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Agricultural practices as promoters of oleocanthal and oleacein availability in virgin olive oils from three olive cultivars

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ABSTRACT

Virgin olive oil (VOO) is one of the greatest differentiating elements within the Mediterranean Diet, mainly due to its health-promoting properties associated with the presence of specific antioxidants—the phenolic compounds (PC). Among all PC in VOO, oleocanthal and oleacein are two of the most important, mainly due to their strong association with health benefits. The presence of these PC in VOO can be induced by many factors, such as agricultural management approaches. To evaluate the relevance of the agronomic practices as inducers of oleocanthal and oleacein in VOO, monovarietal VOO from three cultivars were produced —'Cobrançosa' (COB), 'Arbequina' (ARB), and 'Galega vulgar' (GV)—by a laboratory-scale extraction method, considering two distinct agronomic systems: organic and integrated farming systems. From the obtained VOO, oleocanthal and oleacein were quantified by HPLC-TOFMS. Significantly higher oleocanthal and oleacein concentrations were found in all organic VOO, with COB being the cultivar presenting the highest concentrations, with 561 and 268 mg/kg for oleocanthal, and 348 and 164 mg/kg for oleacein for organic and integrated VOO, respectively. In contrast, GV showed considerably lower values than COB and closer to ARB, with 110 and 0.17 mg/kg for oleocanthal, and 113 and 0.18 mg/kg for oleacein for organic and integrated farming systems, respectively. These results clearly show that both agronomic practices and cultivar can highly influence the chemical and nutritional properties of VOO, with organic farming potentiating the concentration of oleocanthal and oleacein. Of the studied cultivars, COB showed the highest concentration of the target compounds.

1. Introduction

Virgin olive oil (VOO) is a significant differentiating ingredient in the Mediterranean diet (MD), renowned for its health-promoting properties [1,2]. The unsaponifiable fraction of VOO contains several minor but significant compounds that distinctly differentiate it from other vegetable oils. This fraction, comprising around 2 % of its total composition, is extremely abundant in a diverse array of chemical compounds, including tocopherols, aromatic hydrocarbons, sterols, and notably, phenolic compounds (PC). Although hydrophilic, PC are present in VOO and can be categorized into two primary categories: i) the simple phenols, including vanillic, gallic, coumaric and caffeic acids, tyrosol and hydroxytyrosol; and ii) complex phenols, comprising secoiridoids (oleuropein and ligstroside), and the lignans (1-acetoxypinoresinol and pinoresinol) [3]. In recent decades, the phenolic fraction of VOO has garnered increasing scientific interest due to its potential health benefits

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and its role in reducing the risk of chronic diseases [4–6]. Within all PC identified in VOO, oleocanthal and oleacein are prominent secoiridoid derivatives that, like all compounds in the secoiridoid group, undergo a complex biosynthesis and biotransformation process during VOO extraction (Fig. 1). β-glucosidases released during the crushing and malaxation process hydrolyze the glucose moiety of oleuropein and ligstroside to produce their respective aglycone forms, which are subsequently catalyzed by esterases to form oleocanthal and oleacein [7].

Oleocanthal is a well-known PC mainly acknowledged for its anti-inflammatory attributes. Considered homologous to the non-steroidal anti-inflammatory drug (NSAID) ibuprofen, oleocanthal is nowadays categorized as a naturally occurring NSAID [9]. In fact, the way both compounds reduce inflammation is similar [10], with oleocanthal clearly blocking the COX-1 and COX-2 enzymes, leading to anti-inflammatory effects like those of the synthetic NSAID ibuprofen. Oleocanthal has been shown to inhibit COX-1 and COX-2 enzymes even more effectively than ibuprofen, when both are at the same concentration [10], highlighting its importance for health-promoting benefits in the MD [11]. Other studies have also shown oleocanthal as a general health-promoting molecule [12,13], which further strengthens the claim for a regular-based VOO consumption, as adopted in the MD. Similar to oleocanthal, oleacein has also been found to reduce inflammation by lowering COX-2 enzyme activity [14], but its anti-atherosclerotic properties have also been a focus of attention for researchers [15].

