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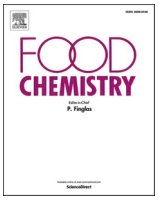

A targeted foodomic approach to assess differences in extra virgin olive oils: Effects of storage, agronomic and technological factors

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ABSTRACT

Extra virgin olive oil (EVOO) quality and composition are mainly affected by genetics, agronomic and technological parameters, undergoing further modifications during storage. In this work, a chemometric approach was applied to study the impact of olive maturity, malaxation time/temperature, and oil storage on the quality and compositional parameters of *Arbequina* EVOO (basic quality indices, volatile and sensory profiles, contents in phenolic compounds, squalene, vitamin E and fatty acids). Storage emerged as the most influential factor, followed by olive maturity and malaxation temperature, while malaxation time had almost no effect. Storage at room temperature had a significant impact on the phenolic profile and quality parameters, mainly the peroxide value and K_{270}. The determination of K_{270}, an indicator of secondary oxidation products, was relevant to analyze the effect of storage conditions. Volatile compounds and fatty acids were good markers of ripeness, and the volatile profile was highly affected by malaxation temperature.

1. Introduction

Extra virgin olive oil (EVOO) is the main lipid source in the Mediterranean diet and plays a key role in its health benefits. Much appreciated for its flavor (Del Giudice et al., 2015), EVOO is consumed throughout the year, despite being a seasonal product. Olives are harvested in autumn, and for example in Spain the harvest is from early October to December. A challenge for producers is therefore to preserve the sensory and healthy properties of EVOO throughout its shelf-life.

EVOO shelf-life has been extensively investigated, both with accelerated (Calligaris et al., 2022) and real-time (Valli et al., 2019) shelf-life tests. Accelerated shelf-life prediction models are based on applying high temperatures to rapidly assess the best-before date (Calligaris et al., 2022). However, this approach is not suitable for determining the changes that take place during storage in real life situations, as certain reactions occur only at high and not at room temperature. In this context, Conte et al. (2020) recently proposed different markers for EVOO conservation: K_{270} for EVOO stored at room temperature and pyropheophytin *a* for EVOO analyzed in stress conditions (high temperatures).

During EVOO storage, 1,2-diglycerides are partially transformed to 1,3-diglycerides (Valli et al., 2019). The phenolic profile of olive oil also undergoes modification during storage. Oleuropein and ligstroside aglycones decrease drastically due to their respective conversion to oleacein and oleocanthal. Additionally, a partial reduction of oleocanthal content is observed as a result of auto-oxidation. In contrast, tyrosol and hydroxytyrosol increase during storage, as their formation by hydrolysis of secoiridoids occurs at a higher rate than their degradation

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(Valli et al., 2019). The volatile profile of EVOO is also transformed, and autooxidation products such as hexanal and octane increase with storage time (Raffo et al., 2015). The volatile profile is particularly influenced by the oil extraction procedures (Kalua et al., 2013), which affect enzyme activity in the lipoxygenase (LOX) pathway, responsible for the formation of several C_6 and C_5 volatile compounds [e.g., hexanal, 1-hexanol, (*E*)-2-hexenal, (*E*)-2-hexen-1-ol, (*Z*)-2-penten-1-ol, and 1-penten-3-one] (Raffo et al., 2015). These changes in phenolic and volatile compounds alter the sensory profile of EVOO; when freshly made it has pungent, bitter, and fruity as main positive sensory attributes, while 13-month-old EVOOs are predominantly ripe and fruity (Sinesio et al., 2015). Finally, chlorophylls are transformed to pyropheophytin *a*, a marker of oxidation (Conte et al., 2020).

Several genetic, agronomic, and technological factors affect EVOO composition and thus influence its evolution during storage. Apart from the cultivar, the growing system (organic *vs.* conventional) (López-Yerena et al., 2019), and the harvest date (López-Yerena et al., 2021) exert a significant influence on the composition. EVOOs with a high concentration of antioxidants have a higher oxidative stability (Ceci et al., 2017), and a longer shelf-life (Castillo-Luna et al., 2021), whereas linoleic acid-rich EVOOs are more prone to oxidation than those richer in oleic acid (Ceci et al., 2017).

Even with the same agronomic parameters, a higher concentration of antioxidants in EVOO can be achieved by modulating extraction conditions, thereby increasing oxidative stability. A higher malaxation temperature (30 *vs.* 20 °C) was found to negatively affect the phenolic concentration, whereas extending the malaxation time by 15 min had no observable effect (30 *vs*. 45 min) (Olmo-Cunillera et al., 2021). The type of decanter (2- or 3-phases) can also affect the antioxidant concentration, which can be higher when a two-phase decanter is used, as the last involves negligible water addition (Sinesio et al., 2015). EVOO composition can also be modified by the use of novel extraction technologies such as ultrasound-assisted methods (Pérez et al., 2021).

In a study of the storage stability of different commercial EVOOs, Castillo-Luna et al. (2021) showed that the shelf-life was influenced by the initial concentration of phenolic compounds. Thus, EVOOs rich in oleacein and oleocanthal are more easily oxidated than those rich in their precursors, oleuropein and ligstroside aglycones. In addition, filtration improves preservation of EVOO quality during storage (Valli et al., 2019).

The use of multivariate statistics has been employed sparingly to study different factors affecting EVOO composition, Kalua et al. (2013) investigated the effect of each production step on phenols and volatile compounds, determining that variety and maturity had a great effect on the phenolic compounds, while the extraction conditions affected higher the volatile compounds. In a recent study, Deiana et al. (2023) studied the effect of storage on fatty acid profile, phenolic composition, and quality parameters through chemometrics, and determined that PV, K270, tyrosol, hydroxytyrosol, and oxidated oleacein were markers of aged EVOO.

In previous research works, authors have studied the effect of different malaxation conditions (3 temperatures and 2 times) on the composition of *Arbequina* EVOO extracted on a laboratory scale (Olmo-Cunillera et al., 2021, 2022). The *Arbequina* EVOO samples were used in the present study to determine the effect of their initial composition on EVOO shelf-life. The oils were stored for 9 months in opaque amber PET bottles in darkness at room temperature and were analyzed every 3 months. Changes in the basic quality parameters, squalene and vitamin E concentrations, as well as the volatile, phenolic, fatty acid and sensory profiles were monitored throughout storage, and analyzed for correlations with agronomic and technological parameters. A chemometric approach was used to determine the effect of storage, ripening index (RI), and malaxation temperatures and time on EVOO quality and composition.

To the best of our knowledge, this is the first study to apply a chemometric targeted approach to analyze such a comprehensive range of EVOO constituents and quality parameters during storage. An unsupervised principal component analysis (PCA) was also carried out to quantify the effect of each parameter on EVOO quality and supervised models were generated to determine the compounds most affected by storage, RI, and malaxation temperature and time.

