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Impact of fresh olive leaves addition during the extraction of Arbequina virgin olive oils on the phenolic and volatile profiles

Ítala M.G. Marx [a,b], Susana Casal [b], Nuno Rodrigues [a], Rebeca Cruz [b], Fátima Peres [c,d], Ana C.A. Veloso [e,f,g], José A. Pereira [a], António M. Peres [a,*]

[a] Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus Santa Apolónia, 5300-253 Bragança, Portugal
[b] LAQV/REQUIMTE, Laboratory of Bromatology and Hydrology, Faculty of Pharmacy, University of Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal
[c] Instituto Politécnico de Castelo Branco, Escola Superior Agrária, 6000-909 Castelo Branco, Portugal
[d] LEAF, Linking Landscape, Environment, Agriculture and Food, Instituto Superior de Agronomia, Universidade de Lisboa, 1349-017 Lisbon, Portugal
[e] Instituto Politécnico de Coimbra, ISEC, DEQB, Rua Pedro Nunes, Quinta da Nora, 3030-199 Coimbra, Portugal
[f] CEB - Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
[g] LABBELS –Associate Laboratory, Braga/Guimarães, Portugal

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ABSTRACT

Leaves incorporation during the extraction of olive oils can enhance their chemical-sensory quality. Thus, leaves from *cvs*. Arbequina or Santulhana were added (1%, w/w) during the extraction of Arbequina oils using an Abencor system, being discussed the impacts on the phenolics and volatiles formation enzymatic pathways. Leaves addition contributed to a significant decrease (P-value < 0.05) of the contents of secoiridoids (−11%), C6-aldehydes (−16%), and ester compounds (–22%). This could be tentatively related to a reduction of the enzymatic activity of secoiridoids biosynthesis and lipoxygenase pathways, promoted by the leaves' addition. Moreover, in the presence of leaves, the oils' total contents of phenolics and volatiles were significantly reduced (−7 and −17%, respectively). Contrary, the incorporation of leaves significantly increased (P-value < 0.05) the contents of C6-alcohols (+37%) and the intensities of the green fruity (+25%) and apple (+30%) sensations.

1. Introduction

The nutritional and health-promoting effects (e.g., antioxidant, anti-inflammatory, cardioprotective, anticancer, antidiabetic and neuroprotective effects) of olives and olive oils are well recognized (Sacchi, Della Medaglia, Paduano, Caporaso, & Genovese, 2017). Virgin olive oils (VOO) are highly appreciated due to the pleasant flavor conferred by volatile compounds and positive taste sensations and also by the effect on the prevention of several important diseases (Campestre, Angelini, Gasbarri, & Angerosa, 2017). In fact, the consumption of polyphenols has been related to the prevention of the lipoperoxidation of low-density lipoproteins (LDL), with an authorized health claim associated with the daily intake of olive oils containing at least 5 mg of tyrosol, hydroxytyrosol and their derivatives per 20 g of olive oil (European Commission Regulation (EU) No 432/2012, 2012). The sensory characteristics, the profiles, as well as the concentrations of phenolics and volatiles are influenced by the olive cultivar, fruit maturity at harvest, agro-climatic conditions, extraction scale, and malaxation conditions (Cecchi, Migliorini, & Mulinacci, 2021). Thus, several strategies have been proposed to enhance the sensory quality and the health-promoting effects of olive oils, including the use of olive leaves (Tarchoune et al., 2019; Sanmartin et al., 2019; Novoselić, Klisović, Lukić, Lukić, & Bubola, 2021; Marx et al., 2022).

The addition of olive leaves during the extraction of olive oil has been described as enhancing pigments, phenolics, and volatiles amounts, positive sensory attributes, and anti-inflammatory, hypoglycemic, antimicrobial, and hypocholesterolemic properties (Sacchi et al., 2017). Some studies evaluated the impact of adding olive leaves (1–10%, w/w), dried or fresh, during laboratory, pilot, or industrial extraction scale, from different cultivars, including Cobrançosa, Oueslati, Neb Jmel, Moraiolo, Leccino, Buža and Arbequina (Malheiro, Casal, Teixeira, Bento, & Pereira, 2013; Malheiro et al., 2017; Sanmartin et al., 2019; Tarchoune et al., 2019; Di Giovacchino, Angerosa, & Di Giacinto, 1996; Novoselić et al., 2021; Marx, Rodrigues, et al., 2021;

* Corresponding author.
E-mail address: peres@ipb.pt (A.M. Peres).

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Marx et al., 2022). Table S1 summarizes the main studies related to the oils' extraction with the addition of olive leaves, at both laboratory and industrial scales. Depending on the study, contradictory effects have been reported. In general, the impact on the chemical-sensory quality of the extracted oils is highly dependent on the cultivar of the olives and leaves, on the relative amount of leaves added, as well as on the extraction scale. However, none of the referred studies discussed the observed trends, namely concerning the phenolic and volatile profiles, based on the possible impact of the leaves' incorporation on the enzymatic pathways responsible for their formation.

The enzymatic and chemical reactions that take place during extraction may be responsible for the reported differences, being dependent on the time and temperature of malaxation, and influencing the final concentrations of phenolics and volatile compounds (Taticchi et al., 2013; Diamantakos, Giannara, Skarkou, Melliou, & Magiatis, 2020).

Hydrophilic phenols, namely secoiridoids, are the most abundant natural antioxidants found in olive oils. The oil's phenolic composition is mainly affected by the activity of hydrolytic enzymes that catalyze the liberation of aglycone secoiridoids from their respective glucoside forms (Servili et al., 2004). Indeed, during the extraction, oleuropein and its biosynthetic precursor, ligstroside, which are abundant in olives, suffer enzymatic hydrolysis mediated by enzymes present in the fruits. β-glucosidase action leads to the formation of ligstroside and oleuropein aglycons, which in turn are converted to the respective monoaldehydic forms (ligstroside and oleuropein aglycone mono-aldehydes) and to different diastereoisomers, due to the ring opening and ring closure of the hemiacetal group. The two dialdehydic forms, oleocanthal and oleacein, are formed by hydrolysis of the methyl ester followed by decarboxylation. The hydrolysis and decarboxylation reactions can occur either enzymatically or chemically due to the acidic aqueous conditions of the extraction (Starec, Calabretti, Berti & Forzato, 2021). Recently, two methylesterase enzymes (elenolic acid methylesterase 1 & 2) have been identified, being responsible for the conversion of the monoaldehydic aglycones forms (from the β-glucosidase action on oleuropein and ligstroside) into oleacein and oleocanthal (Volk et al., 2019). Hydroxytyrosol or tyrosol can also be formed by different pathways during the oil extraction process and storage. One, mediated by esterases, refers to the direct hydrolysis of oleuropein or ligstroside, forming hydroxytyrosol/tyrosol plus an oleoside methyl ester molecule. A second possibility is due to the esterase-catalyzed hydrolysis of aglycon form, producing hydroxytyrosol/tyrosol plus elenolic acid (Johnson, Melliou, Zweigenbaum, & Mitchell, 2018). Finally, acid hydrolysis of oleuropein can also occur, leading to hydroxytyrosol, elenolic acid and glucose (De Leonardis, Aretini, Alfano, MacCiola, & Ranalli, 2008).

