1 # Oleacein and oleocanthal: key metabolites in the stability of 'Corbella'

2 # extra virgin olive oils

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17

18 **Abstract**

19 'Corbella' extra virgin olive oil (EVOO) produced from olives harvested at the reddish to black

20 ripening stage is unstable and easily degraded. The oxidative stability of EVOO depends on its

21 composition, primarily, phenolic compounds and tocopherols which are strong antioxidants, but

22 also carotenoids, squalene, and fatty acids contribute. In this study, with the aim of obtaining

23 more stable oils, the effect of malaxation conditions and olive storage on the composition of

24 'Corbella' EVOO produced in an industrial mill was evaluated. Although a longer malaxation

25 time at a higher temperature and olive storage had a negative effect on the total content of

26 antioxidant components, the antioxidant capacity and oxidative stability of the oil were improved

27 because of a higher concentration of oleacein and oleocanthal. Therefore, for better stability and
28 a longer shelf-life, the content of oleacein and oleocanthal in ‘Corbella’ EVOOs should be
29 enhanced.

30 **Keywords**

31 *Olea Europaea*; oxidation; polyphenols; high-quality; oleic/linoleic; multivariate analysis

32 1. Introduction

33 The cultivation of ‘Corbella’ olives, an ancient cultivar from Catalonia (Spain), has been revived
34 for the production of extra virgin olive oil (EVOO) [1]. Nevertheless, when ‘Corbella” olives are
35 harvested at the reddish to black ripening stage, the resulting oil is unstable and easily degraded.
36 Therefore, there is a need to study the production process to shed light on how the stability of
37 ´Corbella¨ EVOO may be improved to preserve its qualities and extend its shelf life.

38 A serious problem affecting edible oils is lipid oxidation, a major cause of deterioration of
39 chemical, sensory, and nutritional properties. EVOO is highly resistant to oxidative degradation,
40 due to a low content of polyunsaturated fatty acids (PUFAs) and high levels of monounsaturated
41 fatty acids (MUFAs), as well as the presence of phenolic compounds and tocopherols [2].
42 Nevertheless, the variable composition of EVOOs means their resistance to oxidative
43 deterioration also differs. For example, the oils produced from ‘Picual’ olives have a higher
44 oxidative stability than those made from ‘Arbequina’ or ‘Hojiblanca’ cultivars, because they have
45 a lower percentage of linoleic acid and a high phenolic content [3–5].

46 The main factors affecting the fatty acid (FA) profile and triacylglycerol composition of EVOO
47 are the climate in which the olives are cultivated, their cultivar, and stage of maturity when
48 harvested [2]. Parameters of interest are the ratios of MUFA/PUFA and oleic/linoleic acids, which
49 give information about the oxidative stability and rancidity of the oils [6]: the higher the values, the
50 more stable and less rancid they are. The two ratios are correlated, as oleic acid is the main MUFA
51 and linoleic acid the principal PUFA in olive oil. As the autoxidative stability of oleic acid is 10-
52 fold higher than that of linoleic acid [7], olive oils with high oleic and low linoleic acid content are

better from both a nutritional and technological standpoint. Accordingly, the generation of new olive cultivars producing oils with a high oleic/linoleic ratio is a priority in olive breeding programs [6].

The minor unsaponifiable fraction of EVOO contains two main groups of compounds that act as primary inhibitors of oxidation: phenolic compounds and tocopherols. Phenolic compounds are hydrophilic antioxidants only found in olive oils if they are virgin, as they are lost during the refining process. The highest contributors to oxidative stability in EVOO are *o*-diphenols such as hydroxytyrosol and its oleoside forms (oleuropein, oleuropein aglycone and oleacein) [2]. Tocopherols are lipophilic antioxidants that reduce lipid oxidation as well as photooxidation [2]. The major tocopherol in olive oil is *α*-tocopherol, with *β*- and *γ*-tocopherol found in minor amounts. Additionally, a synergistic antioxidant effect exerted by *α*-tocopherol and phenolic compounds was observed [8]. The major constituent of the unsaponifiable fraction in olive oil is squalene, which has a lower antioxidant activity compared to phenolic compounds and *α*-tocopherol. It acts at low or moderate temperatures, and in combination with *α*-tocopherol and phenolic compounds [2].