2. Materials and methods

2.1. Reagents

Cyclohexane and 0.1 N sodium thiosulfate were purchased from Carlo Erba Reagents (Val-de-Reuil, France); acetic acid, chloroform, methanol, acetonitrile, *n*-hexane, 0.5 N sodium methoxide, and 14% boron trifluoride–methanol from Sigma-Aldrich (Madrid, Spain); potassium iodide (KI) from Honeywell Fluka (Buchs, Switzerland); sodium hydroxide pellets, sodium chloride, 1% starch and phenolphthalein from Panreac (Castellar del Vallès, Spain); ethanol 96% from VWR Chemicals (Fontenay-sous-Bois, France); anhydrous sodium sulfate (Na_2SO_4) for gas chromatography (GC) from Scharlau (Sentmenat, Spain). Ultrapure water was obtained using a Milli-Q purification system (Millipore, Bedford, MA, USA).

Oleocanthal (≥95% purity) was purchased from Merck (Darmstadt, Germany); and oleacein and oleuropein aglycone (≥90% and 95% purity, respectively) from Toronto Research Chemical Inc. (ON, Canada). Apigenin and *p*-coumaric (>98% purity) were obtained from Fluka (Buchs, Switzerland), hydroxytyrosol from Extrasynthese (Genay, France), and luteolin (≥96% purity), oleuropein (98% purity), pinoresinol (≥95% purity), squalene, (±)-α-tocopherol (≥96% purity), and tridecanoic acid (C13:0) methyl ester were acquired from Sigma-Aldrich.

The following standards (CAS number and purity percentage in parenthesis) were used for the analysis of volatile compounds and were purchased from Sigma-Aldrich: (*E*)-2-decenal (3913–81-3, ≥95.0%), (*E*)-2-heptenal (18829–55-5, ≥95%), (*E*)-2-hexenal (6728–26-3, ≥97.0%), (*E*,*E*)-2,4-hexadienal (142–83-6, ≥95.0%), (*Z*)-3-hexenyl acetate (3681–71-8, ≥98.0%), 1-hexanol (111–27-3, ≥99.9%), 1-octen-3-ol (3391–86-4, ≥98.0%), 3-methyl-1-butanol (123–51-3, ≥98.5%), 6-methyl-5-hepten-2-one (110–93-0, ≥97.0%), acetic acid (64–19-7, ≥99.8%), ethanol (64–17-5, ≥99.9%), ethyl acetate (141–78-6, ≥99.8%), ethyl propanoate (105–37-3, ≥99.7%), hexanal (66–25-1, 98%), nonanal (124–19-6, ≥95%), octane (111–65-9, ≥99.7%), pentanoic acid (109–52-4, ≥99.8%), and propanoic acid (79–09-4, ≥99.8%). 4-Methyl-2-pentanol (123–51-3, ≥95%) was used as an internal standard (IS).

2.2. EVOO production

Arbequina olives were picked in November 2019 in the orchard of the Institute of Agrifood Research and Technology (IRTA), Mas Bové (Constantí, Spain). The oil was extracted on the same day the olives were harvested, and the RI was calculated by the color of the pulp and skin, as described in a previous study (Olmo-Cunillera et al., 2021). The oils were extracted on a laboratory scale using an ABENCOR® apparatus. Details of the agronomical characteristics and extraction procedure are explained in Olmo-Cunillera et al. (2021). Briefly, a full factorial design was applied to the malaxation conditions, with three levels of temperature (20, 25, and 30 °C) and two of time (30 and 45 min), giving a total of six samples with three replicates each one. In addition, as olives were harvested from different trees, within the same field, different RIs were found for different samples and replicates. The summary of the samples is depicted in the database, in the supplementary material.

2.3. Shelf-life simulation

For the shelf-life simulation, the oils were packed in opaque amber PET bottles (250 mL) with nitrogen in the headspace. Plastic containers

were used because EVOO characteristics are less well preserved in this type of packaging with respect to other materials and thus more changes can be detected in less time (de la Torre-Robles et al., 2019). The oils were stored in a closed cabinet, without light, at the University of Barcelona at room temperature to simulate real life conditions. The temperature was monitored each hour with a thermometer (Model M1, Version 1.3, Tempmate®, Heilbronn, Germany). The mean temperature between 0 and 3 months of storage was 17.6 °C; between 3 and 6 months, 19.5 °C, and between 6 and 9 months, 23.3 °C. Three bottles of each extraction replicate were removed from storage at 0, 3, 6 and 9 months to carry out the analyses. For each sample time, one bottle (250 mL) was sent to the Institute of Agrifood Research and Technology (IRTA) for sensory analysis, those were sent in the same day of sampling without refrigeration and the analysis was carried out between a week from sampling day, before sensory analysis the samples were kept at room temperature in darkness. One aliquot of 5 mL was frozen (-20 °C), to minimize oil transformations, according to other work (Díez-Betriu et al., 2023), and sent to the Department of Agricultural and Food Sciences, Alma Mater Studiorum – Università di Bologna (UNIBO) for volatile analysis, and one was used for the other analyses carried out at the University of Barcelona. Shipments of the frozen samples were carried out under refrigeration conditions, by the fastest courier, to limit any oxidative phenomena during these trips. The quality parameters were analyzed immediately after collecting the samples. For all the other determinations, the samples were stored frozen (−20 °C) until the analysis.

2.4. Quality parameters

Spectrophotometric indices (K_{232}, K_{270} and ΔK), peroxide value, and free acidity were determined following the analytical methodologies established by the International Olive Council, which are also described in detail in our previous study (Olmo-Cunillera et al., 2021).

2.5. Fatty acid profile

Fatty acid methyl esters (FAME) were analyzed as explained elsewhere (Olmo-Cunillera et al., 2022). Briefly, 25 mg of oil were spiked with 100 μL of the IS (C13 tridecanoic acid at 400 ppm). Then, 2 mL of 0.5 N sodium methoxide was added, and the solution was stirred for 30 s and heated at 100 °C for 15 min. The samples were then cooled in an ice bath. Next, 2 mL of 14% boron trifluoride was added to the samples, which were stirred for 30 s, heated at 100 °C for 15 min and cooled in an ice bath. Afterwards, 1 mL of *n*-hexane was added to the samples, stirring for 30 s, and then 2 mL of saturated NaCl was added, again stirring for 30 s. Finally, the samples were centrifuged at 3000 rpm for 7 min and the hexane phase was collected, mixed with anhydrous Na_2SO_4, and left to stand for 5 min, before being collected with a micropipette and stored at −20 °C until analysis. The samples were analyzed by gas chromatography using a Shimadzu, Kyoto, Japan GC-2010 gas chromatograph with a flame ionization detector and a Shimadzu AOC-20i autoinjector. The FAME were separated on a capillary column (40 m × 0.18 mm i.d. × 0.1 μm film thickness) coated with RTX-2330 stationary phase of 10% cyanopropyl phenyl–90% biscyanopropyl polysiloxane from Restek (Bellefonte, USA). The exact chromatographic and detection conditions are detailed in Olmo-Cunillera et al. (2022).

