10).

2.5 Volatile characterization by HS-SPME-GC-MS

The characterization of the volatile fraction of the olive oils was performed by headspace solid-phase microextraction (HS-SPME) gas-chromatography-mass spectrometry (GC/MS).

In 50 mL vials, 3 g of olive oil was spiked with an accurate amount of internal standard (2-methyl-4-pentanol) and volatiles adsorbed to a SPME fiber coated with divinylbenzene/carbonex/polydimethylsiloxane (DVB/CAR/PDMS 50/30 μm) (Supelco, Bellefonte, USA). The vials were conditioned for 5 min at 50°C for an incisive release of the volatile compounds. After this period, the SPME fiber was exposed for 30 min, at the same temperature, for the compound adsorption from the headspace. The conditions used for the volatile extraction were those optimized by Peres et al. [17]. The total procedure was made in duplicate per olive oil sample, with control samples (empty vials regularly with internal standard).

The retained compounds were eluted from the fiber by thermal adsorption for 1 min in the injection port (220°C). The fiber was maintained for further 10 min in the injector port of the chromatography system for cleaning and conditioning for further analyzes. The gas chromatographer used was a Shimadzu GC-2010 Plus equipped with a mass spectrometer Shimadzu GC/MS-QP2010 SE detector. A TRB-5MS (30 m \times 0.25 mm \times 0.25 μm) column (Teknokroma, Spain) was used. The injector was set at 220°C and the manual injections were made in splitless mode. The mobile phase consisted of helium (Praxair, Portugal) at a linear velocity of 30 cm/s and a total flow of 24.4 mL/min. The oven temperatures were the following: 40°C/1 min; 2°C/min until 220°C (30 min). The ionization source was maintained at 250°C with an ionization energy of 70 eV and an ionization current of 0.1 kV. All mass spectra were acquired by electron ionization. The MS spectra fragments were compared with those obtained from a database (NIST 11), and with those of commercial standards acquired from diverse producers. For qualitative purposes, each sample was injected in duplicate. The areas of the chromatographic peaks were determined by integrating the re-constructed chromatogram from the full scan chromatogram using the ion base (m/z intensity 100%) for each compound. For semi-quantification purposes, volatile amounts were calculated by the ratio of each individual base ion peak area to the area of the internal standard base ion peak area and converted to mass equivalents based on the internal standard mass added.

2.6 Statistical analysis

2.6.1 Analysis of variance

An analysis of variance (ANOVA) with Type III sums of squares was performed using the GLM (General Linear Model procedure) of the SPSS software, version 22.0 (IBM

Corporation, New York, U.S.A.). The fulfilment of the ANOVA requirements, namely the normal distribution of the residuals and the homogeneity of variance, were evaluated by means of the Kolmogorov-Smirnov with Lilliefors correction (if $n > 50$) or the Shapiro-Wilk's test (if $n < 50$), and the Levenés tests, respectively. All dependent variables were analysed using a one-way ANOVA with or without Welch correction, depending on whether the requirement of the homogeneity of variances was fulfilled. The main factors studied were the volatile composition and the attributes assessed in the sensory analysis of the olive oils extracted with olive leaves and different malaxation times. If a statistical significant effect was found, means were compared using Tukey's honestly significant difference multiple comparison test or Dunnett T3 test, also depending on whether equal variances could be assumed. All statistical tests were performed at a 5% significance level.

3 Results and discussion

3.1 Sensory analysis

Sensory analysis was performed at three levels: Olfactory, gustatory-olfactory, and olfactory-gustatory sensations. An important result was the total absence of negative attributes (defects) in the 39 samples, independent of the tested malaxation times. The results obtained in the olive oils for each sensation are detailed in Table 2. Starting with the olfactory sensations, and considering the mean values of the central point events (5%–30 min), the values of fruitiness varied between 5.5 (0%–40 min and 10%–20 min) and 6.0 (10%–30 min). According to European legislation, these olive oils could be considered extra-virgin olive oils (EVOO) based only on sensory analysis, due to their zero defects and a median value of fruitiness higher than zero [10]. The parameter named “other fruits” measured the presence of fruit sensation in the olive oil sensory profile. This parameter varied between 1.2 (10%–40 min) and 1.8 (0%–20 and 40 min), suggesting that an elevated quantity of olive leaves and higher malaxation times reduce the perception of “other fruits” in the olive oils. The parameter “green” received lower scores from the panelists in the oils extracted without leaves for 30 min (0%–30 min) (Table 2). In contrast, oils with 10% leaves (20 and 40 min) were among the samples with higher scores. Generally, the addition of olive leaves improved the green sensations, a very important attribute for consumers. Concerning “other sensations” samples with 0%–30 min and those with 10%–40 min reported a lower amount (mean of 1.2), compared to samples with 0%–20 min (mean of 1.8). Evidently, the addition of olive leaves with increased malaxation times could be responsible for a reduction of other sensations perceived. Harmony, measuring the equilibrium between all the perceived parameters, varied between 16.1 and 16.8 points. Oils with 10%–20 min were more

harmonious than those extracted without leaves (0%–30 min) or with higher malaxation times in the presence of leaves (10%–40 min) (Table 2). This means that the addition of olive leaves with a low malaxation time improves the equilibrium and harmony of the olive oils at olfactory sensations. However, as observed in Table 2, if the malaxation time is increased from 20 to 40 min (10% leaves), the harmony immediately reduces from 16.8 to 16.1 points.