Chlorophylls and carotenoids are the pigments responsible for the color of olive oil [9]. In the presence of light, chlorophylls and their derivatives are the most active promoters of photosensitized oxidation in EVOO, contributing greatly to its susceptibility to oxidation [10]. Nevertheless, they show antioxidant effects in the dark [11]. In contrast, carotenoids, especially *β*-carotene, are strong protectors against photosensitized oxidation, acting as singlet oxygen quenchers [10].

Among the principal factors affecting EVOO composition are the cultivar, ripeness, and health of the olive fruits, agroclimatic conditions, the production process, including crushing, malaxation, extraction and filtering, and storage [2]. Maximizing the concentrations of antioxidant components will ensure an oil with higher stability. As the ripening index (RI) of the olives increases, their phenolic content decreases, resulting in oils with lower oxidative stability [4,12–14]; likewise,

79 chlorophylls and carotenoids decrease drastically, while the PUFA levels increase [4,13,14].
80 Furthermore, the storage of olives before oil production increases hydrolytic and oxidative
81 degradation, leading to a depletion in the content of phenolic compounds, tocopherols, and
82 carotenoids, therefore impairing the oil stability, especially when storage is prolonged [15].

83 In a previous pilot study using an ABENCOR system (Abengoa S.A., Seville, Spain), the effect
84 of the RI and malaxation conditions on the phenolic content of 'Corbella' EVOOs was evaluated
85 [12]. Additionally, a targeted metabolic profiling of this ancient olive cultivar was conducted to
86 determine the composition of olives at an early maturation stage [1]. As a continuation of this
87 research, with the aim of improving oil stability and shelf life, the present study analyzed
88 'Corbella' EVOOs produced in an industrial mill under different malaxation conditions using
89 olives of a similar RI (1 to 1.5). The effect of storing the olives for 17 h at ambient temperature
90 on the EVOO composition and oxidative stability was also evaluated.

91 2. Material and methods

92 2.1. Reagents

93 *n*-Hexane, 0.5 N sodium methoxide, 14% boron trifluoride–methanol, Trolox, diphenyl-1-picryl-
94 hydrazyl (DPPH), and Folin–Ciocalteu's reagent were purchased from Sigma-Aldrich (St. Louis,
95 MO, USA); acetic acid, formic acid, methanol, acetonitrile (ACN), N,N-dimethylformamide
96 (DMF), and tertbutylmethylether (TBME) from Sigma-Aldrich (Madrid, Spain); and sodium
97 chloride (NaCl) and sodium carbonate (Na_2CO_3) from Panreac Química SLU (Castellar del
98 Vallès, Spain). Ultrapure water was obtained using a Milli-Q purification system (Millipore,
99 Bedford, MA, USA).

100 Regarding the standards (≥90% purity), oleocanthal was purchased from Merck (Darmstadt,
101 Germany), and oleacein, oleuropein aglycone, and elenolic acid from Toronto Research Chemical
102 Inc. (ON, Canada). Oleuropein, ligstroside, pinoresinol, gallic acid, vanillic acid, caffeic acid,
103 verbascoside, rutin, lutein, *β*-carotene, squalene, and (α)-tocopherol were acquired from Sigma-
104 Aldrich. Apigenin, ferulic acid and *p*-coumaric were obtained from Fluka, and hydroxytyrosol

105 from Extrasynthese (Genay, France). Methyl tridecanoate (C13:0) was used as a standard for the
106 analysis of FAs and was acquired from Sigma-Aldrich.