2.6. Phenolic profile

The phenolic fraction was extracted by a liquid–liquid extraction following the method described in Lozano-Castellón et al. (2020). The phenolic profile was analyzed by ultra-high performance liquid chromatography coupled to electrospray ionization and mass spectrometry in tandem (UPLC-ESI-MS/MS), using two methodologies: one for the main secoiridoids (oleocanthal, oleacein, and oleuropein and ligstroside aglycones) and one for the other phenolic compounds. The method followed for the identification and quantification of the main secoiridoids was validated in a previous study (Lozano-Castellón et al., 2021). The rest of the phenolic fraction was analyzed using the method specified in another study (López-Yerena et al., 2019).

For the identification of phenolic constituents, the extracts were analyzed by HRMS. The exact methodology for the identification, together with the identification data, is provided in the **Supplementary Material 1**. After the identification by HRMS, the available compounds were purchased and the retention time of the standards was compared with the retention time of the EVOO compounds in order to confirm the tentative identifications.

For the separation, a UHPLC Acquity system equipped with a binary pump and autosampler (Waters, Milford, MA, USA) was used. The column was an Acquity UPLC® BEH C18 Column (2.1 × 50 mm, i.d., 1.7 μm particle size) (Waters Corporation®, Wexford, Ireland) with an Acquity UPLC® BEH C18 precolumn (2.1 × 5 mm, i.d., 1.7 μm particle size) (Waters Corporation®). An API 3000 triple-quadruple mass spectrometer (PE Sciex, Framingham, MA, USA) with a turbo ion spray source was used. The exact chromatographic and mass spectrometric parameters are specified in previous studies (López-Yerena et al., 2019; Lozano-Castellón et al., 2021).

Matrix matched calibration was employed to quantify the phenolic compounds, and refined olive oil was used as the blank matrix. The refined olive oil was extracted without the addition of any standards to determine the possible presence of any phenolic compound and was found to be clean. The calibration curves were created using the available standards, or if unavailable, the most similar standard as chemical structure. The standards used to generate the curves, with the analytes quantified with each one in brackets, are: apigenin (apigenin), *p*-coumaric acid (*p*-coumaric acid, *m*-coumaric acid), hydroxytyrosol (hydroxytyrosol, hydroxytyrosol acetate, tyrosol, hydroxytyrosol-*O*-glucoside, hydroxytyrosol lactone), luteolin (luteolin), oleacein (oleacein), oleocanthal (oleocanthal), oleuropein aglycone (oleuropein aglycone, ligstroside aglycone), oleuropein (hydroxyoleuropein aglycone, oleaceinic acid, elenolic acid, hydroxyelenolic acid) and pinoresinol (pinoresinol).

2.7. Volatile profile

The volatile profile was analyzed as described elsewhere (Olmo-Cunillera et al., 2022) following the procedure proposed by Casadei et al. (2021) for the preparation of the IS. The sample was placed in a 20 mL glass vial, closed with a polytetrafluoroethylene septum, and left to equilibrate for 10 min at 40 °C while being shaken. The sample was then subjected to solid-phase microextraction by exposing the fiber to the headspace for 40 min at 40 °C. The volatile fraction was analyzed by gas chromatography-mass spectrometry (GC–MS) (QP2010 Ultra, Shimadzu, Kyoto, Japan) with an autosampler (AOC-5000 plus, Shimadzu, Kyoto, Japan) and a polar-phase capillary column (TG-WAXMS: length 60 m, internal diameter 0.25 mm, and coating 0.50 μm; Thermo Fisher Scientific, Waltham, MA, USA). The exact details of the chromatographic analysis are provided in Olmo-Cunillera et al. (2022). The compounds were tentatively identified by comparing their mass spectra with those reported in the reference library of the instrumental software; the retention times of compounds were compared with those of pure standards, if available, to confirm the identification. The quantification of volatile compounds was carried out by the equation: (Aa / Ais) * Cis where Aa is the area of the analyte, Ais is the area of the IS, and Cis is the exact concentration of the IS. The results are expressed as the mean of three analytical replicates.

2.8. Sensory profile

The sensory analysis of the oils was carried out by the Catalonia Official Tasting Panel in accordance with the regulations established by the International Olive Council (International Olive Council, 2018).

More details about the sensory analysis are provided in Olmo-Cunillera et al. (2022).

2.9. Squalene and vitamin E

For the analysis of squalene and vitamin E (α-tocopherol), 200 μL of the sample was diluted with 800 μL of cyclohexane. The analysis was carried out in a UPLC coupled with a photo-diode array detector using an Aquity UPLC H class system (Waters Corporation®, Milford, MA, USA) equipped with an autosampler, degasser, column thermostat, binary pump, and diode array detector. The chromatographic column was an Atlantis® T3 (2.1 × 100 mm, i.d., 3 m particle size) (Waters Corporation®). The chromatographic and spectrophotometric parameters are detailed elsewhere (Olmo-Cunillera et al., 2021). The compounds were identified by comparing the retention time and the absorbance spectrum with those of the related pure standards. For the quantification, external calibration curves of the pure standards in cyclohexane were generated.

2.10. Chemometrics

For the data analysis, a multivariate statistics approach using chemometrics was applied. The raw dataset was employed for the analysis and exported to SIMCA software v13.0.3.0 (Umetrics, Sweden) or to Metaboanalyst 5.0 (https://www.metaboanalyst.ca/) (Pang et al., 2021). The dataset with the underlying data is provided in the **Supplementary Material 2**.

First, an unsupervised PCA was carried out using SIMCA. The data were auto-scaled using Unit-Variance (UV) scaling, with the formula: (value - mean) / standard deviation. The PCA clusters the samples without group information, which allows the most important factor to be identified: the storage time or the conditions of EVOO production (the RI of olives and malaxation temperature/time).

Then, to assess which variables changed the most during storage, a supervised analysis was carried out, specifically a partial least squares-discriminant analysis (PLS-DA), selecting storage time as the discriminant factor, with each storage time as a class, giving a total of 4 classes in the Y matrix. The X matrix was built with the concentrations of the compounds, except for the sensory analysis, in which the value is directly the value of the taste, which is the percentage of tasters that have recognized one sensory attribute. For this model, the data were scaled using UV scaling and the variables were transformed with a log transformation when needed, using the SIMCA auto-transform option. The list of the variables transformed are in supplementary material. The variables important in the projection (VIP) with a VIP score higher than 1.5 were selected as markers. For model validation, goodness-of-fit (R^2Y) and goodness-of-prediction (Q^2Y) were evaluated, accepting a minimum of Q^2Y prediction ability above 0.5. Hotelling's T2 was used to detect possible outliers, using 95% and 99% confidence limits for suspicious and strong outliers, respectively. In addition, possible outliers were also checked using the residuals normal probability plot, those plots are showed in **Supplementary material 3**. Also, cross-validation of the model was carried out, and ANOVA of cross-validated residuals was conducted to detect if the model was fitted by chance, accepting a *p*-value below 0.01. Finally, a permutation test with 200 permutations was conducted to exclude overfitting.