After olfactory sensations, the gustatory-olfactory sensations were assessed (Table 2; Fig. 1). Regarding fruitiness, values varied between 6.4 (5%–20 min) and 7.0 (0%–40 min, and 10%–40 min). To obtain higher fruitiness, malaxation time should be increased, since higher scores were obtained at 40 min. However, no significant differences were verified with the percentage of olive leaves and malaxation time ($P = 0.306$; Fig. 1). The “sweet” sensation was highly scored in the oils with 0%–40 min and 10%–20 min with a score of 2.5, while samples extracted with 0%–30 min and 10%–40 min received a score of 3.1. Evidently, a clear pattern was not noticed for sweetness regarding the addition of leaves and the increase of the malaxation time, since no significant differences were verified ($P = 0.360$). Bitter sensation was highly scored in the oils with 0%–40 min, while the samples 0%–30 min, 5%–20 min, and 10%–40 min retrieved lower scores, with 1.1 points. Regarding pungent sensation, it appeared that the combination of malaxation time and addition of olive leaves reduced this attribute, since oils with 10%–40 min presented the lowest score of 1.5, while 0%–40 min and 10%–20 min reported the highest pungent sensation with 2.3. Bitter and pungent sensations are mainly related to the extraction of phenolic compounds, mainly secoiridoids [8, 18]. Therefore, increased extraction times are essential to improve these positive attributes, as they were observed mainly in the samples with 0%–40 min. However, the addition of leaves may not increase considerably these two attributes, since different types of phenolic compounds are extracted from the olive leaves and olive paste, mainly secoiridoids in the former, as oleuropein and oleuropein derivatives, like oleuropein aglycon, demethyloleuropein, and the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol and tyrosol from the olive paste [19, 20]. Therefore, malaxation time is more important for these two attributes rather than the quantity of leaves added, probably due to the different phenolic components extracted from leaves and from olive paste.

Green gustative sensations were higher in olive oils with 0%–40 min and 5%–20 min with a score of 1.5 (Table 2; Fig. 1). This means that by adding olive leaves malaxation time can be reduced by 20 min, obtaining the same green sensation in the final olive oil. The lowest scores were reported for oils extracted with 0%–30 min and 10%–30 min, with 1.0 (Table 2; Fig. 1). With respect to “other gustative sensations”, higher scores were obtained in the oils with 0%–40 min and 10%–20 min. Evidently,

Table 2. Sensory profile of olive oils from cv. Cobrançosa extracted with different amounts of olive leaves (0, 5, and 10%) and malaxation times (20, 30, and 40 min) ($n = 3$; mean with the value of standard deviation represented in brackets)

Run	4	8	2	9	1	5	6	12	13	11	3	7	10	
Leaves (%)	0%	0%	0%	5%	5%	5%	5%	5%	5%	5%	10%	10%	10%	
Extraction time (min)	20 min	30 min	40 min	20 min	30 min	30 min	30 min	30 min	30 min	40 min	20 min	30 min	40 min	P-value
Olfactory sensations														
Fruitiness	5.6 a,b (0.66)	5.8 a,b (0.41)	5.5 a,b (0.55)	5.9 a,b (0.20)	5.4 a,b (0.66)	5.3 a (0.42)	5.6 a,b (0.66)	6.0 a,b (0.00)	6.3 b (0.42)	5.9 a,b (0.20)	5.5 a,b (0.55)	6.0 a,b (0.00)	5.8 a,b (0.41)	0.013*
Other fruits	1.8 b (0.26)	1.3 a,b (0.41)	1.8 a,b (0.27)	1.7 a,b (0.41)	1.8 a,b (0.27)	1.4 a,b (0.20)	1.7 a,b (0.26)	1.3 a,b (0.26)	1.3 a,b (0.27)	1.3 a,b (0.26)	1.5 a,b (0.45)	1.6 a,b (0.00)	1.2 a (0.26)	0.003**