107 2.2. Samples

108 The 'Corbella' olive samples were all collected on October 13, 2021. Information about the
109 orchard and the environmental and agronomical conditions are detailed elsewhere [1]. Before the
110 oil production, the olives were washed with water. The olives were crushed using a 5 mm sieve,
111 and the water addition was 10 L/h. The EVOOs were produced in an industrial mill (OLIOMIO
112 200 PROFY, MORI-TEM) by the company MIGJORN (Navàs, Catalonia, Spain) on two
113 consecutive days, October 13 and 14, 2021. The tested variables were temperature (18 and 23 °C)
114 and time (30, 40 and 50 min) of malaxation.

115 Six different EVOOs were produced with the same olive sample on the two days. O1, O2 and O3
116 were produced on October 13 and O4, O5 and O6 were produced the following day. The olives
117 used for the elaboration of O4 – O6 were stored in a tractor trailer at ambient temperature (from
118 14 to 21 °C) for 17 h. To check whether olive storage could have altered the results, O4 was
119 produced using the same malaxation conditions as O1. The EVOO samples were stored at −20
120 °C until the chemical analyses.

121 2.3. Physical characterization of the olives

122 The physical characterization of olives was carried out by the IRTA (Mas Bové) on the same day
123 as the EVOO production, i.e., the characterization was performed twice, on October 13 and 14.
124 The RI was evaluated following the methodology described in Olmo-Cunillera et al. (2023) [1]. The
125 weight of the olives was measured by gravimetric analysis. Additionally, a visual inspection was
126 carried out to determine the condition of the olive samples.

127 2.4. Phenolic extraction and profiling

128 The phenolic compounds underwent liquid-liquid extraction as described in [16]. The quantification
129 was carried out by liquid chromatography coupled to mass spectrometry in tandem mode (LC-

130 MS/MS) following the methodology also described in Olmo-Cunillera et al. (2021) [16]. An Acquity
131 TM UPLC (Waters; Milford, MA, USA) coupled to an API 3000 triple-quadrupole mass
132 spectrometer (PE Sciex, Concord, ON, Canada) with a turbo ion spray source was used. The
133 column and precolumn were an Acquity UPLC® BEH C18 column (2.1 × 50 mm, i.d., 1.7 µm
134 particle size) and Acquity UPLC® BEH C18 Pre-Column (2.1 × 5 mm, i.d., 1.7 µm particle size)
135 (Waters Corporation®, Wexford, Ireland), respectively.

136 The quantification was done with an external calibration curve using refined olive oil with the
137 following standards: apigenin, hydroxytyrosol, *p*-coumaric acid, pinoresinol, oleuropein,
138 ligstroside, oleocanthal, oleacein, oleuropein aglycone, and elenolic acid. Compounds without a
139 corresponding commercial standard were quantified using a phenolic standard with a similar
140 chemical structure.

141 2.5. Fatty acid extraction and profiling

142 FAs were extracted using the method for FA methyl esters (FAME) described in Olmo-Cunillera
143 et al. (2022) [17] with a few modifications. 25 mg of oil was weighed in a 10 mL tube and 40 µL of
144 the internal standard (methyl tridecanoate, C13) was added at 1000 mg/L. Firstly, after the
145 addition of 2 mL of 0.5 N sodium methoxide, the solution was stirred for 30 s and immediately
146 heated at 100 °C for 15 min. The samples were then cooled in an ice bath. Secondly, 2 mL of 14%
147 boron trifluoride was added to the samples, and the solution was again stirred for 30 s and heated
148 at 100 °C for 15 min, before cooling in an ice bath. Thirdly, 1 mL of hexane was added to the
149 samples, and the solution was stirred for 1 min. After the incorporation of 2 mL of saturated NaCl,
150 the samples were stirred again for 30 s. Finally, the samples were centrifuged at 3000 rpm for 7
151 min, and 250 µL of the hexane phase was collected with a micropipette and stored in vials at −20
152 °C until analyzed.