The presence and effective concentration of oleocanthal in VOO is also an important factor when considering the health benefits of daily VOO intake, since not all VOO have the same PC bioavailability. The amount of PC is known to be influenced by some environmental/agriculturally based factors, such as the geographic region where the olives grow, the olive tree cultivar, the agricultural techniques and practices used in the olive groves, the ripeness of the olive fruit, and the VOO extraction process [16]. As mentioned, olive cultivation under different cropping systems may be an important factor for differences in VOO chemical composition. Despite the recent increasing trend in organic farming in the last decades, the integrated system is still the most widely used method of olive grove agricultural management in the Alentejo region (south of Portugal). Integrated farming is characterized by the promotion of natural products for fertilization and disease control, as well as the encouragement of auxiliary fauna for pest control; however, it does not exclude the use of synthetic-based chemicals for plant and pest management when necessary, following specific application protocols [17]. These farming systems are widely used because they allow producers to adopt a hybrid production method, obtaining higher yields and reducing the risks of pest and plague occurrences. On the other hand, organic farming is a more sustainable agricultural system characterized by restricted use of fertilizers and plant protection compounds and, generally, with a more holistic approach to crop production [18]. These differences in agricultural management between organic and integrated farming can lead to variations in the nutritional composition of VOO. To the best of our knowledge, no prior studies have specifically compared oleocanthal and oleacein concentrations in VOO produced under organic versus integrated farming systems. Therefore, this study aims to evaluate and compare the levels of oleocanthal and oleacein concentrations in monovarietal VOO from two Portuguese cultivars, 'Galega vulgar' (GV) and 'Cobrançosa' (COB), and the Spanish cultivar 'Arbequina' (ARB), cultivated under two distinct agricultural management systems: i) organic and ii) integrated.

2. Materials and methods

2.1. Chemicals and reagents

Oleacein (3,4-DHPEA-EDA) ≥ 90.0 % (HPLC) (CAS: 149183-75-5) and oleocanthal (p-HPEA-EDA) ≥ 95.0 % (HPLC) (CAS: 289030-99-5) analytical standards were purchased from Sigma-Aldrich (Steinheim, Germany). Folin & Ciocalteu's phenol reagent was also obtained from Sigma-Aldrich (Steinheim, Germany). Sodium carbonate was purchased from Panreac (Barcelona, Spain). HPLC-grade methanol, acetonitrile, and water were acquired from Merck (Darmstadt, Germany). Formic acid LC-MS grade was purchased from Scharlab (Barcelona, Spain). Individual stock solutions of phenolic compounds were prepared in acetonitrile and stored at −20 °C. The stock solutions were combined into a mixed standard solution of 10 mg/L, from which solvent-based calibration curves were prepared at 10, 50, 100, 500 and 1000 μg/L. Sunflower refined oil was employed to prepare matrix-matched calibration curves at the same concentration levels.

2.2. Olive sampling and VOO production

Olive fruit sampling was performed for three cultivars, 'Galega vulgar' (GV), 'Cobrançosa' (COB), and 'Arbequina' (ARB), in two distinct managed agricultural systems. These cultivars were simultaneously collected from an organic managed olive orchard at Herdade do Esporão (HE), Reguengos de Monsaraz (38°22′48.1″ N, 7°33′38.4″ W), and from two other integrated agricultural systems, Herdade do Malheiro (HM), Vidigueira (38°10′11″ N, 7°44′59″ W), for the cultivars GV and COB, and Herdade da Correia (HC), Vendinha (38°28′42″ N, 7°59′26″ W), for ARB. The site of organic and integrated areas is characterized by the typical Mediterranean climate, with a mean annual rainfall of 544 mm at HE and HC and 384 mm at HM, and a mean annual temperature of 16.8 °C at HE and HC and 17.1 °C at HM. Olive grooves were irrigated during the blooming season and fruit formation. Olive grove age, intensification,