The same process was carried out to assess the effect of malaxation temperature, building the model with 3 classes for the Y matrix: 20, 25 and 30 °C. To examine the impact of the RI, EVOO samples were divided into two groups according to the RI of the olives used for their production: medium and low RI. With the median serving as the mid-point, samples equal to or above the median were designated as having a medium RI, and samples below were designated as having a low RI. In this study, the olives ranged from unripe to partially ripe, with an RI of 1.16 – 2.2 (median 1.44), hence the differences found would be those associated with early stages of maturation. A PLS-DA model using RI as the discriminant factor was built with the same conditions as the previous one, in this case with 2 classes for the Y matrix. Finally, as malaxation time could not be analyzed by a PLS-DA model due to less pronounced effects, an orthogonal-projection to latent structures discriminant analysis (OPLS-DA) was used to determine the markers of malaxation time. This approach uses orthogonal projection to minimize the variability in the descriptor matrix X that is not caused by the discriminant factor (Bylesjö et al., 2006), and therefore, if there is high variability inside the groups, a better separation is achieved compared to the PLS-DA model. For the model 2 classes were used in the Y matrix, 30 and 45 min.

To confirm the marker compounds in each case, *t*-Student or one-way ANOVA tests were used to determine significant differences between groups. To avoid a type I error, a false discovery rate was used, and the threshold was 0.05. *t*-Student was used for malaxation time and the RI, as these had only two groups. ANOVA was used for the other two factors studied, as they had more than two groups. The data were previously scaled to the tests by auto scaling (mean-centered and divided by the standard deviation of each variable). The analyses were carried out using Metaboanalyst 5.0 (Pang et al., 2021).

3. Results and discussion

During the 9-month storage period, the peroxide value, free acidity, spectrophotometric extinction coefficients, and sensory attributes were all within the limits of the European regulation established for EVOOs (European Commission, 2022).

3.1. Multivariate statistics

3.1.1. Unsupervised analysis

The PCA score plots are shown in Fig. 1**a**, colored and shaped by storage time, and in Fig. 1**b**, colored and shaped by RI (low: < 1.44 or medium: ≥ 1.44). The samples were clearly separated by storage time, and principal component (PC) 1 was mainly responsible for the separation. PC2 discriminated between samples obtained from olives with a low or medium RI. Among the successive PCs, PC4 corresponds to malaxation temperature, which did not clearly separate the samples, although a marked tendency was observed. The PCA score plot with PC3 and PC4, colored and shaped by malaxation temperature, is shown in Fig. 1**c**. In this model, PC1 was responsible for 25.8% of the variability, PC2 for 11.5%, and PC4 for 6.34%, indicating that the storage time, which corresponds to PC1, had a greater impact than the RI, PC2, or malaxation temperature, PC3, on EVOO composition. The different malaxation times did not seem to affect the EVOO composition. In our previous study on the effect of malaxation conditions, the RI was the main factor responsible for the variability between freshly produced EVOOs, followed by the malaxation temperature, whereas the malaxation time seemed to have little impact on EVOO composition (Olmo-Cunillera et al., 2021). Sinesio et al. (2015) also reported that different extraction methods (2 or 3-phase decanter) produced EVOOs with variable compositions, although the differences became less pronounced after storage.

3.1.2. Supervised analysis

After the unsupervised PCA, a supervised analysis with each studied parameter was performed. PLS-DA models were built using storage time, RI, and malaxation temperature as discriminant factors. In addition, as the effect of malaxation time was less pronounced, an OPLS-DA model was generated to fit the data and validated, 5 orthogonal components were used. The validation parameters are listed in Table 1. The results of the score plots are shown in Fig. 2 and the results of the validation model are provided in the **Supplementary Material 3**. The marker compounds for each factor are listed in Table 2 together with the VIP score, the *p*-value of the ANOVA or *t*-test, and the values of each variable for every level of the corresponding factor. The rest of VIP scores and p-values are listed in **Supplementary Material 3**.

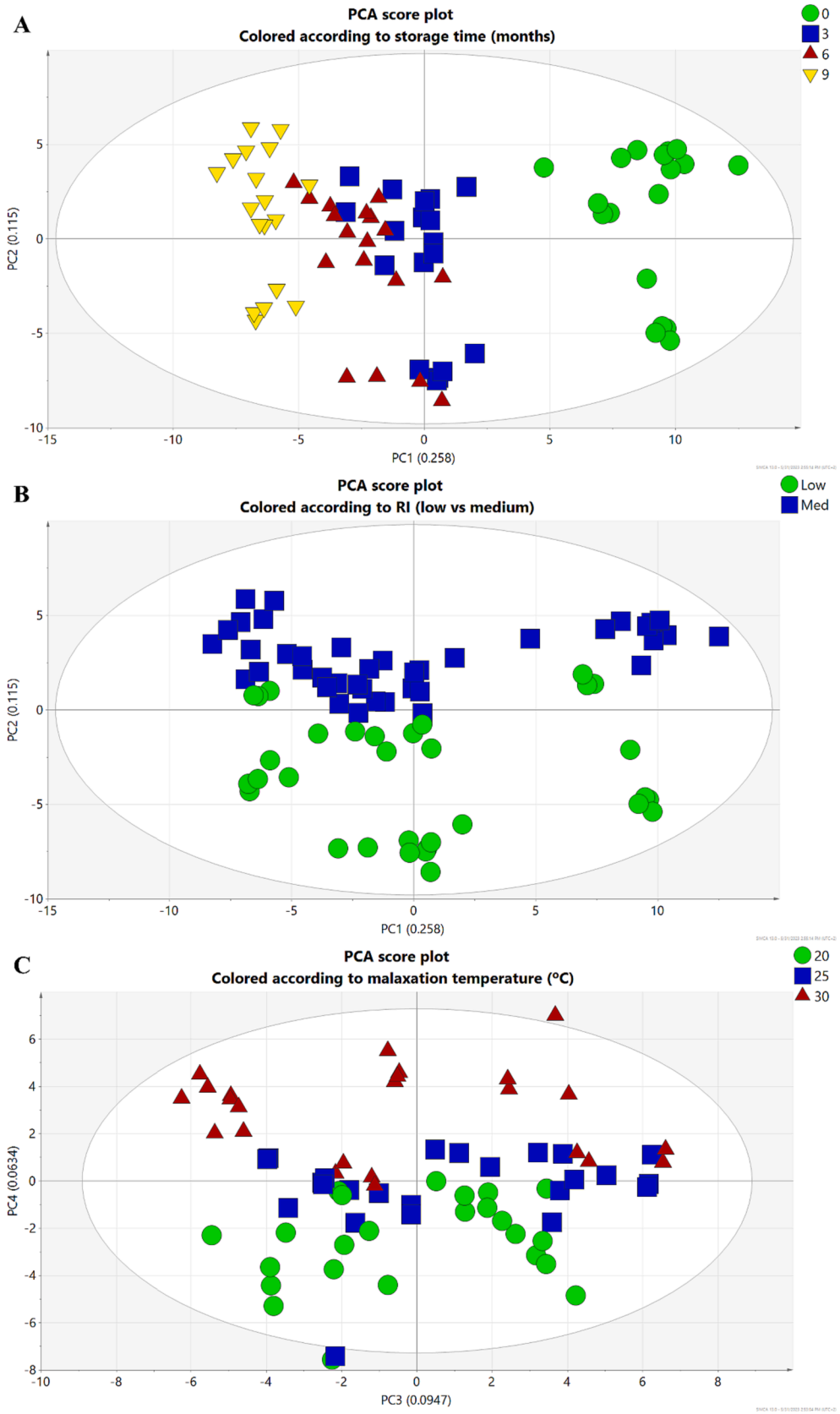

Fig. 1. PCA-score plot. A: PC1 and PC2, colored and shaped according to the different storage time (months). B: PC1 and PC2, colored and shaped according to the different RI low (<1.44) and medium (≥1.44). C: PC3 and PC4, colored and shaped according to the different malaxation temperatures (°C).