Green	1.3 a-c (0.26)	1.0 a (0.00)	1.4 b,c (0.20)	1.4 b,c (0.20)	1.4 b,c (0.20)	1.2 a-c (0.26)	1.4 b,c (0.20)	1.5 c (0.00)	1.5 c (0.00)	1.4 b,c (0.20)	1.4 b,c (0.20)	1.1 a,b (0.00)	1.4 b,c (0.20)	<0.001*
Other sensations	1.8 b (0.27)	1.2 a (0.26)	1.7 b (0.26)	1.5 a,b (0.00)	1.8 b (0.27)	1.6 a,b (0.20)	1.7 b (0.26)	1.3 a,b (0.26)	1.5 a,b (0.00)	1.4 a,b (0.20)	1.7 b (0.26)	1.5 a,b (0.35)	1.2 a (0.26)	<0.001*
Harmony	16.6 a, b (0.49)	16.1 a b (0.20)	16.6 a, b (0.49)	16.2 a b (0.26)	16.0 a b (0.00)	16.4 a, b (0.49)	16.1 a b (0.20)	16.3 a, b (0.41)	16.5 a, b (0.32)	16.2 a b (0.26)	16.8 b b (0.26)	16.3 a, b (0.35)	16.1 a b (0.20)	<0.001*
Σ of olfactory sensations	27.1 c (1.07)	25.4 a (0.80)	26.9 b,c (0.49)	26.7 a-c (0.52)	26.3 a-c (0.68)	25.8 a-c (0.68)	26.4 a-c (0.80)	26.5 a-c (0.45)	27.0 b,c (0.55)	26.3 a-c (0.42)	26.9 b,c (1.02)	26.5 a-c (0.71)	25.7 a, b (0.68)	0.001**
Gustatory-Olfactory sensations														
Fruitiness	6.7 a (0.52)	6.6 a (0.49)	7.0 a (0.00)	6.4 a (0.49)	7.0 a (0.00)	6.5 a (0.55)	7.0 a (0.63)	6.8 a (0.41)	6.8 a (0.42)	6.8 a (0.41)	6.8 a (0.41)	6.8 a (0.71)	7.0 a (0.00)	0.231*
Sweet	2.7 a (0.75)	3.1 a (0.20)	2.5 a (0.55)	3.0 a (0.00)	2.5 a (0.45)	2.8 a (0.61)	2.6 a (0.66)	2.8 a (0.26)	2.8 a (0.27)	2.9 a (0.38)	2.5 a (0.55)	3.0 a (0.71)	3.1 a (0.38)	0.167*
Bitter	1.8 c,d (0.26)	1.1 a (0.20)	2.2 d (0.52)	1.1 a (0.20)	1.8 b,c (0.26)	1.6 a-c (0.20)	1.8 c,d (0.41)	1.8 c,d (0.26)	2.0 c,d (0.00)	1.3 a,b (0.42)	1.7 b-d (0.26)	1.7 b-d (0.00)	1.1 a (0.20)	<0.001*
Pungent	2.2 a-c (0.26)	1.6 a,b (0.58)	2.3 c (0.26)	1.8 a-c (0.42)	2.0 a-c (0.45)	1.9 a-c (0.38)	2.0 a-c (0.45)	1.7 a-c (0.26)	1.9 a-c (0.38)	1.6 a,b (0.38)	2.3 b,c (0.27)	1.8 a-c (0.35)	1.5 a (0.32)	0.002**
Green	1.4 b (0.38)	1.0 a (0.00)	1.5 b (0.00)	1.5 b (0.00)	1.5 b (0.00)	1.3 a,b (0.27)	1.3 a,b (0.26)	1.5 b (0.00)	1.5 b (0.00)	1.3 a,b (0.26)	1.3 a,b (0.26)	1.0 a (0.35)	1.3 a,b (0.26)	<0.001*
Other sensations	1.7 c (0.26)	1.1 a (0.20)	1.8 c (0.27)	1.1 a (0.20)	1.6 b,c (0.20)	1.6 b,c (0.20)	1.6 b,c (0.20)	1.2 a,b (0.26)	1.5 a-c (0.00)	1.1 a (0.20)	1.8 c (0.27)	1.5 a-c (0.35)	1.1 a (0.20)	<0.001*
Harmony	16.5 a (0.55)	16.0 a (0.00)	16.4 a (0.49)	16.1 a (0.20)	16.2 a (0.41)	16.2 a (0.41)	16.2 a (0.41)	16.0 a (0.00)	16.1 a (0.20)	16.1 a (0.20)	16.5 a (0.55)	16.2 a (0.71)	16.0 a (0.00)	0.102*
Σ of gustatory-olfactory sensations	32.9 (0.80)	30.4 a (0.86)	33.7 e (1.13)	30.9 a, b (0.49)	32.6 b-e (0.92)	31.8 a-d (1.29)	32.5 b-e (1.10)	31.8 a-d (0.52)	32.5 b-e (0.32)	31.0 a, b (1.48)	32.8 c-e (0.93)	31.9 a-e (1.77)	31.1 a-c (0.38)	<0.001*
Olfactory-gustatory sensations														
Complexity	7.3 a (0.52)	6.8 a (0.41)	7.2 a (0.41)	7.0 a (0.32)	7.3 a (0.41)	6.9 a (0.20)	6.8 a (0.41)	7.0 a (0.00)	7.0 a (0.00)	7.0 a (0.00)	7.2 a (0.41)	7.0 a (0.71)	7.0 a (0.00)	0.101*
Persistence	7.3 a (0.52)	7.2 a (0.41)	7.7 a (0.52)	7.3 a (0.41)	7.6 a (0.49)	7.0 a (0.63)	7.0 a (0.89)	7.5 a (0.45)	7.1 a (0.20)	7.1 a (0.20)	7.6 a (0.66)	7.4 a (0.00)	7.1 a (0.20)	0.194*
Σ of olfactory-gustatory sensations	14.7 a (0.82)	14.0 a (0.63)	14.8 a (0.75)	14.3 a (0.41)	14.9 a (0.49)	13.9 a (0.66)	13.8 a (1.17)	14.5 a (0.45)	14.1 a (0.20)	14.1 a (0.20)	14.8 a (0.61)	14.4 a (0.71)	14.1 a (0.20)	0.112*
Final score	74.7 d,e (2.27)	69.8 a (1.75)	75.4 e (1.72)	71.9 a-d (0.66)	73.8 b,e (1.68)	71.5 a-d (2.07)	72.8 a-e (2.56)	72.8 a-e (0.75)	73.6 b-e (0.58)	71.3 a-c (1.57)	74.5 c-e (1.58)	72.8 a-e (3.18)	70.8 a, b (1.08)	<0.001*