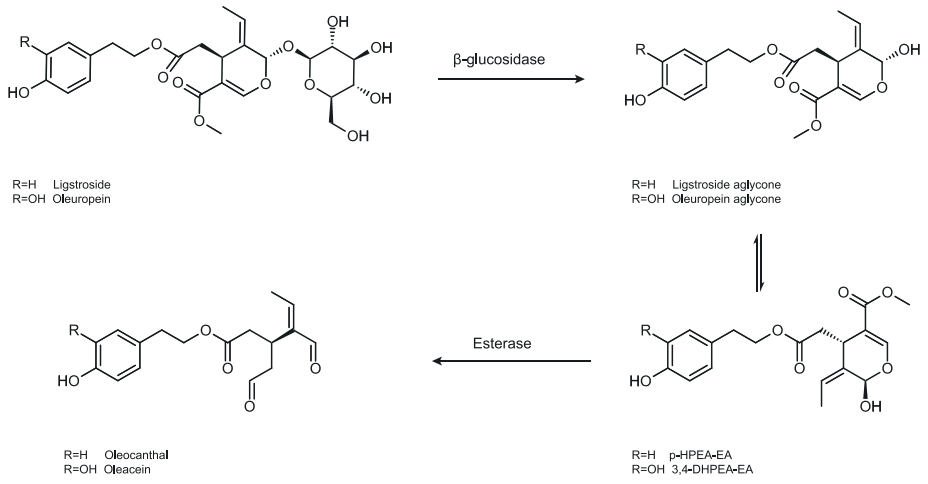

Fig. 1. Biosynthesis pathway of oleocanthal and oleacein during VOO extraction [8].

and altitude are indicated in Table 1.

All samples, from all cultivars, were randomly hand-picked on October 10th, 2021, and VOO produced at the laboratory scale according to a previously published method (VOO lab production) [19]. The obtained VOO were then placed in 50 mL amber glass vials and stored at −20 °C until extraction of the hydrophilic fraction.

2.3. Maturity index determination

Maturity index (MI) was calculated according to the International Olive Council guidelines [20], where 100 fruits were randomly collected and scored from 0 to 7, according to the coloring stage of both skin and flesh, ranging from 0 as skin color deep green to 7 as skin color black with all the flesh purple to the stone. Next, using Equation (1), we calculated the MI value for each ripening stage by considering the number of fruits in each category (from 0 to 7).

$$MI = \frac{A0 + B1 + C2 + D3 + E4 + F5 + G6 + H7}{100} \quad (1)$$

2.4. Extraction of hydrophilic phenolic compounds

The hydrophilic fraction of VOO was extracted by an adaptation to the method proposed by the International Olive Oil Council (COI) [21], where approximately 2.00 ± 0.20 g of VOO was weighed into a 10 mL screw-cap test tube, and 5 mL of a methanol/water (80:20) extraction solution was added. The mixture was then agitated in a vortex for 1 min and sonicated in the ultrasonic bath (Ultrasons H-D, J.P. Selecta, Barcelona, Spain) for 15 min at room temperature. Phase separation was performed by centrifugation (Sigma 2-16P, Osterode am Harz, Germany) for 20 min at 5000 rpm. The methanolic fraction was then collected and diluted 1:100 with methanol/water to match a final solvent composition of 10 % methanol. Finally, the extract was filtered through a 0.45 μm PVDF syringe filter before LC-MS analysis. Triplicates were performed in three independent extractions.

2.5. Liquid chromatography time-of-flight mass spectrometry (HPLC-TOFMS)

Chromatographic analysis was done using a 1290 Infinity HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a reversed-phase C18 analytical column (Zorbax Eclipse Plus 100 mm × 3 mm i.d., 1.8 μm particle size) (Agilent Technologies, Santa Clara, CA, USA) according to a previously described method with slight modifications [32]. A volume of 10 μl of the extract was injected for each sample. Mobile phases A and B were water with 0.1 % formic acid and methanol with 0.1 % formic acid, respectively. The chromatographic method held the initial mobile phase composition (5 % B) constant for 5 min, followed by a linear gradient to 100 % B at 18 min. Then 100 % B remained constant for 5 min. The gradient returns to the initial conditions (5 % B) in 1 min, and the column is equilibrated for 8 min before the next analysis. The total analysis time is 32 min and the flow rate used was 0.4 mL/min. The LC system was controlled by Agilent OpenLab CDS ChemStation software (version A.01.04).