Table 1
Model validation parameters.

Model parameters					
Model type	**Factor**	**Class**	**R2Y (cum)**	**Q2 (cum)**	**p-value CV-ANOVA**
PLS-DA	storage time	Total	0.858	0.742	>1E-32
		0 months	0.961	0.954	
		3 months	0.780	0.570	
		6 months	0.748	0.515	
		9 months	0.942	0.928	
PLS-DA	RI	Total	0.929	0.851	3.47E-22
PLS-DA	malaxation temperature	Total	0.834	0.738	5.35E-27
		20 °C	0.887	0.819	
		25 °C	0.796	0.659	
		30 °C	0.818	0.722	
OPLS-DA	malaxation time	Total	0.927	0.611	2.23E-08

Model validation parameters for the 4 models. R2Y is the proportion of the variation of all the Y explained by the model and Q2 is the proportion of the variation of all the Y that can be predicted by the model. In the case of multi-class models, the data for each class is showed.

All the models clearly separated the groups of samples. In the case of storage time, three and six months were clustered together in the center, and time zero and nine months were separated at each extreme. This indicates that EVOO degradation occurred at the beginning of storage, then seemed to stabilize, before increasing again. During the storage, the period between 6 and 9 months was the hottest (average 23.3 °C) as it corresponded to the end of spring to mid-late summer. The higher temperatures could have contributed to speed the degradation of the oil.

3.2. Marker compounds

3.2.1. Storage

Phenolic compounds as well as the sensory attribute of ripe fruitiness, and two basic quality parameters (peroxide value and K_{270}) can be considered as storage markers as they had higher VIP-scores (>1.5), reflecting a high contribution to the model. Phenolic compounds were the most affected by the storage time, and could potentially be used to identify the state of the oil during shelf-life. Other studies have found that the total phenolic content and peroxide value are the parameters most affected by EVOO storage (Korifi et al., 2016). The sensory attribute of ripe fruitiness peaked at 6 months of storage. Recently, Díez-Betriu et al. (2023) observed a decrease in attributes related to green notes (the opposite of ripe) in EVOO during the first 6 months of storage at room temperature, followed by an increase at 9 months.

Among the phenolic compounds, a marker variable in the model was the group of phenyl ethyl alcohols and derivatives and its individual members, namely tyrosol, hydroxytyrosol and hydroxytyrosol acetate, which are formed by the hydrolysis of secoiridoids. Tyrosol is a degradation product of ligstroside, whereas hydroxytyrosol and its acetate are derived from oleuropein (Lozano-Castellón et al., 2020). During the early stages of storage, the concentration of these compounds increased until their rate of formation was overtaken by their degradation rate, when their concentration decreased. Similar results were reported by Valli et al. (2019), who compared the effect of storage on filtered and clarified EVOOs. In samples clarified with nitrogen, the tyrosol and hydroxytyrosol content increased for the first 8 months and then decreased drastically, whereas in unfiltered EVOOs, such as those in this study, their concentrations increased throughout the first year, probably because the activity of hydrolytic enzymes remained high enough to counteract their degradation.

Other phenolic storage markers found were oleaceinic acid, oleuropein aglycone acid, and hydroxytyrosol lactone, the ester between hydroxytyrosol and the lactone from elenolic acid, which are

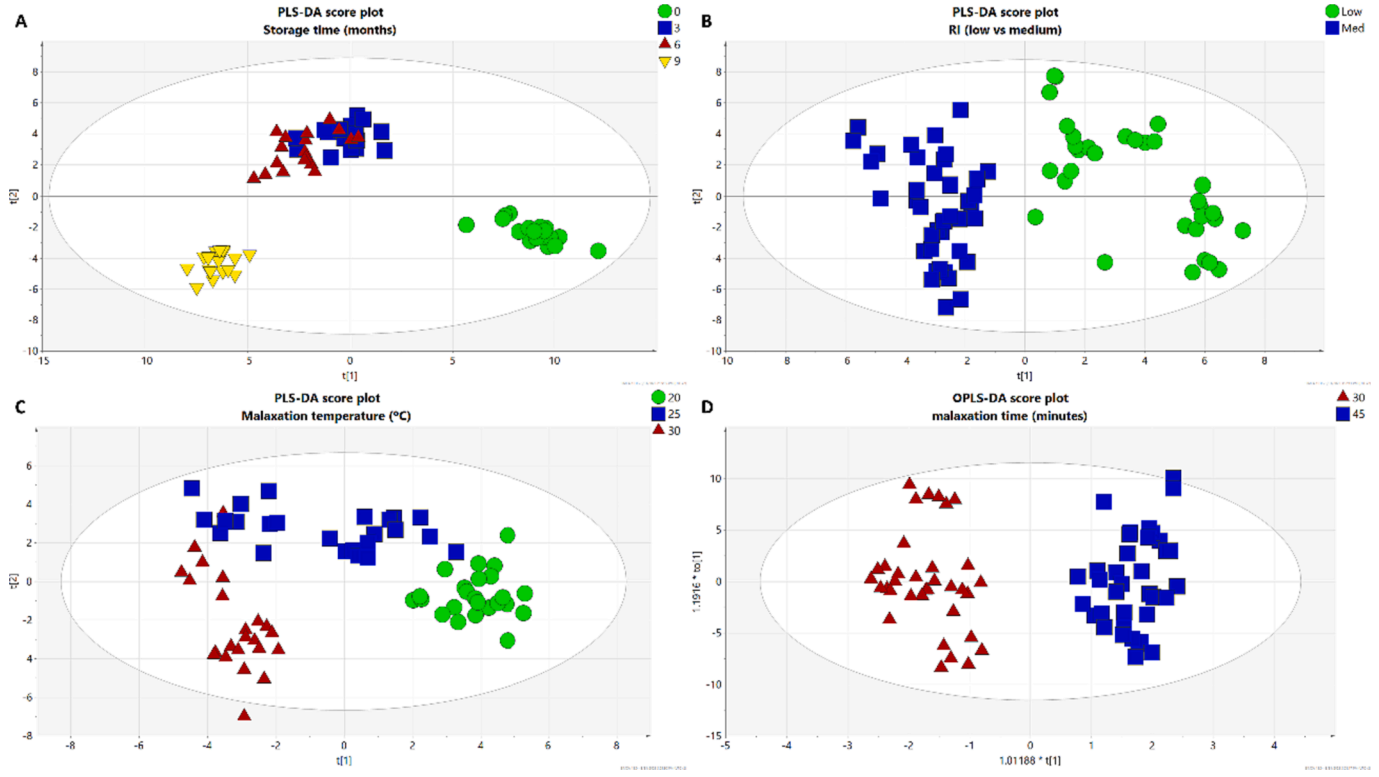

Fig. 2. A: PLS-DA-score plot for storage time (months). B: PLS-DA-score plot for Ripening index, low (<1.44) and medium (≥1.44). C: PLS-DA-score plot for malaxation temperature (°C). D: OPLS-DA-score plot for the malaxation time (minutes).