Volatile compounds from different chemical classes have also been identified in olive oils, playing a key role in the final olive oil quality attributes (Cecchi et al., 2021). The positive olfactory sensations of olive oil are induced by small molecules formed by enzymatic activity, namely of the lipoxygenase (LOX) pathway, which promotes the formation of the C5 and C6 compounds from fatty acids (linoleic and linolenic acids) (Cecchi et al., 2021). Besides the activity of LOX, this pathway involves the sequential activity of hydroperoxide lyases (HPL), alcohol dehydrogenases (ADH), and alcohol acetyl transferases (AAT) (Campestre et al., 2017). These enzymes are naturally found in the olive fruit and start acting just after olives crushing during the olive milling phase (Genovese, Caporaso, & Sacchi, 2021). The sequential action of lipases and lipoxygenases, together with the action of HPL, lead to the formation of C6 aldehydes (e.g., hexanal and (Z)–3–hexenal, related to the green and tomato leaf notes of olive oils) and C9 aldehydes. Subsequently, the isomerization of (Z)–3–hexenal gives rise to (E)–2-hexenal, which presence is related to the herbaceous and pleasant aroma of olive oils, being an olive oil freshness indicator (Genovese et al., 2021). Then, some aldehydes are reduced, due to the ADH activity, being transformed into the respective alcohols and converted into the respective esters by the action of AAT (Campestre et al., 2017; Genovese et al., 2021). In addition, C10 hydrocarbons and C5 alcohols, namely 2-penten-1-ol and 1-penten-3-ol, can be produced from the LnA hydroperoxide through an additional pathway, partly enzymatic, that involves an alkoxyl radical. The subsequent oxidation of C5 alcohols could lead to C5 carbonyl compounds (Campestre et al., 2017; Genovese et al., 2021).

Taking into account the relevance of the abovementioned pathways and the discrepancies reported in the literature regarding the effects of incorporating olive leaves during the oil extraction, the main aim of this study was to discuss the observed changes in the phenolic and volatile profiles. Their impacts at the sensory level were also studied, relating them with the known enzymatic pathways. For that, Arbequina oils were extracted in an Abencor laboratory unit, with fresh leaves (1%, w/w) from two cultivars (Arbequina and Santulhana). Arbequina is one of the most spread cultivars around the world (Tous, 2018), while *cv.* Santulhana is a Portuguese olive cultivar mainly cultivated in traditional olive groves located in the region of Trás-os-Montes (northeast Portugal) (Rodrigues, Baptista, Casal, & Pereira, 2018). This latter cultivar was chosen aiming to contribute to its valorization and preservation.

2. Materials and methods

2.1. Olives and olive oil extractions

Olives from Arbequina cultivar, ripeness index between two and three, were picked by hand from trees in mid-November 2019 from an orchard located in the region of Trás-os-Montes (northeast Portugal). Olive tree leaves from Arbequina were also collected from the same trees and at the same time during the fruit harvest. Santulhana leaves were picked by hand from other olive orchard located in the same Portuguese region. The leaves from both cultivars were separated from branches, carefully washed, removed the excess of water, weighed, and crushed, using a shredder knives Moulinex® equipment.

Olive oils were extracted in a laboratory oil extraction system (Abencor analyser; MC2 Ingenieria y Sistemas S.L., Seville, Spain), located at the School of Agriculture of the Polytechnic Institute of Castelo Branco, Portugal (schematization in Fig. 1). The olives were crushed with a hammer mill equipped with a 4 mm sieve at 1000 × g. Crushed olive leaves (1%, w/w) were added and mixed with the milled olives. After the homogenization of both matrices, the malaxation step was initiated. The malaxation of the pastes was performed for 30 min at 30 °C, and the centrifugation was carried out at 2500 × g for 1 min. After centrifugation, the olive oil was separated by settling in a graduated cylinder. Fig. 1 describes in detail each of the three different types of obtained Arbequina oils, i.e., oils extracted without leaves incorporation, and oils extracted after the incorporation of olive tree leaves from Arbequina or Santulhana. Five independent extractions were performed for each type of oil, totalizing 15 independent olive oils (3 Arbequina olive oil types × 5 independent extractions). Water traces in the extracted oils were removed with anhydrous sodium sulfate, filtered through a cellulose filter, and stored protected from light in amber glass bottles (~100 mL) until analysis. The samples were stored at 18–22 °C and analyzed after a 6-month storage period to fully develop the oils' sensory attributes.

2.2. Olive oil physicochemical analysis

Free acidity (FA, in % oleic acid), peroxide value (PV, in mEq O_2/kg) and specific coefficients of extinction at 232 and 268 nm (K_{232}, and K_{268}), were evaluated according to the EU Regulation (Annexes II and IX in Commission Regulation (EEC) N° 2568/91 and amendments).

2.3. Olive oil phenolic compounds profile

The phenolic profile of the olive oil samples was established following the guidelines of the International Olive Council (IOC)