The HPLC equipment was connected to a time-of-flight mass spectrometer Agilent TOF 6220 (Agilent Technologies, Santa Clara, CA, USA) equipped with an electrospray interface operating in negative ionization mode, using the following operation parameters: capillary voltage, 2500 V; nebulizer pressure, 40 psig; drying gas, 9 L/min; gas temperature, 325 °C; skimmer voltage, 65 V; octopole rf, 250 V; and fragmentor voltage, 140 V. LC-TOFMS accurate mass spectra were recorded across the range 50–1000 *m/z*. Accurate mass measurements of each peak from the total ion chromatograms were obtained by means of an automated calibrant delivery system using a dual-nebulizer electrospray source that introduces the flow from the outlet of the chromatograph together with a low flow of a calibrating solution (calibrant solution A, Agilent Technologies), which contains the internal reference masses (purine (C5H4N4 at *m/z* 121.050873) and HP-921 [hexakis-(1H,1H,3H-tetrafluoropentoxy)-phosphazene] (C18H18O6N3P3F24) at *m/z* 922.009798). Agilent MassHunter Data Acquisition software (version B.04.00) was used for method development and full-scan data acquisition. Agilent MassHunter Qualitative Analysis and Quantitative TOF Analysis software (version 10.0) were used for data processing.

To evaluate the analytical method, calibration curves of oleocanthal and oleacein at five concentration levels were prepared in the range of 10–1000 μg L^{-1}. Refined sunflower oil extracts were used to prepare matrix-matched calibration curves at the same concentration levels, considering the final dilution factor (1:100). Good linearity was obtained for both compounds, with correlation coefficients higher than 0.9998. Limits of quantification (LOQs) and limits of detection (LODs) were established as the minimum analyte concentration corresponding to a signal-to-noise ratio (S/N) = 10 and (S/N) = 3, respectively. They were calculated experimentally using the lowest concentration level of matrix-matched solutions. The LOQ and LOD for oleocanthal were 32.1 μg L^{-1} and 9.6 μg L^{-1}, and 5.3 μg L^{-1} and 1.6 μg L^{-1} for oleacein. The results obtained were appropriate for the quantification of these compounds in real oil samples. However, they could be improved using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system [32].

2.6. Data analysis

For the statistical analyses of the experimental data, analysis of variance (ANOVA) was applied with the Fisher test for a confidence level of 95 %. All analyses were performed using XLSTAT software (version 2022.4.1).

3. Results and discussion

Various agronomic practices and operational conditions during VOO extraction significantly influence its PC concentrations. Key factor include edaphoclimatic conditions, cultivar specificity, and fruit ripening stages, among others [22]. The extraction process involves the promotion of PC in VOO, particularly secoiridoid derivatives like oleocanthal and oleacein, primarily during the hydrolysis of oleuropein, demethyloleuropein, and ligstroside, which are catalyzed by endogenous β-glucosidases released during the mechanical processing of the fruits [23]. The extraction process is a crucial step to consider when characterizing PC in VOO. Consequently, to minimize the variability introduced by this process, all VOO were obtained using the same extraction process, which consisted on a previously established

Table 1
Characterization of olive groves (Herdade do Esporão – HE, Herdade do Malheiro – HM, and Herdade da Correia – HC) from the three cultivars 'Galega vulgar', 'Cobrançosa' and 'Arbequina' by age (year of implementation), intensification (trees/ha), and average field altitude from sea level (m).

Cultivar	Arbequina		Galega vulgar		Cobrançosa	
Management	Organic	Integrated	Organic	Integrated	Organic	Integrated
Olive grove	HE	HC	HE	HM	HE	HM
Year	2006	2010	<2000	2000	2006	2000
Intensification	250	1200	50	250	250	250
Altitude	210 ± 20	190 ± 10	210 ± 20	155 ± 10	210 ± 20	155 ± 10

laboratory-scale VOO extraction method [19].