Table 2
Marker compounds of each studied factor.

Storage time				Mean (mg/kg of oil) ± standard deviation			
	Compound	VIP value	p-value	0 months	3 months	6 months	9 months
	Tyrosol	2.02068	2.16E-84	nd	1.20 ± 0.03	1.26 ± 0.04	nd
	Hydroxytyrosol	2.00732	2.83E-51	0.60 ± 0.12	1.31 ± 0.08	1.27 ± 0.10	nd
	Phenolic alcohols	1.94496	7.67E-44	4.1 ± 0.5	5.12 ± 0.10	5.14 ± 0.10	2.17 ± 0.10
	3,4-DHPEA-AC	1.8993	1.29E-32	2.17 ± 0.05	2.62 ± 0.07	2.61 ± 0.09	2.17 ± 0.10
	HOA II	1.81731	2.79E-37	1.37 ± 0.11	1.55 ± 0.11	1.43 ± 0.06	2.19 ± 0.10
	Lactone ester with OHTY	1.76137	4.75E-44	3.6 ± 0.4	4.2 ± 0.5	3.8 ± 0.6	18 ± 2
	Luteolin	1.69777	2.09E-39	1.55 ± 0.07	1.41 ± 0.07	1.45 ± 0.08	0.89 ± 0.06
	Peroxide value	1.6245	3.08E-29	4.77 ± 1.19	4.83 ± 1.1	9.44 ± 1.67	15 ± 2
	K_{270}	1.61781	1.36E-06	0.12 ± 0.03	0.12 ± 0.02	0.16 ± 0.02	0.16 ± 0.02
	HDCM-OA	1.58618	4.19E-37	1.18 ± 0.07	1.31 ± 0.07	1.42 ± 0.09	2.6 ± 0.3
	Ripe*	1.51474	0.000213	0	0	0.75	0
RI							
	Compound	VIP value	p-value	Low RI (<1.44)	Medium RI (>1.44)		
	C16:1 n-7	2.7595	3E-19	1.15 ± 0.1	1.50 ± 0.11		
	C18:1 n-9	2.36916	8.03E-12	70.0 ± 1.3	68.1 ± 0.4		
	C16:0	2.30088	6.22E-11	15.0 ± 0.5	15.8 ± 0.2		
	∑ C_5 LnA-Alc.	2.27514	1E-10	0.66 ± 0.08	0.51 ± 0.06		
	C20:0	2.22384	4.17E-10	0.392 ± 0.008	0.375 ± 0.010		
	Hexanal	2.20428	5.88E-10	0.42 ± 0.18	0.8 ± 0.2		
	∑ C_6 LA-Ald.	2.20428	5.88E-10	0.42 ± 0.18	0.8 ± 0.2		
	C18:2 n-6	2.14602	2.72E-09	9.79 ± 0.8	10.7 ± 0.3		
	C18:3 n-3	2.08643	1.77E-08	0.573 ± 0.011	0.54 ± 0.03		
	1-Penten-3-olo	2.03617	4.76E-08	0.37 ± 0.08	0.27 ± 0.05		
	1-Hexanol	1.96658	1.73E-07	0.21 ± 0.10	0.35 ± 0.08		
	C22:0	1.93015	3.09E-07	0.120 ± 0.002	0.116 ± 0.003		
	(*E*)-3-Hexen-1-ol	1.73073	3.18E-05	0.5 ± 0.3	0.9 ± 0.3		
	∑ C_6 LnA-Alc.	1.72987	2.55E-05	0.6 ± 0.3	1.1 ± 0.3		
	A-tocopheroltoco	1.70069	2.55E-05	181 ± 13	162 ± 17		
	(*E*,*E*)-2,4-Hexadienal	1.55505	0.000185	0.18 ± 0.06	0.24 ± 0.06		
	Pentanal	1.5196	0.000359	0.28 ± 0.13	0.18 ± 0.07		
	∑ C_5 LnA-Ald.	1.5196	0.000359	0.28 ± 0.13	0.18 ± 0.07		
	Apigenin	1.50885	0.03821	2.4 ± 0.3	2.2 ± 0.2		
Malaxation temperature							
	Compound	VIP value	p-value	MT 20 °C	MT 25 °C	MT 30 °C	
	Hexyl acetate	2.50062	3.17E-11	0.06 ± 0.02	0.11 ± 0.02	0.05 ± 0.03	
	∑ C_6 LA-Est.	2.50062	3.17E-11	0.06 ± 0.02	0.11 ± 0.02	0.05 ± 0.03	
	Hexanal	2.09625	5.12E-12	0.89 ± 0.17	0.5 ± 0.2	0.43 ± 0.13	
	∑ C_6 LA-Ald.	2.09625	5.12E-12	0.89 ± 0.17	0.5 ± 0.2	0.43 ± 0.13	
	C18:3 n-3	1.89582	2.19E-06	0.54 ± 0.03	0.54 ± 0.03	0.58 ± 0.01	
	∑ C_5 LnA-Alc.	1.89078	1E-08	0.48 ± 0.05	0.61 ± 0.11	0.65 ± 0.07	
	1-Penten-3-one	1.85833	5.5E-08	0.39 ± 0.08	0.51 ± 0.11	0.60 ± 0.12	
	∑ C_5 LnA-Ket.	1.85833	5.5E-08	0.39 ± 0.08	0.51 ± 0.11	0.60 ± 0.12	
	(*Z*)-3-Hexen-1-ol, acetate	1.78932	2.08E-06	0.29 ± 0.12	0.36 ± 0.13	0.17 ± 0.06	
	∑ C_6 LnA-Est.	1.78932	2.08E-06	0.29 ± 0.12	0.36 ± 0.13	0.17 ± 0.06	
	1-Penten-3-olo	1.70875	2.08E-06	0.24 ± 0.04	0.33 ± 0.09	0.36 ± 0.06	
	C16:1 n-9	1.62776	0.002535	0.135 ± 0.006	0.13 ± 0.003	0.135 ± 0.004	
	C17:1	1.62705	0.000304	0.31 ± 0.03	0.29 ± 0.02	0.28 ± 0.02	
	∑ C_6 LnA-Alc.	1.62135	9.61E-05	1.1 ± 0.3	0.6 ± 0.3	0.7 ± 0.3	
	(*E*)-3-Hexen-1-ol	1.62055	5.98E-05	1.0 ± 0.3	0.5 ± 0.3	0.6 ± 0.3	
Malaxation time							
	Compound	VIP value	p-value	Mt 30 min	Mt 45 min		
	Apigenin	3.2081	0.001631	2.2 ± 0.2	2.4 ± 0.2		
	Hexyl acetate	2.67998	0.019168	0.08 ± 0.04	0.06 ± 0.03		
	∑ C_6 LA-Est.	2.67998	0.019168	0.08 ± 0.04	0.06 ± 0.03		
	1-Hexanol	2.59889	0.023756	0.33 ± 0.12	0.24 ± 0.1		
	(*Z*)-3-Hexen-1-ol, acetate	1.90193	0.001631	0.33 ± 0.16	0.21 ± 0.06		
	∑ C_6 LnA-Est.	1.90193	0.001631	0.33 ± 0.16	0.21 ± 0.06		