For each sensory attribute assessed, within the same line, 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.

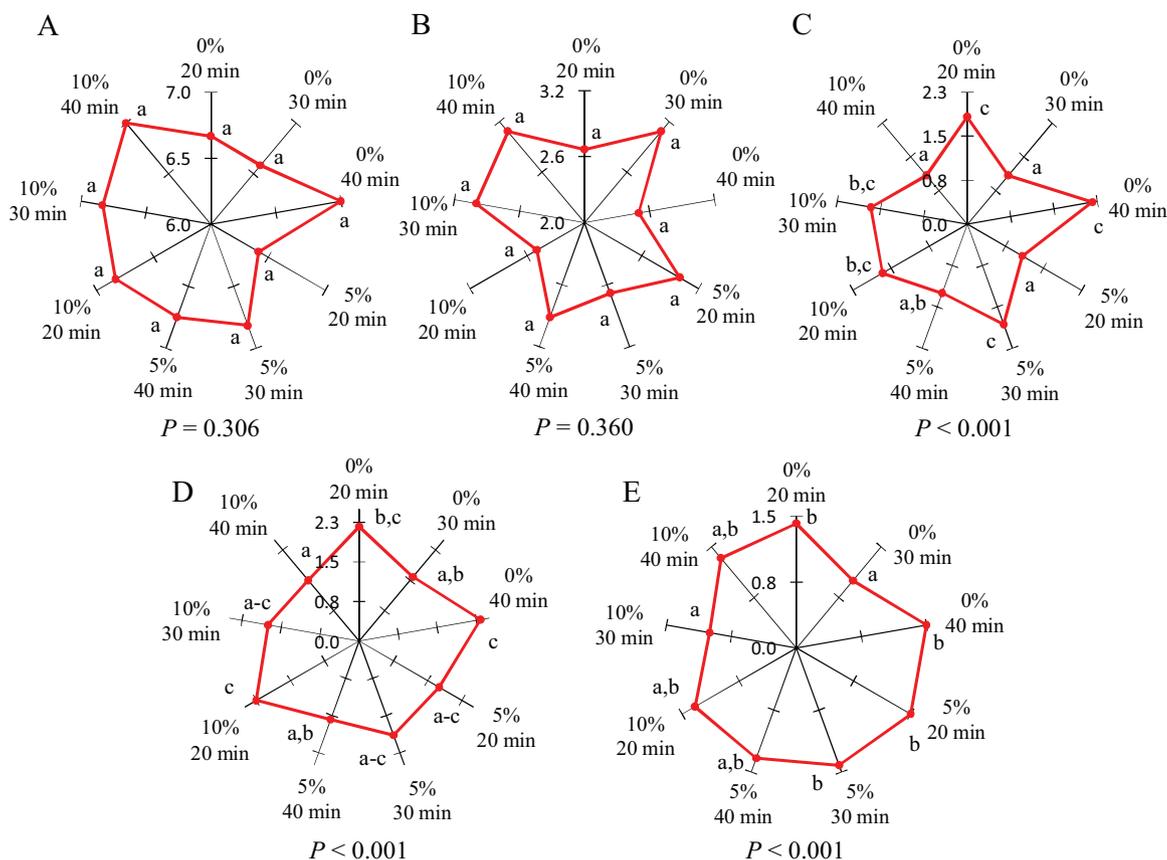


Figure 1. Chromatographic profiles of olive oils from cv. Cobrançosa extracted with different percentages of olive leaves at 30 min of malaxation time (I.S. – internal standard). Volatile compounds: 1 – (*Z*)-3-hexenal; 2 – (*E*)-2-hexenal; 3 – (*Z*)-3-hexen-1-ol; 4 – hexanol; 5 – 2-heptanone; 6 – (*E,E*)-2,4-hexadienal; 7 – 3-ethyl-1,5-octadiene (I); 8 – 3-ethyl-1,5-octadiene (II); 9 – benzaldehyde; 10 – (*Z*)-3-hexenyl acetate; 11 – hexyl acetate; 12 – limonene; 13 – benzyl alcohol; 14 – β -ocimene; 15 – octanal; 16 – nonanal; 17 – phenylethyl alcohol; 18 – dodecane; 19 – decanal; 20 – α -copaene; 21 – caryophyllene; 22 – α -farnesene.

with the addition of 10% of leaves, we can also reduce the malaxation time in 20 min to achieve the same score for “other sensations.” However, regarding gustative harmony, higher scores were obtained with lower malaxation times, 20 min, with and without leaves (0 and 10%, respectively) (Table 2). Increasing malaxation time generally reduces harmony, since some other attributes may be extracted in higher amounts, like green sensation (Table 2).

Relative to “final sensations,” only two parameters were evaluated: Complexity and persistence, as shown in Table 2. Complexity presented the highest and lowest scores in the oils without leaves addition, depending on the malaxation time, with higher complexity at 20 min and a score of 7.3, which was reduced to 6.8 for 30 min of malaxation. Samples with leaves addition all had higher scores than the control ones, varying between 7.0 and 7.2 points, independent of the malaxation times. Regarding persistence, higher scores were reported in oils without leaves and higher malaxation times (0%–40 min), with 7.7, while the oils with 5 and 10% leaves,

both with 40 min, reported lower persistence (7.1). Knowing that persistence is mostly associated with bitter and pungent sensations, the higher scores at 0%–40 min were expected. This observation, as already explained, is related to the different types of phenolic compounds extracted from the olive leaves and olive paste.

Overall, and pertaining to the total final scores of the panelists, values ranged from 69.8 (0%–30 min) and 75.4 (0%–40 min and 10%–20 min). Within the oils with leaves addition, those extracted with 10% of leaves during 20 min presented the highest score of 74.5 (Table 2). This result suggests that the addition of olive leaves may allow a reduction on the malaxation time without loss of sensory attributes, but that higher malaxation times might compromise the achieved attributes.

The addition of olive leaves to Italian olives also improved the sensory characteristics of olive oils [21]. The main improvements were verified in the green, fruity, and bitter taste, in accordance with our work. Furthermore, these

Table 3. Volatile profile (mg/kg of oil; values in bold are expressed as $\mu\text{g}/\text{kg}$ of oil) of olive oils from cv. Cobrançosa extracted with different amounts of olive leaves and malaxation times ($n = 3$; mean with the value of standard deviation represented in brackets)