Table 1 outlines several factors in the olive grove that may affect the phenolic content in VOO. In the present study, edaphoclimatic conditions shouldn't greatly affect the results, because both organic and integrated orchards considered for this study are located in the same region. As for cultivar and fruit ripening, these factors were also minimized since the same cultivars and similar maturity indexes (MI) were used for comparison. Table 2 presents the MI values, ranging from 3 to 4, with MI 3 indicating fruits that have half of their surface turning red/purple and MI 4 indicating fruits that are black on the outside but have white flesh inside. It is well-established that fruit ripening affects the overall phenolic composition of VOO [24], as previously reported studies show, lower amounts of polyphenols are detected with the advancement of the ripening stage [25].

Regarding other agronomic factors related to the differences presented by the distinct olive orchards, namely the age of the orchard, some reports can be found in the literature relating this factor with the concentration of polyphenols in VOO [26]; for instance, El Chami et al. [27] reported, for the cultivar 'Baladi' in two distinct regions, a negative correlation between PC in VOO and tree ageing when comparing adult with centenary trees. Nevertheless, other researchers have reported contradictory results [28]. Thus, we are led to believe that other parameters may contribute more to the presence and relative concentration of PC in VOO than tree age, namely cultivar specificity and agriculture management system. As for the altitude, positive correlations between the concentration of polyphenols and increasing altitude have been reported in the bibliography [29,30]. Although, to find this factor as a relevant influencing parameter to influence the presence of PC in VOO, altitude differences should be higher than 200 m [30]. In our study, differences in altitude were lower than 50 m; therefore, this factor was not considered as relevant for the focus of this study.

Considering the above, we can assert with confidence that all major sources of variability were effectively mitigated. This allows us to identify the selected variables—cultivar and agricultural practices—as the primary factors influencing the presence and relative concentration of the target PC, oleocanthal and oleacein. According to the literature, liquid chromatography coupled to mass spectrometry (LC-MS) using a methanol-water gradient is suitable for determining oleocanthal and oleacein in VOO [32]. These deprotonated molecules $[M-H]^-$ were detected by high-resolution mass spectrometry in negative ion mode. The identification of each compound was based on the comparison of the retention time against commercial standards. Quantification involved interpolating the chromatographic peak area into corresponding matrix-matched calibration curves. Fig. 2 exemplifies the identification of oleocanthal and oleacein in VOO from the COB cultivar produced under organic farming, showcasing their chromatograms and specific mass spectrum.

The two target PC were identified using the deprotonated $[M-H]^-$ ion, with m/z values of 303.1238 for oleocanthal and 319.1187 for oleacein (Fig. 2). The soft ionization provided by ESI-TOFMS analysis facilitated the detection of the intact molecules without fragmentation. However, previous studies have described the characteristic fragmentation patterns of oleacein and oleocanthal through tandem mass spectrometry (MS/MS) analysis [31]. Specifically, oleacein exhibits a characteristic ion at m/z 123 corresponding to the hydroxytyrosol fragment, while oleocanthal yields two fragments at m/z 137 and m/z 119 corresponding to tyrosol and its major fragment, respectively.

Table 2
Measurement of maturity index (MI) for three olive cultivars: 'Arbequina' (ARB), 'Galega vulgar' (GV), and 'Cobrançosa' (COB), obtained by two different production systems, organic and integrated.

	ARB	GV	COB
Organic	3.56	4.14	3.85
Integrated	2.92	3.67	3.34

Additionally, both oleocanthal and oleacein share some characteristic fragments in their MS/MS spectra, which are typical of the structure of these secoiridoid derivatives.