* Values for ripe sensory attribute are expressed as the median for each storage time, as it is a discontinuous variable. Nd: not detected. 3,4-DHPEA-AC: 2-(3,4-Dihydroxyphenyl) ethyl acetate. HOA II: hydroxyoleuropein aglycone (isomer II), oleuropein aglycone acidic form. Lactone ester with OHTY: Lactone from Elenolic acid forming an ester with hydroxytyrosol. HDCM-OA: hydroxydecarboxymethyl oleuropein aglycone, oleaceinic acid. ∑ C_5 LnA-Alc: sum of the C_5 volatile alcohols derived from the α-linolenic acid. ∑ C_6 LA-Ald: sum of the C_6 volatile aldehydes derived from the linoleic acid. ∑ C_6 LnA-Alc: sum of the C_6 volatile alcohols derived from the α-linolenic acid. ∑ C_5 LnA-Ald: sum of the C_5 volatile aldehydes derived from the α-linolenic acid. ∑ C_6 LA-Est: sum of the C_6 volatile esters derived from the linoleic acid. ∑ C_5 LnA-Ket: sum of the C_5 volatile ketones derived from the α-linolenic acid. ∑ C_6 LnA-Est: sum of the C_6 volatile esters derived from the α-linolenic acid.

degradation products of oleuropein formed during storage. The acids of oleuropein and ligstroside aglycone were previously proposed as markers of EVOO oxidation during accelerated storage simulation, in which the oil was stored at 60 °C for 7 weeks in darkness (Lerma-García et al., 2009). Another marker of storage was the flavonoid luteolin, whose concentration decreased over time due to degradation.

Finally, two quality parameters, the peroxide value and K_{270}, were considered as marker compounds. The peroxide value reflects the initial primary autooxidation products, whereas the absorbance constant at 270 nm represents subsequent degradation products (Tarapoulouzi et al., 2022). These variables are therefore well-known to serve as markers of storage. The peroxide value and the K_{270} increased over time

but without exceeding the legal limit of the extra virgin category (20 mEq O_2/kg and K_{270} lower than 0.22, respectively). As other studies have shown, during prolonged storage, the peroxide value initially increases but finally decreases due to degradation (Li et al., 2014). The monitoring of K_{270} values is potentially a straightforward, quick and cheap option to track the deterioration of EVOO during non-heated storage, as also evidenced by the work of El Yamani et al. (2022).

In summary, certain phenolic compounds, as well as peroxide and K_{270} values were the primary markers of storage.

3.2.2. Ripening

The EVOOs analyzed in the present study were produced with *Arbequina* olives at early stages of maturation: either green olives with a RI of below 1.44 or green/partially spotted olives with a RI between 1.44 and 2.2. The marker compounds of the RI were fatty acids and volatile compounds, as well as α-tocopherol and the flavone apigenin. The percentage of fatty acids C16:1 n-7, C16:0, and C18:2 n-6 was found to increase with the RI, whereas C18:1 n-9, C18:3 n-3, C20:0, and C22:0 decreased. Yu et al. (2021) recently reported similar results for *Arbequina* EVOO, in which the percentage of C18:1 n-9, C18:3 n-3, and C22:0 decreased with ripeness and C16:1 n-7, C16:0, and C18:2 n-6 increased. The synthesis of fatty acids is active during the ripening process. Oleic acid is synthesized from acetyl-CoA by fatty acid synthase I and III (FAS I/III) followed by FAS II and stearoyl-ACP 9-desaturase (Caporaso, 2016), and then transformed into linoleic acid by oleate desaturase. In *Arbequina* olives, oleate desaturase may be more active than the previous enzymes, leading to a higher final concentration of linoleic acid compared to oleic acid.

Regarding the volatile compounds, C_5 compounds in the EVOO increased with the RI, while C_6 decreased. The C_5 marker compounds detected were 1-penten-3-ol and pentanal, and the C_6 tracers were hexanal, 1-hexanol, (*E*)-3-hexen-1-ol, and (*E*,*E*)-2,4-hexadienal. These volatile compounds are mainly related to positive attributes: soft green aromas are characteristic of 1-penten-3-ol, hexanal is related to green apple and grass aromas (Luna et al., 2006), and (*E*,*E*)-2,4-hexadienal is generally associated with ripe fruity sensory notes (Luna et al., 2006) although both hexanal and (*E*,*E*)-2,4-hexadienal are formed by oxidation (Vichi et al., 2003). All these C6 compounds, except for (*E*,*E*)-2,4-hexadienal), are derived from LOX pathway, in which linoleic and α-linolenic acids are converted to 13-hydroperoxide, whose derivatives can be cleaved by hydroperoxide lyase (HPL) to form C_6 aldehydes. Also, 13-hydroperoxide derived from α-linolenic acid can be degraded via an alkoxy radical to C_5 compounds (Angerosa et al., 2004). The activity of LOX and HPL during ripening is not constant (Padilla et al., 2010), increasing at the beginning and decreasing toward the end of the process. Linoleic acid and α-linolenic acids are transformed into 13-hydroperoxide during the early stages of ripening, when HPL is highly active, so there is a greater tendency for 13-hydroperoxide to be transformed into C_6 rather than C_5 volatile compounds. When the ripening process advances this tendency is reverted, the activity of HPL decreases and then the C_5 volatile compounds increase and the C_6 content is reduced (Padilla et al., 2010).

However, enzyme activity in olives can vary with the variety (Niu et al., 2022). For example, in the *Sayali* cultivar, oleic acid and linoleic acid were found to increase during ripening, whereas the concentration of C_6 volatile compounds in the EVOO seemed not to be affected by the RI of the olives (Nsir et al., 2017).