Run	4	8	2	9	1	5	6	12	13	11	3	7	10	
Leaves (%)	0%	0%	0%	5%	5%	5%	5%	5%	5%	5%	10%	10%	10%	
Extraction time (min)	20 min	30 min	40 min	20 min	30 min	30 min	30 min	30 min	30 min	40 min	20 min	30 min	40 min	P-value
(Z)-3-hexenal	2.62 a-c (0.28)	2.45 a,b (0.12)	2.76 a-c (0.79)	4.24 b-d (1.56)	4.25 b-d (0.35)	4.48 b-d (1.74)	3.59 a-d (0.74)	3.70 a-d (0.30)	4.66 c,d (0.18)	1.90 a (0.90)	5.59 d (2.15)	4.55 c,d (1.18)	2.93 a-c (0.50)	<0.001*
(E)-2-hexenal	8.83 a,b (1.56)	8.67 a,b (2.36)	7.68 a (1.04)	10.4 a,b (3.00)	9.37 a,b (1.95)	8.78 a,b (1.20)	8.91 a,b (0.41)	11.7 a,b (3.03)	11.1 a,b (3.51)	12.5 a, b (3.15)	10.9 a,b (4.12)	11.9 a,b (4.31)	14.1 b (3.74)	0.020*
(Z)-3-hexen-1-ol	0.09 a,b (0.02)	0.09 a,b (0.02)	0.05 a (0.01)	0.10 a,b (0.02)	0.12 b (0.03)	0.12 b (0.01)	0.11 b (0.02)	0.13 b (0.02)	0.10 a,b (0.03)	0.14 b (0.04)	0.12 b (0.03)	0.13 b (0.04)	0.12 b (0.04)	<0.001*
Hexanol	0.70 a (0.03)	0.64 a (0.10)	0.70 a (0.07)	0.59 a (0.15)	0.66 a (0.06)	0.73 a (0.05)	0.69 a (0.01)	0.59 a (0.20)	0.66 a (0.15)	0.67 a (0.11)	0.68 a (0.10)	0.60 a (0.15)	0.64 a (0.11)	0.461*
2-Heptanone	0.31 a,b (0.02)	0.32 a,b (0.03)	0.35 a,b (0.04)	0.30 a,b (0.05)	0.32 a,b (0.01)	0.32 a,b (0.04)	0.38 b (0.16)	0.29 a,b (0.04)	0.28 a,b (0.01)	0.29 a, b (0.03)	0.33 a,b (0.04)	0.28 a,b (0.03)	0.27 a (0.03)	<0.001*
(E,E)-2,4-hexadienal	1.21 d (0.05)	0.13 a (0.01)	0.14 a (0.03)	0.23 a,b (0.10)	0.26 a,b (0.03)	0.30 b,c (0.06)	0.25 a, b (0.02)	0.20 a,b (0.08)	0.26 a,b (0.09)	0.26 a, b (0.13)	0.34 b,c (0.08)	0.35 b,c (0.13)	0.44 c (0.06)	<0.001*
3-Ethyl-1,5-octadiene (I)	1.07 a,b (0.05)	1.08 a-c (0.10)	1.05 a (0.08)	1.26 c-e (0.14)	1.39 d,e (0.08)	1.21 c (0.03)	1.17 a-c (0.04)	1.12 a-c (0.05)	1.09 a-c (0.04)	1.07 a-c (0.09)	1.41 e (0.07)	1.24 c,d (0.05)	1.18 b,c (0.04)	<0.001*
3-Ethyl-1,5-octadiene (II)	1.16 a,b (0.04)	1.15 a (0.07)	1.13 a,b (0.08)	1.27 b (0.14)	1.44 c (0.19)	1.23 a,b (0.04)	1.20 a,b (0.06)	1.16 a,b (0.09)	1.15 a,b (0.06)	1.14 a (0.10)	1.43 c (0.08)	1.26 a,b (0.04)	1.21 a,b (0.03)	<0.001*
Benzaldehyde	1.48 a-c (0.08)	1.53 a-c (0.10)	1.30 a (0.37)	1.66 a-d (0.32)	1.24 a (0.12)	1.66 a-d (0.29)	1.54 a-c (0.18)	1.90 c,d (0.20)	2.02 d (0.12)	2.07 d (0.05)	1.44 a,b (0.34)	1.48 a-c (0.17)	1.86 b-d (0.31)	<0.001*
(Z)-3-hexenyl acetate	2.12 a-c (0.71)	1.79 a,b (0.58)	2.44 a-c (0.49)	3.29 b,c (1.22)	1.97 a-c (0.70)	3.19 b,c (1.52)	3.73 c (1.54)	2.07 a-c (0.99)	2.95 a-c (0.49)	1.82 a, b (0.89)	3.48 b,c (0.42)	3.73 b,c (1.26)	1.23 a (0.23)	<0.001*
Hexyl acetate	0.14 a-c (0.05)	0.13 a,b (0.03)	0.15 a-d (0.04)	0.18 a-d (0.08)	0.12 a,b (0.06)	0.21 b-d (0.10)	0.27 d (0.11)	0.07 a (0.00)	0.21 c-d (0.03)	0.14 a-c (0.07)	0.16 a-d (0.08)	0.27 c,d (0.06)	0.09 a,b (0.01)	<0.001*
Limonene	0.09 b (0.03)	0.07 a,b (0.02)	0.06 a,b (0.02)	0.07 a,b (0.03)	0.06 a,b (0.03)	0.09 b (0.02)	0.07 a,b (0.02)	0.07 a,b (0.02)	0.07 a,b (0.02)	0.07 a, b (0.03)	0.04 a (0.00)	0.05 a,b (0.00)	0.08 b (0.02)	<0.001**
Benzyl alcohol	0.37 a-c (0.03)	0.39 a-c (0.12)	0.28 a (0.10)	0.45 a-d (0.06)	0.31 a (0.06)	0.43 a-d (0.10)	0.38 a-c (0.06)	0.52 b-d (0.12)	0.55 c,d (0.08)	0.55 c, d (0.07)	0.33 a,b (0.12)	0.47 a-d (0.09)	0.60 d (0.19)	<0.001*
β -ocimene	0.22 a-d (0.03)	0.22 a-d (0.07)	0.14 a (0.06)	0.26 b-d (0.10)	0.17 a,b (0.08)	0.29 b-d (0.06)	0.23 a-d (0.04)	0.23 a-d (0.07)	0.35 d (0.04)	0.34 d (0.03)	0.20 a-c (0.02)	0.28 b-d (0.08)	0.31 c,d (0.06)	<0.001*
Octanol	0.02 a-d (0.00)	0.02 a-c (0.00)	0.01 a (0.00)	0.02 a-d (0.00)	0.02 a,b (0.00)	0.02 b-c (0.01)	0.02 a-d (0.00)	0.02 b-d (0.00)	0.03 c-e (0.00)	0.03 d, e (0.00)	0.02 a-d (0.01)	0.02 b-e (0.00)	0.03 e (0.01)	<0.001*
Nonanal	0.09 a-c (0.01)	0.06 a (0.01)	0.07 a (0.02)	0.08 a,b (0.02)	0.08 a,b (0.00)	0.13 b-d (0.04)	0.11 a-d (0.01)	0.10 a-c (0.03)	0.09 a-c (0.01)	0.16 d, e (0.02)	0.10 a-c (0.03)	0.14 c,d (0.02)	0.22 e (0.07)	<0.001*
Phenylethyl alcohol	0.14 a (0.02)	0.15 a (0.03)	0.14 a (0.04)	0.24 a-d (0.06)	0.17 a (0.03)	0.28 b-d (0.08)	0.24 a-d (0.02)	0.24 (0.05)	0.32 (0.07)	0.33 c-e (0.06)	0.20 a,b (0.02)	0.35 d,e (0.07)	0.43 e (0.09)	<0.001*
Dodecane	0.05 a,b (0.01)	0.05 a,b (0.02)	0.02 a (0.01)	0.07 b (0.02)	0.04 a,b (0.00)	0.04 a,b (0.02)	0.05 a,b (0.02)	0.04 a,b (0.00)	0.07 b (0.02)	0.06 b (0.01)	0.03 a,b (0.01)	0.04 a,b (0.01)	0.19 c (0.06)	<0.001*
Decanal	n.d. (1.06)	2.69 a-c (1.06)	n.d. (0.75)	2.42 a,b (0.47)	2.68 a-c (0.47)	3.02 a-c (1.35)	2.25 a-c (0.41)	3.72 a-c (0.88)	2.61 a,b (0.71)	4.25 c (1.06)	2.62 a-c (0.50)	3.94 b,c (1.20)	3.60 a-c (0.88)	<0.001*
α -Copaene	0.01 b (0.00)	2.04 a (0.21)	3.84 a (1.90)	2.61 a (0.63)	8.47 b (2.81)	0.01 c (0.00)	0.02 c (0.00)	0.01 c (0.00)	0.02 d (0.00)	0.02 d (0.00)	9.62 b (1.06)	0.01 c (0.00)	3.92 a (1.37)	<0.001*
Caryophyllene	5.50 a,b (0.42)	5.86 a,b (0.99)	5.25 a,b (1.66)	8.37 b-d (1.91)	4.68 a (0.81)	8.09 b-d (1.85)	8.30 b-d (0.68)	7.68 a-c (1.20)	0.01 d,e (0.00)	0.01 e,f (0.00)	6.44 a,b (1.46)	0.01 c-e (0.00)	0.02 f (0.00)	<0.001*
α -Farnesene	0.14 a,b (0.02)	0.15 a,b (0.04)	0.05 a (0.00)	0.19 b (0.04)	0.08 a,b (0.01)	0.16 a,b (0.03)	0.15 a,b (0.02)	0.33 c (0.12)	0.44 c,d (0.09)	0.48 d (0.08)	0.10 a,b (0.04)	0.20 b (0.01)	0.34 c (0.13)	<0.001*