153 Fast GC analyses were performed on a Shimadzu GC-2010 Gas Chromatograph (Shimadzu,
154 Kyoto, Japan) equipped with a flame ionization detector and a Shimadzu AOC-20i Autoinjector.
155 Separation of fatty acid methyl esters was carried out on a capillary column (40 cm × 0.18 mm

156 i.d. x 0.1 μm film thickness) coated with an RTX-2330 stationary phase of 10% cyanopropyl
157 phenyl - 90% biscyanopropyl polysiloxane from Restek (Bellefonte, USA). Operating conditions
158 are described in Olmo-Cunillera et al. (2022) [17].

159 The concentration of each FA was calculated considering the area and concentration of the
160 internal standard, applying the following equation,

161 $(A_i \times C_{IS}) / (A_{IS} \times M_S)$, (1)

162 where A_i is the area of the FA; C_{IS}, the concentration of the internal standard; A_{IS}, the area of the
163 internal standard, and M_S, the mass of the sample. The percentage of composition was calculated
164 by dividing the area of the FA between the area of the sum of all identified FAs and multiplying
165 by 100.

166 2.6. Determination of carotenoids, chlorophylls, *α*-tocopherol, and squalene

167 The determination of the carotenoids (lutein and *β*-carotene), chlorophylls, *α*-tocopherol (vitamin
168 E) and squalene was done with a 200:800 (v/v) (EVOO:TBME) dilution in amber vials and
169 performed by LC [16]. An Acquity TM UPLC coupled to a photodiode detector (PDA) (Waters
170 Corporation®; Milford, MA, USA) was used. The column was a YMCTM C30 (250 × 4.6 mm,
171 i.d., 5 μm particle size) (Waters Corporation®, Milford, MA, USA). The mobile phases were
172 TBME:methanol (8:2 v/v) (A) and methanol (B). An increasing linear gradient (v/v) of A was
173 used (t (min), %A) as follows: (0, 10); (10, 25); (20, 50); (25, 70); (35, 90); (43, 94); (45, 19);
174 (55, 10). The method had a constant flow rate of 0.6 mL/min, and an injection volume of 10 μL.
175 The absorbance was measured at 450 nm for carotenoids (lutein and *β*-carotene) and at 210 nm
176 for *α*-tocopherol and squalene.

177 For the quantification of each compound, an external calibration curve of the corresponding
178 commercial standard was employed (lutein, *β*-carotene, chlorophyll, *α*-tocopherol, and squalene).

179 2.7. Extraction and determination of the antioxidant capacity (DPPH free radical scavenging
180 assay) and oxidative stability (Rancimat)

181 The extraction method for the DPPH assay was as follows. A sample of 0.5 g of EVOO was
182 dissolved in 1 mL of hexane in a 10 mL centrifuge tube and shaken for 30 s. A total of 2 mL of
183 methanol:H_2O (8:2) was added, and the samples were shaken again for 30 s. Afterwards, the two
184 phases were separated using centrifugation at 3000 rpm and 4 °C for 4 min. The methanolic
185 fraction was collected in another centrifuge tube and underwent a second cleaning with 1 mL of
186 hexane, whereas the hexane fraction was again treated with 2 mL of methanol:H_2O (8:2) to
187 recover the remaining phenolic compounds. All tubes were shaken for 30 s and centrifuged at
188 3000 rpm and 4 °C for 4 min. The methanolic phases were recovered together and stored at −20
189 °C until the TPC and DPPH analysis.

190 The DPPH radical scavenging activity assay was performed based on the reduction of the DPPH•
191 radical by antioxidants, as described in Olmo-Cunillera et al. (2023) [1] . Results were expressed as
192 µg of Trolox equivalents (TE) per g of oil for DPPH. Trolox was used as the standard to prepare
193 a calibration curve for DPPH (linearity range: 5–100 µg/mL, R^2 >0.927).

194 The oxidative stability was evaluated with the Rancimat method [18]. This technique measures the
195 oxidative stability of oils and fats in accelerated conditions and is based on the induction of sample
196 oxidation by exposure to high temperatures and air flow. Therefore, the longer the induction time,
197 the more stable the sample. A mass of 3 g of EVOO sample was heated at 120 °C with a constant
198 air flow of 20 L/h. The results were expressed as the induction time of oxidation (in hours),
199 measured with the Rancimat 743 apparatus (Metrohm Co., Basilea, Suiza). The induction time of
200 oxidation is the time required to cause a sudden change in the conductivity of an aqueous solution
201 where the volatile compounds resulting from the oil oxidation are collected.