From Fig. 3-A, we can see that all organically produced VOO presented significantly higher concentrations of oleocanthal, with COB being the cultivar showing the highest concentrations in both cultivation systems, with 561 ± 48 and 268 ± 23 mg/kg of VOO, respectively, for organic and integrated production. Considering other reports from the literature [8,32], our study showed oleocanthal concentrations for COB comparatively higher, regardless of the cultivation method. For instance, Karkoula et al. [32] reported for the Greek 'Koroneiki' cultivar the highest oleocanthal concentration of 355.0 ± 12.1 mg/kg. For the same 'Koroneiki' cultivar, Sánchez de Medina et al. [32] quantified 537 ± 59 mg/kg of oleocanthal from samples collected in a different region and year, demonstrating the variability within the cultivar and indicating that different agronomic and edaphoclimatic conditions may lead to differences in oleocanthal concentration in VOO. For the Italian cultivar 'Bianchera', Starec et al. [8] showed oleocanthal concentrations of 170.0 ± 1.5 mg/kg, while Sánchez de Medina et al. [32] reported values of 153 ± 17 and 67 ± 7 mg/kg for the Spanish 'Picual' and 'Arbequina' cultivars, respectively. Our study found that the amount of oleocanthal in ARB from organic farming (104.3 ± 22.3 mg/kg) is similar to other reports [32], suggesting that ARB has a considerably lower oleocanthal concentration when compared to other cultivars [32].

Considering that oleacein shares the same biosynthesis pathway as oleocanthal, the same samples were also characterized in terms of oleacein concentrations to further correlate with oleocanthal quantifications (Fig. 3). For both cultivation systems, similar to the oleocanthal findings, COB also had a significantly higher concentration of oleacein, with 164 ± 12 and 350 ± 26 mg/kg of VOO for integrated vs. organic systems, respectively. When comparing all cultivars, the samples from organic farming also showed significantly higher (p-value <0.05) oleacein concentrations. The results clearly indicate that VOO produced under an organic farming system may present higher concentrations of oleocanthal and oleacein, revealing that in fact the applied agricultural practices produce fundamental differences in the chemical composition of VOO, especially when considering the phenolic content, as expressed in this work by the target compounds oleocanthal and oleacein.

Considering that the biosynthesis of oleocanthal is regulated by the oleuropein/ligstroside degradation process (Fig. 1), and these secoiridoid compounds are produced by the secondary metabolism of the olive tree, it is expected that the farming practices carry a considerable weight in this process. Research has demonstrated that organic farming practices significantly increase the concentrations of oleocanthal and oleacein in VOO [33]. As is known, the PC synthesized by the secondary metabolism, such as oleuropein and its derivatives, play an important role in the defensive mechanism of the plant against external agents [34]. Thus, in organic systems, due to the absence of synthetic pesticides, plants are exposed to higher stress levels caused by external agents; therefore, a natural increase in the production of these defense substances is also expected [35]. Furthermore, the lower levels of bioavailable nitrogen observed in organic farming, in contrast with integrated systems where synthetic fertilizers are applied, restrict plant growth and thus enhance the production of the secondary metabolites. As demonstrated by Fernández-Escobar et al. [36], the use of more nitrogen fertilizers directly reduces the polyphenol content in VOO, which is consistent with our findings comparing VOO from organic and integrated farming systems. So [33,37],agronomic practices play a decisive role in the presence of the target PC in VOO, but this only becomes a major factor when other relevant variables are attenuated [37], as we also showed to be the case for this study. Nevertheless, further investigation must be performed to affirm COB as a cultivar with high potential for oleocanthal and oleacein, mainly within organic production. Therefore, in our future work, we plan to monitor the levels of oleocanthal and oleacein during the ripening process for the various studied cultivars.