We found that α-tocopherol decreased in the EVOO produced from olives of medium RI. Previous studies have reported that vitamin E concentration in EVOO differs with the RI, diminishing or increasing according to the cultivar (Zhang et al., 2023). Finally, a decrease in apigenin was also associated with the medium RI. In previous studies by our group, an opposite trend was found in *Corbella* EVOO (López-Yerena et al., 2021). However, in the present study, the difference between the low and medium RI was minimum.

3.2.3. Malaxation temperature

The marker compounds of the malaxation temperature were volatile compounds and three fatty acids, namely C18:3 n-3, C16:1 n-9 and C17:1. The concentration change of those compounds between different samples is able to discern between the different levels of this factor (20, 25 and 30 °C). Other studies have shown that malaxation conditions can change the percentage of the fatty acids found in low concentration, such as C16:1 and C18:2 (Reboredo-Rodríguez et al., 2014).

Among volatile compounds, the markers of malaxation temperature were LOX-derived C_5 and C_6 compounds. During EVOO extraction, particularly during malaxation as this is the longest process, the enzymes in the olives react, affecting the profile of volatile and phenolic compounds. The activity of the enzymes depends on the malaxation temperature, resulting in different reactions and consequently a variable final composition. In the present study, the changes in the volatile profile were more meaningful than the differences in the phenolic profile, as no phenolic markers of the malaxation temperature were identified. Kalua et al. (2013), who used chemometrics to investigate the effect of each production step on the flavor-related compounds of EVOO (phenols and volatile compounds), concluded that the extraction procedure had a greater impact on the volatile compounds compared to the impact on phenolic compounds.

The concentration of C_5 compound markers, the sum of C_5 alcohols, 1-penten-3-one and 1-penten-3-ol, increased with the malaxation temperature, whereas the C_6 compounds hexanal and (*E*)-3-hexen-1-ol decreased. Furthermore, the concentration of hexyl acetate and (*Z*)-3-hexen-1-ol acetate reached a maximum at the intermediate temperature (25 °C). The highest temperature seemed to favor the transformation of α-linolenic acid-derived 13-hydroperoxyde to a pentene radical instead of the C_6 enzymatic pathway, which could explain the increase in 1-penten-3-one and 1-penten-3-ol at 30 °C. In contrast, at 20 °C, the C_6 enzymatic pathway seemed to be preferred. Moreover, at 25 °C the activity of enzymes from the early steps of the LOX pathway increased (Angerosa et al., 2004). Thus, at this temperature, early pathway C_6 compounds (hexanal and (*E*)-3-hexen-1-ol) were further transformed, and their concentration decreased, whereas the concentration of compounds produced in later steps of the LOX pathway (hexyl acetate and (*Z*)-3-hexen-1-ol acetate) increased.

In addition, as the temperatures applied were all low, all the marker compounds were related with positive attributes, except for (*E*, *E*)-2,4-hexadienal.

3.2.4. Malaxation time

The marker compounds of the malaxation time were the flavonoid apigenin and some C_6 volatile compounds. Indeed, the concentration of apigenin increased with the malaxation time. During malaxation, the precursor of apigenin (apigenin glucoside) present in olives can be transformed to apigenin by β-glucosidase (López-Yerena et al., 2021). Hence, increasing the time will lead to an increase in apigenin concentration as there is more time for the reaction to occur.

On the other hand, the content of volatile marker compounds from the LOX pathway, namely hexyl acetate, 1-hexanol and (*Z*)-3-hexen-1-ol acetate, decreased with longer malaxation due to enhanced enzymatic activity and degradation. Shorter malaxation times are optimal to obtain EVOOs with high amounts of volatile compounds related to pleasant aromas (Angerosa et al., 2001).

In summary, storage time substantially modified the phenolic profile and quality parameters of the EVOO. During this period, the oxidative status of the oil increased, with a loss of the main phenolic compounds and an increase in their oxidated forms such as oleacein and oleuropein aglycone acids. The peroxide value and the extinction coefficients at 232 and 270 nm also increased. On the other hand, the fatty acid profile of the EVOO was mainly affected by the maturity of the olives used for its production. Green *Arbequina* olives produced an EVOO richer in oleic acid, while olives picked after the first stages of ripening produced an oil with more linoleic acid and less oleic acid. Finally, the volatile

compounds were changed by storage, RI, and malaxation conditions, although they were not markers of storage time. During storage, the changes in the phenolic profile and quality parameters were so pronounced that alterations in the volatile profile were less important in the discriminant model. All the legal parameters of the oil samples evaluated in this study fell within the related limits for the extra virgin category (European Commission, 2022). In EVOO produced from olives at an early stage of ripening, the content of LOX-derived C_6 volatile compounds was higher than those produced from olives at more advanced stages, with lower concentrations of LOX-derived C_5 compounds. A higher malaxation temperature increased the content of C_5 compounds and decreased the C_6 compounds.

4. Conclusions

According to the results of this study, storage time had a higher impact than the RI and malaxation conditions on the herein analysed EVOO composition. Storage time corresponded to PC1 of the PCA, which is responsible of the 25.8% of variability between samples. Then, ripening index and malaxation temperature were related to PC2 and PC4 and explained the 11.5% and 6.34% of variability, respectively. Finally, malaxation time did not correspond to any PC, hence its impact on EVOO composition was minor.

EVOO degradation occurred mainly during the first months of storage, and then seemed to stabilize until the sixth month, when the degradation increased, coinciding with a seasonal increase in room temperature. The RI of the olives also had a high impact on EVOO composition, whereas the effect of the malaxation temperature was limited and that of the malaxation time seemed to be hidden by the effects of the other parameters. The best marker to assess the oxidative status of the EVOO during storage was found to be K_{270}, that can be easily exploited thanks to its simplicity of analysis. Volatile compounds and fatty acids were proper markers of ripeness, and the volatile profile was highly affected by malaxation temperature.

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CRediT authorship contribution statement

Julián Lozano-Castellón: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Alexandra Olmo-Cunillera:** Methodology, Validation, Investigation, Data curation, Writing – review & editing. **Enrico Casadei:** Methodology, Validation, Investigation, Data curation, Writing – review & editing. **Enrico Valli:** Methodology, Validation, Investigation, Data curation, Writing – review & editing. **Inés Domínguez-López:** Validation, Investigation, Data curation, Writing – review & editing. **Eleftherios Miliarakis:** Validation, Investigation, Data curation. **Maria Pérez:** Methodology, Supervision, Writing – review & editing. **Antònia Ninot:** Methodology, Validation, Investigation, Data curation, Writing – review & editing. **Agustí Romero-Aroca:** Methodology, Validation, Investigation, Resources, Data curation, Writing – review & editing, Supervision. **Alessandra Bendini:** Methodology, Validation, Resources, Data curation, Writing – review & editing, Supervision. **Rosa M. Lamuela-Raventós:** Methodology, Validation, Resources, Writing – review & editing, Project administration. **Anna Vallverdú-Queralt:** Methodology, Validation, Resources, Writing – review & editing, Project administration.

Declaration of Competing Interest

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

Data availability

The data is provided in the supplementary file 2

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137539.

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