For each volatile compound, within the same line, 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.

authors found that 3% of olive leaves addition retrieved higher organoleptic evaluation, compared to 1, 2, and 5%. In the case of Tunisian olive cultivars, the addition of 3% olive leaves to olives also improved the fruity, bitter, and green sensations [3].

In our study, the most influenced parameters that were improved by the addition of olive leaves were those from olfactory and gustatory-olfactory sensations, showing that the volatile compounds have a key role on the acceptability and classification of the olive oils. Therefore, the volatile fraction of cv. Cobrançosa olive oils extracted with different percentages of olive leaves and with different malaxation times was also evaluated, as detailed in the next section.

3.2 Volatile composition

The volatile composition of cv. Cobrançosa olive oils extracted with olive leaves and different malaxation times was characterized, and the profile obtained is presented in Table 3. Overall, 22 volatiles (Fig. 2; Table 3) were found in the olive oils, distributed by seven chemical classes: Aldehydes, alcohols, esters, ketones, terpenes (monoterpenes and sesquiterpenes hydrocarbons), alkanes, and alkenes. The addition of olive leaves and the

application of different malaxation times influenced quantitatively, but not qualitatively, the volatile compounds present in the olive oils (Fig. 2; Table 3). Total volatiles ranged from 18.5 mg/kg (0%–40 min) and 26.3 mg/kg (10%–40 min). Evidently, for total volatiles, the main factor was the percentage of leaves added; for all the malaxation times tested, the addition of leaves positively and significantly influenced the total volatiles ($P = 0.003$ for 20 min; $P < 0.001$ for 30 min; and $P = 0.001$ for 40 min of malaxation). Among the 22 volatiles, only the content of hexanol was not affected by the tested conditions ($P = 0.461$), as this alcohol is a minor component of the volatile profile of the olive oils obtained, ranging from 0.59 and 0.73 mg/kg.

The volatile composition of the obtained olive oils was mainly composed by GLV's (green leaf volatiles), mainly (*E*)-2-hexenal. This compound content varied between 7.7 and 14.1 mg/kg, respectively, in oils extracted with 0%–40 min and 10%–40 min (Table 3), as the increase associated with the presence of leaves was significant for both 5% and 10% amounts ($P = 0.004$) (Fig. 3A). This aldehyde comprised between 40 and 53% of the total volatiles mass quantified in the 39 olive oils analyzed. At 40 min of malaxation, oils with 10% of leaves reported nearly double the amount of (*E*)-2-hexenal (14.1 mg/kg) compared to 0% oils (7.68 mg/kg).

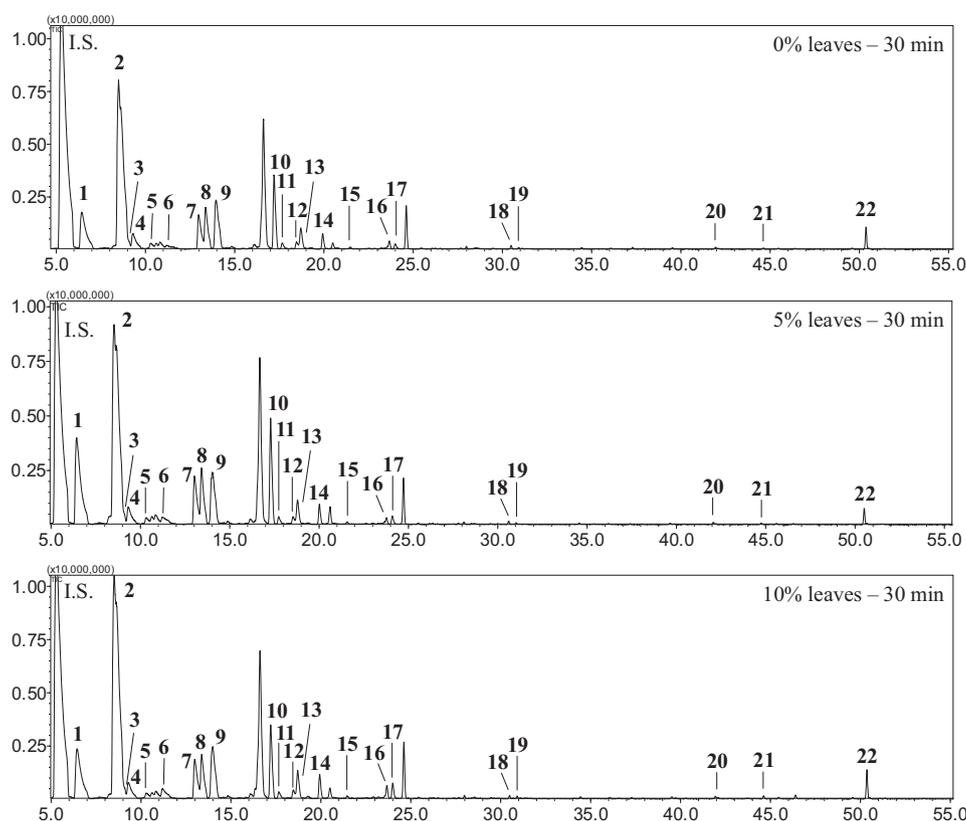


Figure 2. Gustatory-olfactory sensations of cv. Cobrançosa olive oils extracted with different percentages of olive leaves and malaxation times. A – Fruityness; B – Sweet; C – Bitter; D – Pungent; E – Green. In the same sensory attribute, different letters differ significantly ($P < 0.05$).