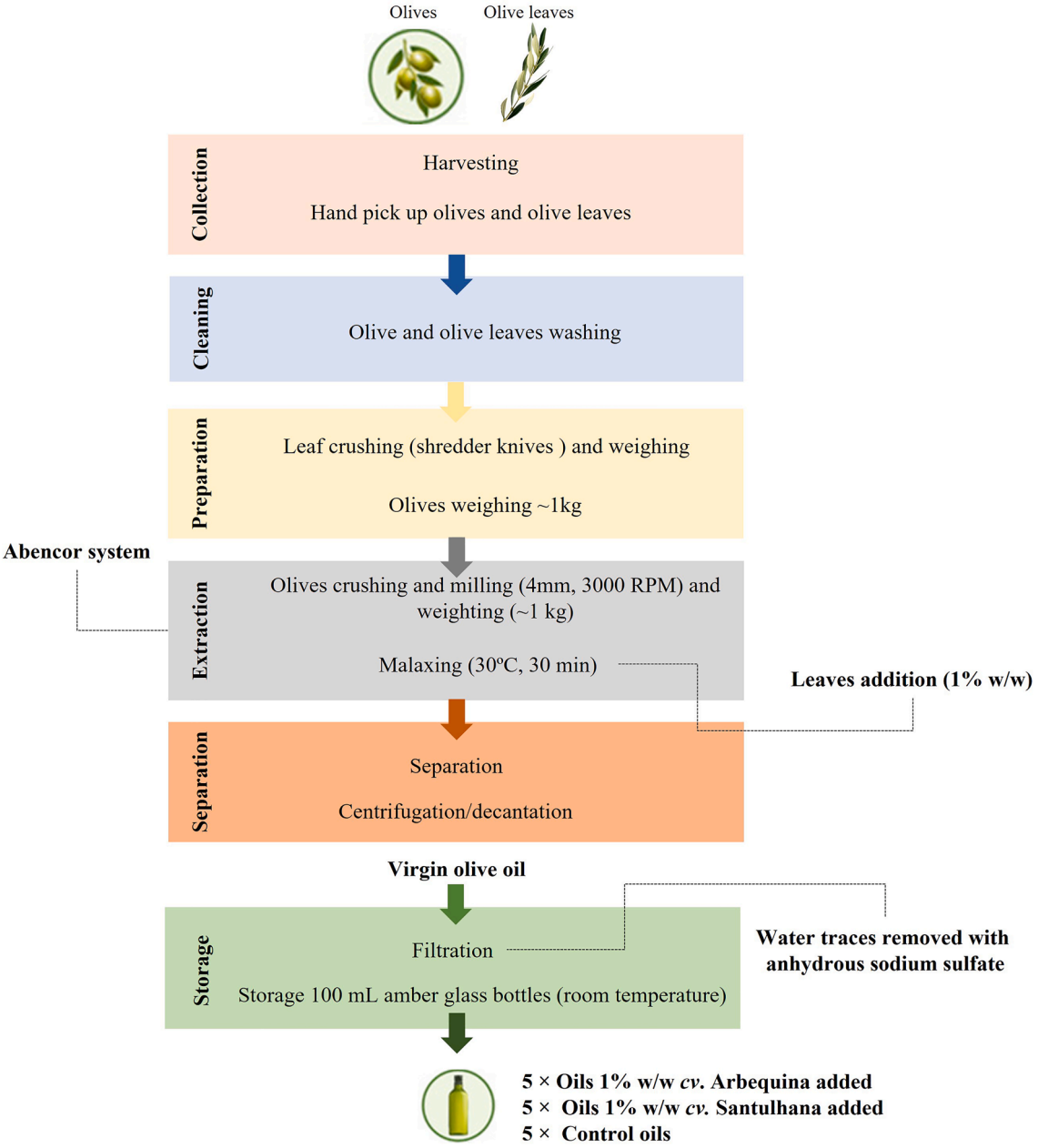

Fig. 1. Technological process of olive oil extraction at laboratory scale (Abencor System).

(International Olive Council, 2017), with minor modifications as previously described (Marx, Casal, et al., 2021). Methanolic extracts (80% v/v), from duplicate extractions of each sample, were injected in a high-performance liquid chromatography with diode-array detection (HPLC-DAD) system (Jasco, Japan) comprising a data transmitter (LC–NetII/ADC), two integrated pumps (PU–4180), an auto-sampler (AS–4050), a column oven (ECOM Eco2000, Czech Republic), and the DAD (MD–4010). Separation was accomplished on a pentafluorophenyl column (Kinetex 2.6 μm PFP 100 Å; LC length 100 mm; internal diameter: 4.60 mm) from Phenomenex (Spain), at 35 °C, using an eluent gradient with water and acetonitrile, both with 0.1% of formic acid, at 1.0 mL/min. Peaks were identified based on the retention times (RT) and UV/Vis spectra (200–600 nm), by comparison with pure standards as previous described by Marx et al. (2022). Results were expressed as mg of tyrosol equivalents per kg of olive oil, using the experimental data recorded at 280 nm, following the IOC guidelines (International Olive Council, 2017). Peaks related to oleuropein and ligstroside derivatives were tentatively identified based on the literature data (Klen, Wondra, Vrhovšek, & Vodopivec, 2015).

2.4. Olive oil total hydroxytyrosol and tyrosol derivatives after acid hydrolysis

The total content of hydroxytyrosol and tyrosol derivatives of the olive oils was chromatographically determined after acid hydrolysis of secoiridoids, following the methodology proposed by Romero and Brenes (2012).

2.5. Olive oil 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). The SPME fiber used was a divinylbenzene/carbonex/polydimethylsiloxane (DVB/CAR/

PDMS 50/30 μm) (Supelco, Bellefonte, USA) and the gas chromatographer used was a Shimadzu GC 2010 Plus (Japan) equipped with a mass spectrometer Shimadzu GC-MS QP 2010 SE detector (Japan), as previously described (Malheiro et al., 2017). Separation was accomplished on a TRB-5MS (30 m × 0.25 mm × 0.25 μm) column (Teknokroma, Spain). All mass spectra were acquired by electron ionization, being the spectra fragments identified by comparison with the database of the NIST 11 Library (National Institute of Standards and Technology, Gaithersburg, MD, USA) and with the spectra of commercial standards. The areas of the chromatographic peaks were determined by integrating the reconstructed chromatogram from the full scan chromatogram using the ion base (m/z intensity 100%). In this study, the volatile compounds were identified based on the linear retention index (LRI) and the structural information. For each compound the Kovats retention index was calculated using a commercial standard mixture of *n*-alkanes (C6-C20, Sigma Aldrich, Germany), and the identification was confirmed by comparing the LRI obtained and those reported in the literature (Adams, 2007). For semi-quantification purposes, the amounts of the identified volatiles were calculated considering the ratio of each base ion peak area to the area of the internal standard (IS: 4-methyl-2-pentanol, 98% purity, Sigma Aldrich, Germany) base ion peak area, assuming a response factor equal to one. The amounts were converted to mass equivalents based on the IS mass used (i.e., mg of IS equivalent per kg of olive oil), following the methodology proposed by Malheiro et al. (2017).

2.6. Olive oil sensory evaluation

The sensory evaluation of the olive oils was performed by a trained sensory panel following the methodologies described by the EU standard methods (European commission delegated regulation (EU) 2015/1830, 2015). The analysis was performed by a trained panel from the School of Agriculture of the Polytechnic Institute of Bragança, Portugal (Rodrigues, Casal, Peres, Baptista, & Pereira, 2020). The descriptive analysis followed the recommendations of the IOC (International Olive Council, 2005) with some modifications as previously reported by Rodrigues et al. (2020).

2.7. Statistical analysis

The composition of the three different Arbequina olive oils (extracted without or with the addition of Arbequina or Santulhana leaves) were analyzed using the one-way ANOVA followed by the Tukey's post-hoc multi-comparison test. When only two types of olive oils were compared, the *t*-Student test was applied. All statistical analyzes were performed using the open-source statistical program R (version 3.6.2), at a 5% significance level.