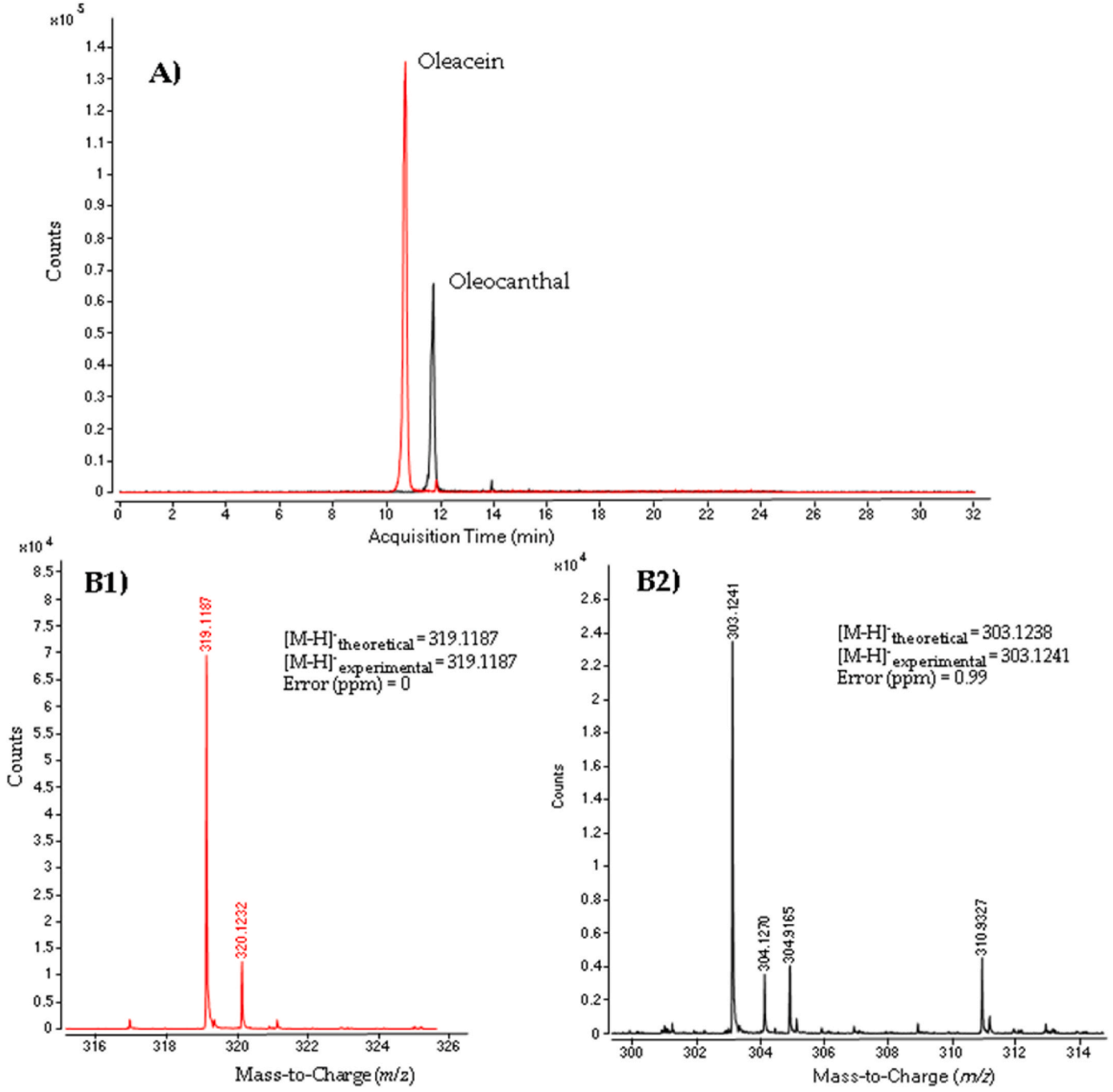

Fig. 2. (A) Extracted ion chromatograms of oleacein ($m/z = 319.1187$) and oleocanthal ($m/z = 303.1238$); (B1) accurate mass spectrum at retention time 10.67 min for oleacein identification; (B2) accurate mass spectrum at 11.72 min for oleocanthal identification. Expressed results from 'Cobrançosa' cultivar from the organic farming system.

4. Conclusions

Obtained results showed significantly higher concentrations of both oleocanthal and oleacein when applying organic farming. This suggests that the agronomic differences between both integrated and organic production systems substantially influence the VOO's chemical and nutritional profiles. Consequently, organically produced VOO may potentiate the well-known health benefits associated with the MD. Current knowledge provides a key foundation in farming practices, chemical properties, and economic aspects for promoting the health benefits of consuming monovarietal VOO, especially from the 'Cobrançosa' cultivar, a traditional Portuguese cultivar, when produced organically, due to its oleocanthal and oleacein biochemical profile.