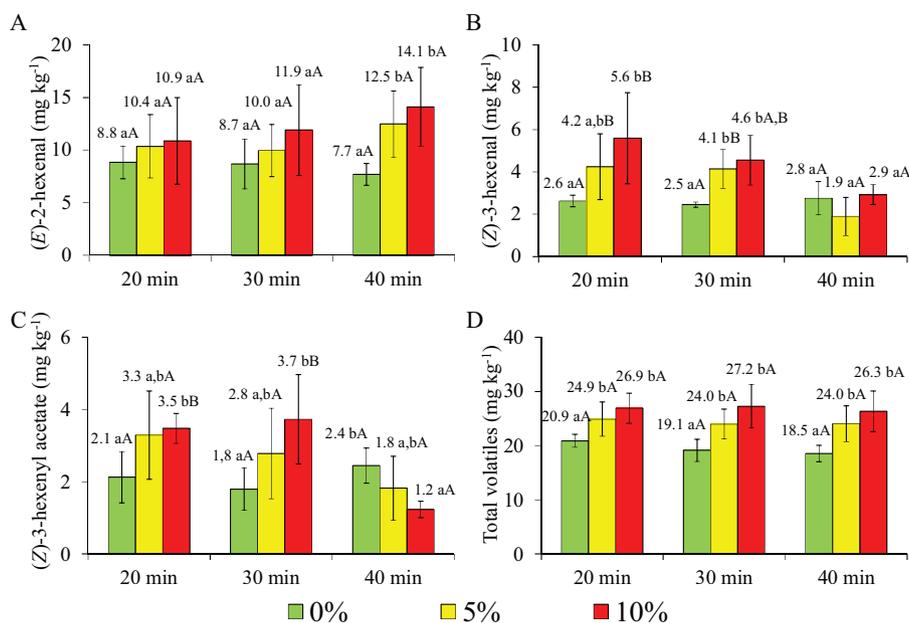


Figure 3. Volatile changes in (*E*)-2-hexenal (A), (*Z*)-3-hexenal (B), (*Z*)-3-hexenyl acetate (C), and total volatiles (mg/kg) (D) in olive oils from cv. Cobrançosa extracted with different amounts of olive leaves and malaxation times. Mean values of different percentages of olive leaves with a specific malaxation time with different capital letters differ significantly ($P < 0.05$). Mean values of a specific percentage of leaves at different malaxation times with different minor letters differ significantly ($P < 0.05$).

Similar observations were verified by Di Giovacchino et al. [21] with 5% of leaves and 60 min of malaxation. Ammar et al. [3] reported an increase of about 30% in (*E*)-2-hexenal by adding 3% of leaves to Chemlali olives.

The increase of (*E*)-2-hexenal is related to the LOX pathway (lipoxygenase), by which polyunsaturated fatty acids (PUFA) are oxidized and latter cleaved by the hydroperoxide lyase, leading to the formation of aldehydes [22]. The main aldehydes formed during LOX are hexenal and (*Z*)-3-hexenal, but through the action of isomerase (*Z*)-3-hexenal yields (*E*)-2-hexenal. Therefore, through the addition of olive leaves, the enzymatic production of (*E*)-2-hexenal is enhanced. The inclusion of olive leaves during olive oil extraction may increase the levels of PUFA in the olive paste, since olive leaves are rich in PUFA [23], thus enhancing the enzymatic action and LOX pathway.

Another aldehyde that was influenced by the addition of olive leaves and the malaxation time was (*Z*)-3-hexenal (Table 3), also formed under the LOX pathway. The cv. Cobrançosa olive oils (*Z*)-3-hexenal content was significantly influenced by the addition of leaves with 20 and 30 min of malaxation time ($P = 0.015$ and $P < 0.001$, respectively), ranging from 1.9 mg/kg (5%–40 min) to 5.6 mg/kg (10%–20 min) (Table 3 and Fig. 3B). Malaxation time did not affect the (*Z*)-3-hexenal content in the control olive oils ($P = 0.565$), but a significant reduction ($P < 0.001$ and $P = 0.019$, respectively) was observed with 5% and 10% of leaves. In the oils with 10% of leaves, losses of 1 mg/kg were observed from 20 to 30 min of malaxation, and of 1.7 mg/kg from 30 to 40 min (Fig. 3B).

(*E*)-2-hexenal and (*Z*)-3-hexenal are connoted with green leaves, grassy, green, apple-like, leaf-like, and fresh cut grass sensations [24]. Therefore, their presence in olive oil is important for the overall sensory characteristics of the oil. Such a hypothesis was also verified by Di Giovacchino et al. [21], reporting that leaves addition improved the “fresh-cut grass” sensation in the final olive oil. Additionally, these compounds can be used as a substrate for the formation of other volatiles [25–27]. When (*Z*)-3-hexenal is reduced by alcohol dehydrogenase, it forms (*Z*)-3-hexen-1-ol. This alcohol, also a GLV, was present in low amounts in our olive oils, ranging from 50 and 140 $\mu\text{g}/\text{kg}$ (Table 3). However, it is important because, in the LOX pathway, this alcohol leads to the formation of (*Z*)-3-hexenyl acetate by esterification, due to the action of alcohol acetyltransferase. (*Z*)-3-hexenyl acetate is one of the volatiles responsible for the characteristic odor of green banana in some olive oils, as well as fruity and green leaves [24]. This ester content varied between 1.2 mg/kg (10%–40 min) and 3.7 mg/kg (10%–30 min) (Fig. 3C). Depending on the malaxation times applied, the addition of olive leaves caused variable results: (*Z*)-3-hexenyl acetate content increased significantly with the addition of leaves at 20 and 30 min ($P = 0.030$ and $P = 0.042$ respectively), while for 40 min of malaxation, the addition of leaves significantly reduced this ester content ($P = 0.011$). Regarding the malaxation times, only in the oils with 10% of leaves was a significant change in accordance with the malaxation time verified ($P < 0.001$), while in the control oils and 5% leaves oils, no significant differences were detected

($P=0.207$ and $P=0.107$ respectively). From the results obtained, it can be retained that high malaxation times reduced the presence of (*Z*)-3-hexenyl acetate in the final olive oil when leaves were added.