3. Results and discussion

3.1. Physicochemical quality parameters of Arbequina oils extracted with or without leaves incorporation

The physicochemical quality data (FA, PV, K_{232} and K_{268}) of the Arbequina olive oils extracted without (control) and with addition of leaves from Arbequina or Santulhana were determined. The results obtained showed that all extracted oils fulfilled the legal thresholds for extra virgin olive oil (EVOO) classification (FA (% oleic acid): 0.08 to 0.1; PV (mEq O2/kg): 0.75 to 0.99; K_{232}: 1.80 to 1.90; and K_{268}: 0.04 to 0.05), in agreement with previous works (Tarchoune et al., 2019; Novoselić et al., 2021).

3.2. Phenolic profiles and contents of hydroxytyrosol and tyrosol derivatives of Arbequina oils extracted with or without leaves incorporation

The phenolic profiles established for the studied olive oils were similar, independently of incorporating leaves or not during the extractions, although the contents of the identified phenolics were highly different (Table 1). Seven phenolic compounds were identified and quantified in all Arbequina oils, including vanillic acid and vanillin, as well as those associated with the health claim of European Food Safety Authority (EFSA) (European Commission Regulation (EU) No 432/2012, 2012), namely hydroxytyrosol, tyrosol, oleuropein, oleacein, oleocanthal and derivatives of oleuropein and ligstroside. These latter compounds, formed along the biosynthetic pathway of secoiridoids, were the most abundant phenolics in the studied oils. It should be remarked that the phenolic profile of the control oils is in-line with those previously reported for Arbequina oils, although differences occur at the content levels, as expectable (Bajoub, Ajal, Fernández-Gutiérrez, & Carrasco-Pancorbo, 2016; Marx et al., 2022). In this study, the peaks related to oleuropein and ligstroside derivatives were tentatively identified following the IOC guidelines (International Olive Council, 2017) and literature data (Klen et al., 2015).

Our work showed that the incorporation of olive leaves during the extraction of the olive oil led to a significant decrease of the total phenolics contents (an average reduction of 7.3% compared to the control oils). This fact was mainly due to the reduction of the oleacein and oleocanthal contents (an average decrease of 30 and 23%, respectively, compared to the control oils).

Aglycone isomers, oleacein and oleocanthal are produced from the same substrates, oleuropein and ligstroside. However, aglycone isomers

Table 1
Phenolic compounds contents (mean ± standard deviation, mg of tyrosol equivalents/kg of oil) for the Arbequina olive oils studied and the estimated total contents of hydroxytyrosol and tyrosol derivatives (mean ± standard deviation; mg of hydroxytyrosol and tyrosol derivatives/kg of oil) in the hydrolyzed olive oils.

Phenolic compounds	Without leaves addition (control)	With Arbequina leaves	With Santulhana leaves	*P*-value[1]
Hydroxytyrosol	0.9 ± 0.2^{b}	0.6 ± 0.1^{c}	1.3 ± 0.1^{a}	<0.0001
Tyrosol	1.1 ± 0.5^{a}	1.0 ± 0.2^{a}	1.2 ± 0.2^{a}	0.3493
Vanillic acid	2.1 ± 0.1^{b}	2.2 ± 0.3^{b}	2.5 ± 0.4^{a}	0.0451
Vanillin	4.4 ± 0.2^{c}	5.2 ± 0.4^{b}	6.3 ± 0.2^{a}	<0.0001
Oleuropein	1.4 ± 0.1^{a}	1.0 ± 0.2^{b}	0.9 ± 0.1^{c}	<0.0001
Oleacein	53.7 ± 0.9^{a}	36.2 ± 2.2^{b}	38.9 ± 1.2^{c}	<0.0001
Oleocanthal	10.8 ± 0.7^{a}	8.1 ± 0.3^{b}	8.6 ± 1.1^{b}	<0.0001
ΣOleuropein derivatives	81.5 ± 3.3^{a}	82.2 ± 2.4^{a}	82.8 ± 2.2^{a}	0.5563
ΣLigstroside derivatives	37.7 ± 1.7^{a}	35.4 ± 1.2^{b}	35.2 ± 1.9^{b}	0.0036
$\Sigma_{\text{phenolic alcohols}}$	2.0 ± 0.6^{ab}	1.6 ± 0.2^{b}	2.4 ± 0.3^{a}	0.0010
$\Sigma_{\text{phenolic acids}}$	2.1 ± 0.1^{b}	2.2 ± 0.3^{b}	2.5 ± 0.4^{a}	0.0451
$\Sigma_{\text{phenolic aldehydes}}$	4.4 ± 0.2^{c}	5.2 ± 0.4^{b}	6.3 ± 0.2^{a}	<0.0001
$\Sigma_{\text{secoiridoid derivatives}}$	185.2 ± 5.7^{a}	163.0 ± 3.8^{b}	166.4 ± 3.6^{b}	<0.0001
$\Sigma_{\text{identified phenolics compounds}}$[2]	193.7 ± 5.8^{a}	172 ± 3.4^{c}	177.7 ± 3.8^{b}	<0.0001
$\Sigma_{\text{non-identified phenolic compounds}}$[3]	46.7 ± 4.3^{a}	46.1 ± 5.3^{a}	50.1 ± 2.6^{a}	0.1002
$\Sigma_{\text{total phenolic content}}$[4]	240.4 ± 7.9^{a}	218.2 ± 8.2^{c}	227.7 ± 3.1^{b}	<0.0001
Acid hydrolysis[5]				
Hydroxytyrosol derivatives	105.8 ± 9.8^{a}	71.7 ± 3.8^{b}	75.5 ± 6.1^{b}	<0.0001
Tyrosol derivatives	36.1 ± 5.6^{a}	26.1 ± 1.5^{b}	33.0 ± 1.8^{a}	<0.0001
ΣHydroxytyrosol and tyrosol derivatives	142.0 ± 14.9^{a}	97.8 ± 4.2^{b}	108.5 ± 5.9^{c}	<0.0001

[1] *P*-values for the one-way ANOVA. Different letters in the same row show statistically differences from the given mean (*P*-value < 0.05); n = 5.
[2] Sum of identified phenolic content.
[3] Sum of non-identified phenolic content.
[4] Sum of $\Sigma_{\text{identified phenolics compounds}}$ and $\Sigma_{\text{non-identified phenolic compounds}}$.
[5] Acid hydrolysis based on the HPLC-DAD quantification.