202 2.8. Statistical analysis and multivariate analysis

203 All the analyses were done in triplicate. Statgraphics Centurion 18 software, version 18.1.13 and
204 RStudio, version 2022.12.0 Build 353 (R Project for Statistical Computing version 4.2.2) were
205 used to perform the analysis of variance. First, the normality of data and the homogeneity of
206 variance were tested by the Saphiro-Wilk test and Levene's test, respectively. An analysis of

207 variance of two factors (two-way ANOVA) with a Tukey test was applied to evaluate the effect
208 of the malaxation conditions on the oil samples O1, O2, O3, O5 and O6 when the assumptions of
209 normality and homogeneity of variance were met ($p \geq 0.05$). If any of these assumptions were not
210 met ($p < 0.05$), a nonparametric statistical test was applied (Kruskal-Wallis with a pairwise Mann–
211 Whitney U as a post-hoc test). To evaluate the effect of the olive storage time in the tractor trailer
212 on the EVOO samples O1 and O4, a one-way ANOVA with Tukey test was used when the
213 assumptions of normality and homogeneity of variance were met ($p \geq 0.05$). If any of these
214 assumptions were not met ($p < 0.05$), a nonparametric statistical test was applied (Kruskal-Wallis
215 with Bonferroni correction). In addition, a two-way ANOVA was performed to determine
216 possible interactions between the malaxation factors (temperature and time).

217 For the multivariate analysis, the software used was SIMCA 13.0.3.0 (Umetrics, Umeå, Sweden).
218 All the composition data (content of phenolic compounds, FAs, carotenoids, chlorophylls, α-
219 tocopherol, and squalene) as well as the Rancimat and DPPH data were included. An
220 unsupervised approach, specifically a principal component analysis (PCA), was performed. The
221 data were standardized with UV-scaling and mean-centering.

222 3. Results and discussion

223 3.1. Physical characterization of the olives

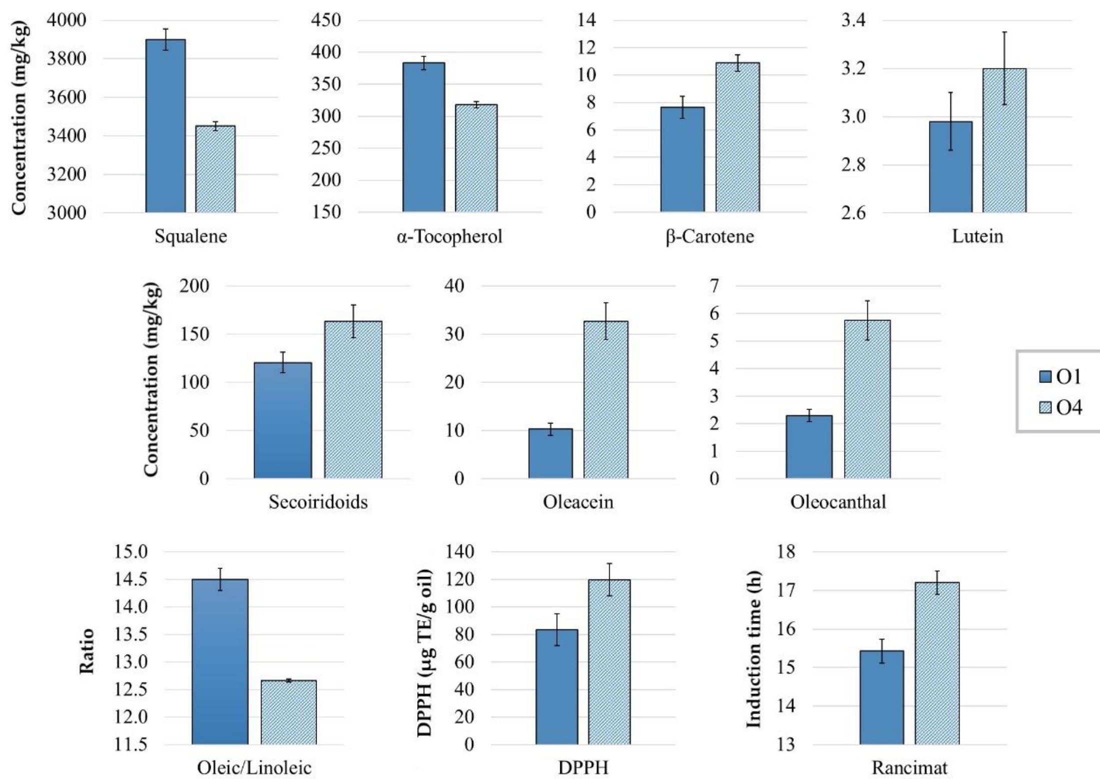
224 The olive samples used to produce EVOO on either of the two days of production had very similar
225 physical characteristics. The RI of the olives processed on October 13 and 14 was 1.14 ± 0.11 and
226 1.20 ± 0.05, and the weight 1.83 ± 0.23 g and 1.80 ± 0.17 g, respectively. Overall, all the samples
227 were in good condition, although some olives had suffered minor damage due to the harvesting
228 machine employed. The damage was a bit more noticeable after 17 h of storage.