3.3 Response surface models

Figure 4A–C present the response surface plots, showing the effect of malaxation time and percentage of leaves mass on the major volatiles of olive oils, particularly (*E*)-2-hexenal and (*Z*)-3-hexenal, as well as on total volatiles. The fitted second-order polynomial equations for (*E*)-2-hexenal, (*Z*)-3-hexenal, and total volatiles are presented in Eqs. (2), (3), and (4), respectively, after neglecting the non-significant terms at 5% of significance, with coefficients of determination (R^2) of 0.781, 0.846, and 0.885, respectively, and adj- R^2 of 0.624, 0.737, and 0.802, respectively.

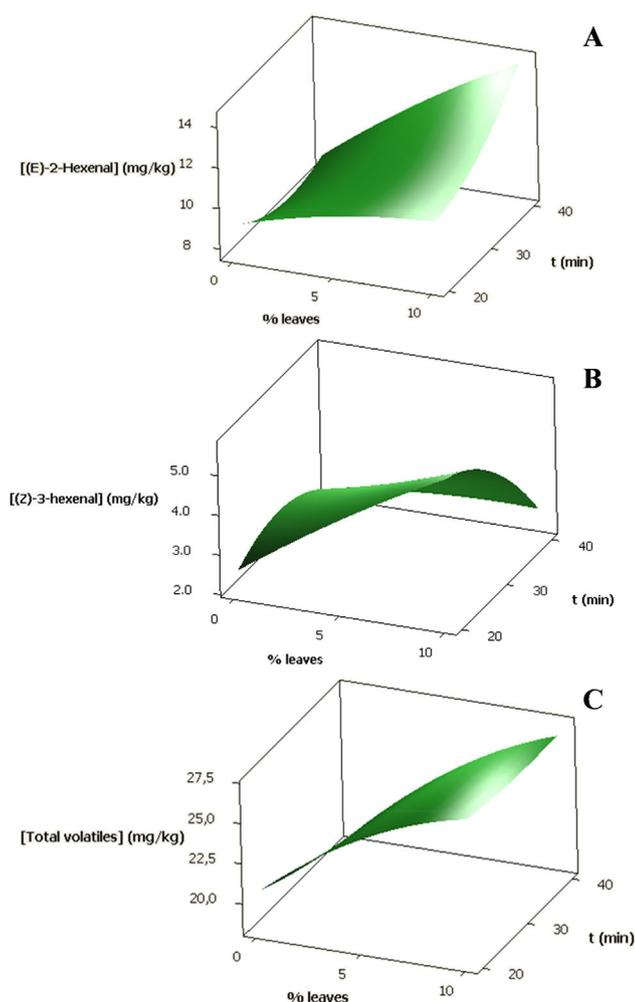


Figure 4. Response surface plots of (*E*)-2-hexenal (A), (*Z*)-3-hexenal (B), and total volatiles (C), in function of percentage of olive leaves added during the extraction process and malaxation time.

$$Y_{(E)\text{-2-hexenal}} = 10.152 + 1.951X_1 \quad (2)$$

$$Y_{(Z)\text{-3-hexenal}} = 3.990 + 0.874X_1 - 0.811X_2 - 0.699X_1X_2 \quad (3)$$

$$Y_{\text{Total Volatiles}} = 24.055 + 3.680X_1 \quad (4)$$

with X_1 = percentage of leaves and X_2 = malaxation time.

Considering the R^2 values, our results indicate that more than 78% of variation in the contents of these compounds can be explained by the independent variables studied, namely, percentage of leaves and malaxation time. Furthermore, the lacks-of-fit were not significant, with P -values of 0.773, 0.281, and 0.937, respectively, indicating the suitability of the models to accurately predict the variation.

The regression analysis of the experimental data showed that the percentage of leaves always had a significant positive linear effect on the contents of (*E*)-2-hexenal, (*Z*)-3-hexenal, and total volatiles, in line with the previously observed data (Fig. 3A, B and D). On the contrary, malaxation time had a significant negative linear effect on (*Z*)-3-hexenal, as a significant negative interaction was also found between both independent variables. Moreover, for this compound, the effects of malaxation time and percentage of leaves were similar due to the similarity of the absolute values of both factors coefficients (0.874 vs. 0.811).

These results showed that the contents of (*E*)-2-hexenal, (*Z*)-3-hexenal, and total volatiles increase as leaf percentage increases from 5 to 10%, while malaxation time did not have a pronounced effect, with the exception of (*Z*)-3-hexenal.

4 Conclusions

With the present work, it was concluded that the addition of olive leaves to olives during the extraction process of olive oil was not responsible for the appearance of sensory defects, and improved both olfactory and gustative-olfactory sensations, mainly fruity and green attributes. It was concluded that this observation was related to the improvement of the volatile fraction of olive oils, mainly (*E*)-2-hexenal, (*Z*)-3-hexenal, and (*Z*)-3-hexenyl acetate. Malaxation time has a determinant part in the final volatile content and sensory attributes, particularly in the presence of leaves, with increased contact times inducing potential volatiles loss, for instance, of (*Z*)-3-hexenyl acetate.

The addition of olive leaves to olives for the extraction of differentiated and sensorial improved olive oils could be considered at percentages between 5 and 10%. However, malaxation time should be kept for up to 30 min, since 40 min causes the loss of important sensory attributes in the final olive oils. Olive leaves, a sub-product of olive mill industry, could be used as powerful odorants for olive sector,

namely to improve the sensory perceptions of olive oils with lower sensory attributes and extracted from ripe olives.

Ricardo Malheiro thanks FCT, POPH-QREN and FSE for the Post-Doctoral grant (SFRH/BPD/111901/2015).

The authors have declared no conflict of interest.

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