are produced by hydrolysis due to the activity of β-glucosidase, while oleacein and oleocanthal are formed by the enzymatic action of β-glucosidase and methylesterase (Taticchi et al., 2013). The reduction trend of oleacein and oleocanthal followed the same relative reduction proportion of the total concentration of phenolic compounds (Table 1). To better discuss these interrelationships, the *f* index (Miho, Moral, López-González, Díez, & Priego-Capote, 2020), which is the ratio between the concentration of aglycones and the sum of oleacein and oleocanthal contents, was calculated. The control oils presented the lowest values (*f* index = 1.8) in comparison with the *cv.* Arbequina oils extracted with their own leaves or extracted with *cv.* Santulhana leaves (*f* index = 2.7 or 2.5, respectively). So, all extracted oils had higher amounts of aglycone isomers, being those extracted with leaves richer in these compounds, allowing inferring a possible inhibition/reduction of the enzymatic activity of methylesterase. In fact, these findings may corroborate the hypothesis that the presence of leaves could negatively impact the ring-opening process of oleuropein and ligstroside aglycones, keeping them closed. This would have a negative impact on the enzymatic cascade pathway involving the methylesterase, which activity, responsible for the formation of oleacein and oleocanthal compounds from aglycones in the open form, could have been reduced due to the lack of substrate. The leaves impact could be related to their composition or/and due to some microorganisms, that are naturally present in leaves (Varanda, Materatski, Landum, Campos, & Félix, 2019). On the other hand, *cv.* Arbequina has been described as a cultivar with a phenolic profile with a secoiridoids predominance in oleacein and oleocanthal (Miho et al., 2020). Thus, the observed reduction trend could be related to a genetic factor, and so, different results may be found for other olive cultivars.

Oleacein and oleocanthal reductions are in-line with previous works conducted at industrial (Marx et al., 2022), and laboratory (Novoselić et al., 2021) scales for Arbequina and Buža oils, respectively, after incorporating different amounts of leaves. On the other hand, increasing trends of the oleacein or total secoiridoid contents have also been reported by Marx et al. (2022), for Arbequina oils industrially extracted after the incorporation of Arbequina leaves or by Tarchoune et al. (2019) and Sanmartin et al. (2019). It should be remarked that, although the control oils (extracted without leaves incorporation) obtained in this study, using an Abencor system were richer in phenolics, namely in secoiridoid derivatives, than those extracted at an industrial scale (Marx et al., 2022). For both extraction scales, the contents of the secoiridoid derivatives represent a similar percentage of the total content of identified phenols (~96–98%). Likewise, for oils extracted at both laboratory and industrial scales, the content of oleacein is approximately 30% of the total amount of secoiridoid derivatives. The higher oleacein amount found in oils extracted in the laboratory compared to those industrially extracted can be partially explained by the higher malaxation temperature (30 and 22 °C, for laboratory and industrial extractions, respectively). High temperatures enhance enzymatic activity, as discussed by several researchers, and contribute to increasing the partition coefficient of hydrophilic phenols between the oil and the water phase (Diamantakos et al., 2020). Although some studies (at industrial or laboratory scale) were conducted with the same cultivar and relative amount of added leaves, it is important to emphasize that laboratory extraction (Abencor system) cannot fully mimic the industrial extraction, even at similar malaxation conditions, and so, different oil compositions are expected (Di Giovacchino, Costantini, Ferrante, & Serraiocco, 2002). Variables such as harvesting method, batch size, leaf incorporation method, malaxation time, and temperature, greatly influence the physicochemical composition of the oils. Thus, to minimize the heterogeneity of the raw materials (olives and leaves), to better control the leaves incorporation into the olive paste and the particle size of the grinded leaves, the extraction was carried out on at laboratory scale (Abencor system). Furthermore, the leaves grinding method at laboratory scale allowed obtaining smaller leaf particles compared to the industrial process, increasing the contact surface between the crushed leaves and the olive paste and thus, promoting a high interaction level. Regarding the free forms of hydroxytyrosol and tyrosol compounds, the gross contents found in the studied oils were of the same order of magnitude, regardless of the leaves incorporation or not, and the relative proportion of these two phenolic compounds on the total amount of phenolics, increased. Apparently, these findings would contradict the hypothetical reduction of methylesterase activity, responsible for the formation of oleacein and oleocanthal from their precursors (open-form aglycones). However, as previously discussed, these phenolic alcohols can also be formed due to enzymatic or chemical (acid) hydrolysis pathways of oleuropein or ligstroside (De Leonardis et al., 2008; Johnson et al., 2018), which the leaves' incorporation may have promoted.

In which concerns the secoiridoid peaks, identified and quantified as oleuropein and ligstroside derivatives, following the literature proposed by Klen et al. (2015), a slight increase in their relative proportions (taking into account the total phenolic content) was observed in the studied oils, which could be attributed to the extraction with leaves (an average increase of 5 and 0.2%, respectively), although the gross amounts were of the same order of magnitude (Table 1).

Table 1 also shows the secoiridoid fraction, after acid hydrolysis, for the studied oils, comprising the sum of the hydroxytyrosol and tyrosol derivatives contents. The incorporation of leaves from the two cultivars decreased the total amounts of hydroxytyrosol and tyrosol derivatives compared to the control oils (reduction of 31 and 24% for Arbequina and Santulhana leaves addition, respectively). This is in-line with the results previously discussed regarding the sum of alcohol and secoiridoid contents of the oils extracted without or with the incorporation of leaves, determined following the IOC guidelines and the literature (International Olive Council, 2017; Klen et al., 2015). In fact, a good correlation could be established between the contents assessed using the two chromatographic methods (*R*-Pearson = 0.998).

3.3. Effect of leaves addition during oil extraction on the volatile profile

The HS-SPME-GC-MS method allowed identifying 25 volatile compounds belonging to seven volatile classes (acids, alcohols, aldehydes, esters, hydrocarbons, ketones, and terpenes), in the Arbequina oils evaluated. Among them, six classes were common in all oils analyzed, being the ketone class only detected in oils extracted with Santulhana leaves. Table 2 shows the mean estimated contents of the volatiles detected. The most abundant chemical class was aldehydes, in agreement with the literature data for Arbequina oils (Cavalli, Fernandez, Lizzani-Cuvelier, & Loiseau, 2004; Marx, Rodrigues, et al., 2021).

Considering the most abundant volatiles and their role at the olfactory level, the focus was centered on the volatiles related with the LOX pathway (i.e., C5 and C6 volatile compounds). Indeed, for the studied Arbequina oils (extracted with or without leaves incorporation), the C5 and C6 compounds represented more than 78% of total volatile compounds (compounds identified assuming a minimum similarity threshold of 85%).

Only 2-penten-1-ol, which belongs to the C5 group, was detected. This compound was found in all oils at low concentrations (0.31 to 0.36 mg/kg), being its concentration not affected by the addition of leaves (Table 2). The low concentrations found agree with the literature for olive oils of different cultivars (Campestre et al., 2017). The 2-penten-1-ol compound is derived from C5 aldehyde 2-pentanal, through the enzymatic activity of ADH. However, in the present work, it was not possible to identify this aldehyde in any oil. Moreover, it is notable that the amounts of this compound are not significantly different when comparing samples extracted with or without the addition of leaves (Table 2).