CRediT authorship contribution statement

Miguel D. Ferro: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Irene Caño-Carrillo:** Resources, Methodology, Investigation. **Bienvenida Gilbert-López:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation. **Alfonso Fernández-García:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Maria J. Cabrita:** Writing – review & editing, Supervision, Resources. **José M. Herrera:** Writing – review & editing, Supervision,

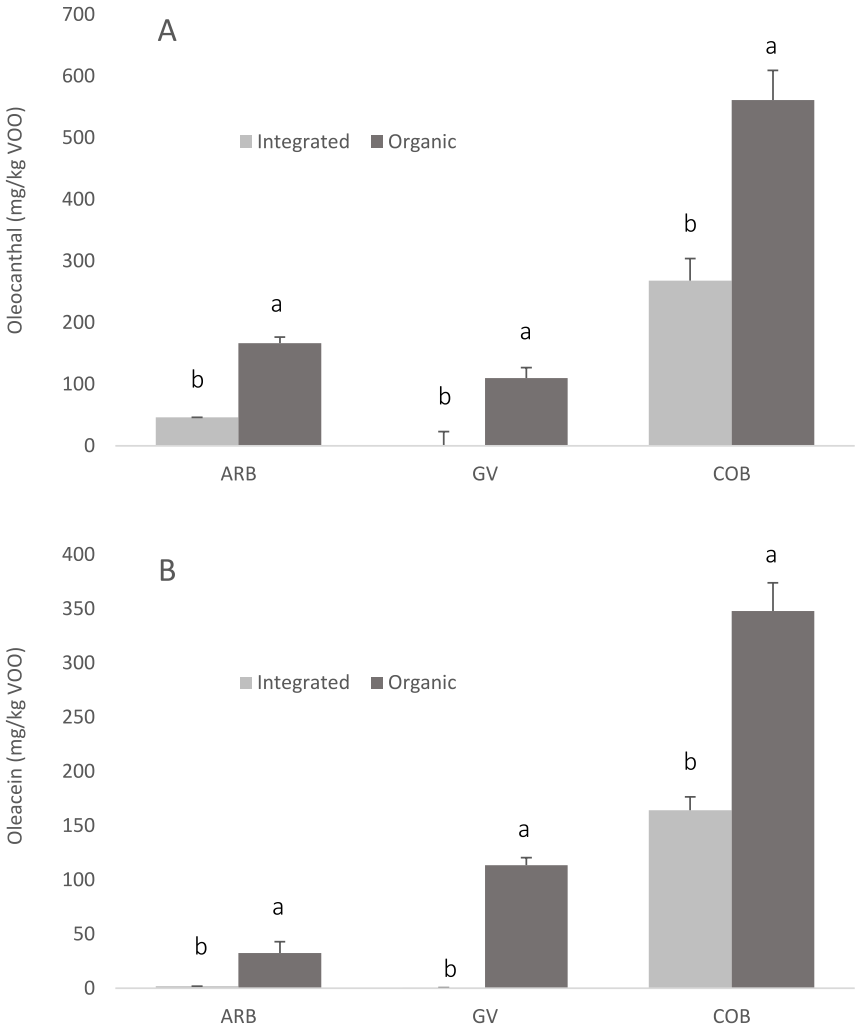

Fig. 3. Oleocanthal (A) and oleacein (B) quantification (mg/kg of VOO) of VOO from cultivars 'Arbequina' (ARB), 'Galega vulgar' (GV), and 'Cobrançosa' (COB) from organic (dark grey) and integrated (light grey) cultivation systems.
[a-b]: ANOVA performed for production system, within each cultivar, for oleacein and oleocanthal. Different superscripts in a row differ significantly (p-value <0.05).

Resources. **Maria F. Duarte:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization.

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Declaration of competing interest

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Data availability

Data will be made available on request.

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