229 3.2. Effect of olive storage on EVOO composition and oxidative stability

230 The EVOO samples O1 and O4 were produced under the same malaxation conditions (18 °C and
231 30 min) but on different days. O1 was produced on the same day the olives were harvested and

232 O4 the following day, after the olives had been stored for 17 h in a tractor trailer at ambient
233 temperature.

234 The olive storage had a negative effect on the content of α-tocopherol and squalene (Figure 1), a
235 positive effect on the secoiridoid content, and no effect on the total FAs (Table S1), in agreement
236 with a previous report [15]. These changes can be expected, as olive storage enhances the activity
237 of hydrolytic and oxidative enzymes [19]. Additionally, carotenoids (lutein and β-carotene)
238 increased (Figure 1), whereas chlorophyll levels were unaltered (Table 1).



239

240 **Figure 1.** Concentration (mg/kg oil) of squalene, α-tocopherol, β-carotene, lutein, secoiridoids, oleacein,
241 and oleocanthal in the EVOO samples O1 and O4, as well as the oleic/linoleic ratio, antioxidant capacity
242 by DPPH (µg TE/g oil), and oxidative stability by Rancimat (induction time (h)). O1 was produced on the
243 day the olives were harvested, and O4 on the day after harvesting with stored olives. Both EVOOs were
244 malaxed at 18 °C for 30 min. Results are expressed as mean ± standard deviation, n = 9. All variables
245 differed significantly ($p < 0.05$) between samples.

246 **Table 1.** Concentration of phenolic compounds (mg/kg), carotenoids (lutein and β-carotene), chlorophylls,
247 α-tocopherol (vitamin E), and squalene (mg/kg), and antioxidant capacity (DPPH) (µmg TE/g olive fruit),
248 and oxidative stability (Rancimat (h)) of the EVOO samples. All results are expressed as mean ± standard
249 deviation, n = 9. Different letters/numbers mean significant differences ($p < 0.05$) between samples, with
250 increasing letters/numbers indicating increasing values. Letters are used for the malaxation study, and
251 numbers for the olive storage study.