The content of C6 aliphatic compounds represented more than 78% of the total content of volatiles of the Arbequina oils (with or without leaves addition). All studied oils contained C6 alcohols (e.g., (E)-3-hexen-1-ol, (Z)-2-hexen-1-ol and 1-hexanol), C6 aldehydes (e.g., (E)-2-

Table 2
Volatile compounds contents (mean ± standard deviation, mg/kg[1]) for each Arbequina olive oils, without or with the incorporation of Arbequina or Santulhana leaves (1%, w/w).

Volatile group	Volatile Compounds[1]	LRI[2]	Without leaves addition (control)	With Arbequina leaves	With Santulhana leaves	*P*-value[3]
	Acid					
	acetic acid	1013	1.53 ± 0.20^{a}	1.29 ± 0.14^{b}	1.06 ± 0.10^{c}	<0.0001
	Alcohols					
C5 alcohol	2-penten-1-ol	775	0.36 ± 0.06^{a}	0.31 ± 0.08^{a}	0.33 ± 0.10^{a}	0.1530
	1-hexanol	871	0.55 ± 0.17^{a}	0.84 ± 0.07^{a}	0.68 ± 0.43^{a}	0.0760
	(E)-2-hexen-1-ol	869	0.22 ± 0.03^{b}	0.33 ± 0.03^{a}	0.33 ± 0.016^{a}	0.0130
C6 alcohols	(Z)-3-hexen-1-ol	857	0.13 ± 0.03^{a}	0.13 ± 0.02^{a}	0.16 ± 0.02^{a}	0.040
non-LOX alcohols	benzyl Alcohol	1029	n.d	0.21 ± 0.04	0.18 ± 0.05	0.1763
	1-octanol	1072	n.d	0.10 ± 0.02	0.12 ± 0.01	0.0453
	phenylethyl alcohol	1113	0.36 ± 0.16^{a}	0.41 ± 0.08^{a}	0.34 ± 0.07^{a}	0.3311
	1-nonanol	1171	n.d	0.07 ± 0.01	0.09 ± 0.02	0.0060
	Aldehydes					
C6 aldehydes	(E)-2-hexenal	854	115.9 ± 7.7^{a}	99.4 ± 4.9^{b}	94.6 ± 11.6^{b}	<0.0001
non-LOX aldehydes	4-pentenal,2-methyl	803	3.32 ± 0.26^{a}	3.02 ± 0.23^{b}	2.65 ± 0.28^{c}	<0.0001
	(E,E)-2,4-hexadienal	913	0.51 ± 0.15^{a}	0.48 ± 0.06^{a}	0.56 ± 0.17^{a}	0.4377
	benzeneacetaldehyde	1046	n.d	0.13 ± 0.03^{a}	0.10 ± 0.01^{b}	0.0251
	nonanal	1105	1.26 ± 0.47^{a}	1.39 ± 0.19^{a}	1.13 ± 0.14^{a}	0.1810
	decanal	1207	n.d	0.04 ± 0.01	n.d	——
	Ester					
C6 Esters	(Z)-3-hexen-1-yl, acetate	1006	5.71 ± 0.70^{a}	4.83 ± 0.51^{b}	4.11 ± 0.22^{c}	<0.0001
	Hydrocarbons					
	pentene dimer isomer I	940	4.86 ± 0.51^{a}	4.33 ± 0.69^{ab}	4.06 ± 0.91^{b}	0.0567
	pentene dimer isomer II	947	4.57 ± 0.42^{a}	3.92 ± 0.56^{b}	3.46 ± 0.69^{b}	0.0007
	pentene dimer isomer III	996	14.50 ± 1.54^{a}	12.41 ± 1.22^{b}	10.74 ± 0.89^{c}	<0.0001
	Dodecane	1202	0.55 ± 0.32^{a}	0.17 ± 0.08^{b}	0.12 ± 0.03^{b}	<0.0001
	Ketone					
	2-octanone	990	n.d	n.d	0.45 ± 0.16	——
	Terpenes					
	D-limonene	1023	0.53 ± 0.17a	0.16 ± 0.04b	0.16 ± 0.03b	<0.0001
	β-*cis*-ocimene	1050	n.d	0.06 ± 0.01	n.d	——
	β-curcumene	1393	0.15 ± 0.12^{a}	0.14 ± 0.02^{a}	0.15 ± 0.06^{a}	0.915
	α-farnesene	1506	0.49 ± 0.40^{a}	0.35 ± 0.11^{a}	0.27 ± 0.12^{a}	0.1732
	Σ_{Acids}	——	1.53 ± 0.20^{a}	1.29 ± 0.14^{b}	1.06 ± 0.10^{c}	<0.0001
	$\Sigma_{\text{C5 Alcohols}}$	——	0.36 ± 0.06^{a}	0.31 ± 0.08^{a}	0.33 ± 0.10^{a}	0.1530
	$\Sigma_{\text{C6 Alcohols}}$	——	0.90 ± 0.18^{b}	1.30 ± 0.06^{a}	1.17 ± 0.58^{ab}	0.0455
	$\Sigma_{\text{non-LOX Alcohols}}$	——	0.36 ± 0.16^{b}	0.79 ± 0.13^{a}	0.73 ± 0.10^{a}	<0.0001
	$\Sigma_{\text{C6 Aldehydes}}$	——	115.9 ± 7.7^{a}	99.4 ± 4.9^{b}	94.6 ± 11.6^{b}	<0.0001
	$\Sigma_{\text{non-LOX Aldehydes}}$	——	5.08 ± 0.51^{a}	5.07 ± 0.24^{a}	4.44 ± 0.33^{b}	0.0008
	$\Sigma_{\text{C6 Esters}}$	——	5.71 ± 0.70^{a}	4.83 ± 0.51^{b}	4.11 ± 0.22^{c}	<0.0001
	$\Sigma_{\text{Hydrocarbons}}$	——	24.49 ± 2.15^{a}	20.83 ± 2.34^{b}	18.38 ± 2.40^{b}	<0.0001
	Σ_{Ketones}	——	——	——	0.45 ± 0.16	——
	Σ_{Terpenes}	——	1.17 ± 0.64^{a}	0.71 ± 0.14^{b}	0.59 ± 0.16^{b}	0.0057
	$\Sigma_{\text{Total C5 compounds}}$	——	0.36 ± 0.06^{a}	0.31 ± 0.08^{a}	0.33 ± 0.10^{a}	0.1530
	$\Sigma_{\text{Total C6 compounds}}$	——	122.5 ± 8.4^{a}	105.6 ± 5.2^{b}	99.9 ± 12.1^{b}	<0.0001
	$\Sigma_{\text{Total Volatile compounds}}$[4]	——	155.5 ± 10.9^{a}	134.5 ± 7.3^{b}	125.8 ± 14.6^{b}	<0.0001

[1] Peaks identification performed by comparing the mass spectra with NIST 11 Library database, being set a minimum similarity of 85%. Contents were expressed as mg of IS equivalents/kg of olive oil.
[2] LRI = Linear Retention Index.
[3] *P*-values for the one-way ANOVA (comparison 3 groups) or *t*-Student test (comparison among the mean values of only 2 groups). Different letters in the same row show statistical differences from the given mean (*P*-value < 0.05); n = 5.
[4] Sum of volatile compounds (similarity ≥ 85%), expressed in equivalents of internal standard. n.d: not detected.

hexenal) and C6 esters (e.g., (Z)-3-hexen-1-yl, acetate), which were formed from the LA and LnA through the LOX pathway (Cecchi et al., 2021). The highest concentrations of the C6 compounds related with the LOX pathway, was found in the control oils (without leaves) (122.5 ± 8.4 mg/kg, Table 2). Indeed, leaf addition during the oils' extractions decreased the content of these compounds (reduction of 14 to 18%).

The (E)-2-hexenal, described as a freshness marker in vegetable oils (Cavalli et al., 2004), was the sole C6 aldehyde detected, accounting for the majority of the volatiles found in the Arbequina oils and representing more than 94% of the total content of the C6 volatile group. The addition of olive leaves during the malaxation process also promoted a significant but similar decrease in the (E)-2-hexenal amount (−14 and −18% for leaves of *cvs.* Arbequina or Santulhana, respectively), not evidencing any leaf cultivar dependency. A similar reduction trend was previously reported by Sanmartin et al. (2019), for oils extracted in a pilot unit with 3% (w/w) of olive leaves. Contrary, Marx, Rodrigues, et al., 2021), reported an increase trend in oils industrially extracted with the addition of *cv.* Santulhana leaves, while a decrease trend was observed for oils extracted with the addition of *cv.* Arbequina leaves.

As pointed out, the addition of leaves decreased the amount of C6 aldehydes in the same proportion of decreased volatiles (Table 2). This fact may indicate that the incorporation of leaves during the extraction seemed to interfere in the LOX pathway, probably in the enzymatic conversion of 13-L-hydroperoxides from LnA, whose decomposition is catalyzed by HPL, determining the formation of the (Z)–3–hexenal, which isomerization gives rise to (E)–2-hexenal.

Regarding C6 alcohols, three compounds were identified (Table 2), namely 1-hexanol, derived from LA, as well as (E)-2-hexen-1-ol and (Z)-3-hexen-1-ol derived from LnA, due to the ADH enzyme that transformed the respective aldehydes into alcohols. Globally, the incorporation of Arbequina or Santulhana leaves significantly increased the contents of this C6 group, compared to the control oils, leading to a rise of 30 and 23%, respectively.

Table 3 presents the relative abundances (in %) of the main

Table 3
(E)-2-hexenal percentage distribution of the main metabolites coming from LnA oxidation in Arbequina olive oils.

Arbequina olive oils	Main metabolites from LnA oxidation (%)			
	(E)-2-hexenal	(E)-2-hexen-1-ol	(Z)-3-hexen-1-ol	(Z)-3-hexen-1-ol, acetate
Without leaves addition (control)	75	0.14	0.08	3.7
With Arbequina leaves	74	0.25	0.10	3.6
With Santulhana leaves	75	0.26	0.13	3.3

metabolites arising from the LnA oxidation, determined in the Arbequina oils. As can be inferred, the relative percentage of (E)-2-hexenal is similar in the Arbequina oils extracted with or without leaves addition, although a slightly higher conversion of (E)-2-hexenal into (Z)-2-hexen-1-ol was observed in oils extracted with leaves. This could be tentatively attributed to a possible slight increase of the ADH enzymatic activity in oils extracted with leaves. Regarding the (E)-3-hexen-1-ol and the respective (Z)-3-hexen-1-yl, acetate, it could be observed a slightly higher conversion of the C6 alcohol into the respective C6 ester in control oils (extracted without leaves incorporation), which suggested that the presence of leaves may have a probable negative effect on the AAT enzymatic activity.

The synthesis of VOO volatile compounds may also depend on the level of the HPL activity cleaving the polyunsaturated fatty acid hydroperoxides produced during the oil extraction process (Sánchez-Ortiz, Pérez, & Sanz, 2013). The volatile compounds detected in VOO are usually related to the LOX pathway, being synthesized during the milling step of the oil extraction due to the cells' disruption. After being synthetized, the volatiles abundance in oils is mainly regulated by the partition phenomena that occurr between the oily and aqueous phases. This HPL activity is rapidly inactivated during the milling step, in this sense, the most of the VOO volatile compounds produced through the LOX pathway are synthesized in the milling step of the oil extraction process (Sánchez-Ortiz et al., 2013). The apparent lack of synthesis of volatiles during malaxation remains unclear but can be tentatively related to the deactivation of the enzymes of the LOX pathway by some compounds present in the olive paste, which in the present study, may be strengthened by the addition of 1% (w/w) of olive leaves during the oil extraction.

3.4. Effect of leaves addition during oil extraction on the sensory sensations

Sensory evaluation of the studied Arbequina oils allowed perceiving 13 different positive gustatory and ten positive olfactory attributes. The number of attributes and its intensities differs for oils extracted without or with olive leaves incorporation (Table 4). On the contrary, no defect sensation was perceived.

According to the Table 4, it is possible to note that some attributes, as cherry and dry hay grass sensations (both gustatory and olfactory) were perceived only in Arbequina oils extracted without leaves. This fact could be related to the presence in higher intensities of other attributes, such as dry fruits, apple, fresh herbs, and tomato leaves in oils extracted with leaves added, which may have interfered with the perception of the attributes mentioned above. On the other hand, it is possible to note that the addition of Santulhana leaves promoted a more pronounced decrease in banana gustatory sensation, as well as a significant decrease in the harmony of the oil, both gustatory and olfactory (Table 4).

The incorporation of leaves during the oil extraction significantly increased the intensities of gustatory attributes of green fruity (P-value < 0.05), decreased the pungency (P-value < 0.05), and did not influence the oils' bitterness (P-value >0.05) (Table 4). Other studies (Di Giovacchino et al., 1996; Malheiro et al., 2013; Marx et al., 2022) also reported that olive leaves addition during the oil extraction could improve some sensory characteristics of the extracted oils. Oleacein and oleocanthal are the main compounds responsible for the oils' gustatory attributes of bitterness and pungency, respectively (Andrewes et al., 2003). In our study, the concentration of oleacein and oleocanthal decreased in oils extracted with leaves (Table 1) which probably influenced the bitterness and pungency changes, more evident for oils extracted with Santulhana leaves. Furthermore, the addition of Santulhana leaves during the extraction significantly increased the sensation of sweetness (Table 4), which is probably due to a slight decrease in bitterness. In general, the results of this study agree with those reported by Novoselić et al. (2021), for oils extracted with leaves.

Table 4
Intensities of gustatory and olfactory attributes (mean ± standard deviation) of the studied Arbequina olive oils.

Sensory attributes perceived by the sensory panel[1]	Without leaves addition (control)	With Arbequina leaves	With Santulhana leaves	P-value[2]
Gustatory attributes				
Green fruity	5.2 ± 0.7^{b}	6.6 ± 0.4^{a}	6.3 ± 0.3^{a}	<0.0001
Sweet	5.5 ± 0.7^{b}	5.7 ± 0.4^{ab}	6.3 ± 0.7^{a}	0.011
Bitter	1.2 ± 0.3^{a}	1.3 ± 0.2^{a}	1.0 ± 0.2^{a}	0.9681
Pungent	2.6 ± 0.3^{a}	2.5 ± 0.4^{a}	1.9 ± 0.4^{b}	0.0002
Apple	3.9 ± 0.4^{b}	5.4 ± 0.4^{a}	5.5 ± 0.4^{a}	<0.0001
Banana	6.1 ± 0.6^{a}	6.4 ± 0.8^{a}	3.2 ± 0.4^{b}	<0.0001
Tomato	4.1 ± 0.5^{b}	4.1 ± 0.6^{b}	5.5 ± 1.2^{a}	0.0007
Dry fruits	1.8 ± 0.5^{b}	3.2 ± 0.6^{a}	3.5 ± 1.2^{a}	<0.0001
Tomato leaves	5.1 ± 0.5^{a}	5.4 ± 0.8^{a}	4.9 ± 0.4^{a}	0.1915
Cherry	3.1 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	——
Dry hay grass	1.1 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	——
Fresh herbs	2.7 ± 0.6^{b}	3.9 ± 0.5^{a}	3.7 ± 0.4^{a}	<0.0001
Cabbage	3.9 ± 0.6^{a}	3.6 ± 0.6^{a}	3.4 ± 0.7^{a}	0.2716
Harmony	7.8 ± 0.5^{a}	7.3 ± 0.6^{a}	5.9 ± 0.5^{b}	0.0054
Olfactory attributes				
Green fruity	5.0 ± 0.3^{b}	6.2 ± 0.4^{a}	6.3 ± 0.3^{a}	<0.0001
Apple	4.5 ± 0.5^{b}	5.5 ± 0.3^{a}	5.3 ± 0.5^{a}	<0.0001
Tomato	5.4 ± 0.5^{a}	6.0 ± 1.3^{a}	6.1 ± 0.6^{a}	0.1387
Dry fruits	2.5 ± 0.4^{a}	2.1 ± 0.3^{a}	2.5 ± 0.5^{a}	0.0049
Banana	3.5 ± 0.5^{a}	3.7 ± 0.7^{a}	3.8 ± 0.5^{a}	0.3620
Tomato leaves	3.8 ± 0.6^{b}	5.8 ± 0.4^{a}	5.3 ± 0.6^{a}	<0.0001
Cherry	3.9 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	——
Dry hay grass	1.5 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	——
Fresh herbs	3.1 ± 0.4^{c}	4.4 ± 0.3^{a}	3.8 ± 0.7^{b}	<0.0001
Cabbage	5.8 ± 0.4^{a}	4.0 ± 0.8^{b}	4.4 ± 1.0^{b}	<0.0001
Harmony	8.3 ± 0.4^{a}	7.9 ± 0.4^{a}	7.2 ± 0.4^{b}	<0.0001
Global				
Persistence	7.7 ± 0.4^{a}	6.5 ± 0.5^{b}	6.8 ± 0.4^{b}	0.0002
Complexity	5.9 ± 0.5^{a}	4.8 ± 0.5^{b}	5.0 ± 0.4^{b}	0.0085

[1] Intensity scale: from 0 (absence of attribute: not perceived by the panelists) to 10 (maximum attribute intensity).

[2] P-values for the one-way ANOVA; Different letters in the same row show statistically differences from the given mean (P-value < 0.05) following the IOC regulations (International Olive Council, 2005); n = 5.

For the olfactory sensations, the profile of the Arbequina oils extracted without leaves addition (control) was in-line with the literature (Sánchez-Rodríguez et al., 2020; Marx, Rodrigues, et al., 2021). On the other hand, the incorporation of olive leaves during extraction significantly increased the positive notes of green fruity, apple and tomato leaves (P-value < 0.0001), and in a less extent, the tomato and banana sensations. These trends can be associated with the higher concentrations of C6 alcohols, responsible for the characteristic fruity aroma, in oils extracted with leaves (Table 3).

4. Conclusions

The results pointed out that the incorporation of fresh olive leaves

(1%, w/w) during oils' extraction highly influenced the phenolic, volatile, and sensory profiles of *cv.* Arbequina oils. Moreover, it was possible to hypothesize some of the observed trends in terms of the enzymatic pathways responsible for the formation of the main phenolics and volatiles, namely, due to the secoiridoid biosynthesis and LOX pathways. Indeed, it could be concluded that the incorporation of leaves promoted a significant decrease of the alcohol and secoiridoid phenolic compounds, independently of the cultivar. This trend could be attributed to a hypothetically activity reduction of the methylesterase enzyme, responsible for the formation of oleacein and oleocanthal, probably due to the lack of their precursors (open-form aglycones). In fact, *cv.* Arbequina is a cultivar for which oleacein and oleocanthal are the predominant secoiridoids, thus, it is important to remark that the referred reduction trend could be related with genetic factors of this cultivar, being possible to observe different behaviors for other cultivars. Regarding the volatile compounds, namely of the compounds derived from the polyunsaturated fatty acids by the LOX pathway (C5 and C6 compounds), the results demonstrated that the leaves addition led to a significant reduction of the total C6 compounds, which may be tentatively attributed to a possible negative impact of the leaves on the enzymatic activity of hydroperoxide lyases. On the other hand, oils extracted with leaves showed higher values of C6 alcohols, which may be tentatively associated with a higher enzymatic activity of alcohol dehydrogenases, responsible for the conversion of aldehydes to alcohols. Oils extracted without leaves presented higher amounts of C6 esters, probably attributed to a partial inactivation of the alcohol acetyl transferases. Additionally, Arbequina and Santulhana leaves addition improved some positive sensory attributes, such as the green fruity and apple sensations, probably related to the increase of the C6 alcohols volatile compounds. Finally, it could be stated that, in general, the addition of olive leaves (1%, w/w) during Arbequina oil extraction in an Abencor unit promoted a reduction of the contents of phenolics and volatiles related with the secoiridoid biosynthesis and lipoxygenase pathways, respectively. On the other hand, this practice allowed improving some positive sensory notes.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.133327.

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