Sample ID	O1	O2	O3	O4	O5	O6
Production date	13/10/2021	13/10/2021	13/10/2021	14/10/2021	14/10/2021	14/10/2021

Malaxation temperature (°C)	18	18	18	18	23	23
Malaxation time (min)	30	40	50	30	30	40
Phenolic compounds (mg/kg)						
Sum of phenolics	165.90 ± 18.31 [a,1]	163.81 ± 16.06 [a]	191.21 ± 9.11 [a]	180.87 ± 17.26 [1]	174.77 ± 25.67 [a]	169.10 ± 3.50 [a]
Secoiridoids	120.68 ± 10.80 [a,1]	134.97 ± 18.74 [ab]	170.82 ± 7.49 [c]	163.40 ± 16.91 [2]	157.60 ± 23.08 [bc]	152.82 ± 3.69 [bc]
Ligstroside aglycone	11.83 ± 1.72 [a,1]	12.94 ± 1.89 [a]	13.45 ± 1.51 [a]	12.60 ± 1.57 [1]	12.01 ± 1.56 [a]	11.50 ± 0.75 [a]
Oleuropein aglycone	82.72 ± 7.47 [a,1]	87.52 ± 12.49 [ab]	105.92 ± 8.59 [bc]	103.75 ± 12.05 [2]	109.39 ± 18.27 [c]	91.59 ± 3.84 [abc]
Oleocanthal	2.30 ± 0.22 [a,1]	3.92 ± 0.33 [b]	6.90 ± 0.37 [d]	5.76 ± 0.71 [2]	3.97 ± 0.22 [b]	5.64 ± 0.47 [c]
Oleacein	10.31 ± 1.23 [a,1]	16.14 ± 1.51 [b]	36.05 ± 2.95 [d]	32.72 ± 3.78 [2]	23.22 ± 2.21 [c]	35.53 ± 2.12 [d]
Hydroxyelenolic acid	9.72 ± 1.03 [b,2]	5.70 ± 0.48 [a]	5.54 ± 0.76 [a]	5.34 ± 0.77 [1]	5.37 ± 0.62 [a]	5.38 ± 1.08 [a]
Oleocanthalic acid	1.18 ± 0.07 [b,1]	0.94 ± 0.09 [a]	1.18 ± 0.09 [b]	1.15 ± 0.08 [1]	0.93 ± 0.07 [a]	1.08 ± 0.08 [ab]
Hydroxyoleuropein aglycone	2.90 ± 0.26 [c,2]	1.98 ± 0.17 [a]	1.74 ± 0.05 [a]	1.76 ± 0.13 [1]	1.86 ± 0.12 [a]	1.77 ± 0.18 [a]
*Secoiridoid derivatives**						
Elenolic acid	552.70 ± 48.29 [d,2]	298.86 ± 35.16 [c]	225.64 ± 25.36 [ab]	205.00 ± 13.83 [1]	275.12 ± 20.61 [bc]	195.50 ± 28.83 [a]
Phenolic alcohols	5.96 ± 0.73 [c,2]	4.96 ± 0.26 [b]	4.62 ± 0.45 [b]	3.68 ± 0.67 [1]	4.13 ± 0.53 [ab]	3.33 ± 0.24 [a]
Hydroxytyrosol	2.97 ± 0.43 [b,2]	2.38 ± 0.36 [ab]	3.01 ± 0.37 [b]	2.17 ± 0.35 [1]	2.57 ± 0.36 [b]	1.86 ± 0.19 [a]
Hydroxytyrosol acetate	2.99 ± 0.32 [b,2]	2.58 ± 0.32 [b]	1.67 ± 0.21 [a]	1.53 ± 0.22 [1]	1.55 ± 0.18 [a]	1.47 ± 0.08 [a]
Flavonoids	3.78 ± 0.47 [c,2]	3.15 ± 0.32 [b]	2.60 ± 0.04 [a]	2.49 ± 0.05 [1]	2.49 ± 0.13 [a]	2.45 ± 0.14 [a]
Apigenin	2.43 ± 0.31 [c,2]	2.01 ± 0.24 [b]	1.49 ± 0.04 [a]	1.39 ± 0.04 [1]	1.37 ± 0.08 [a]	1.37 ± 0.12 [a]
Luteolin	1.45 ± 0.16 [c,2]	1.23 ± 0.03 [b]	1.11 ± 0.01 [ab]	1.10 ± 0.03 [1]	1.12 ± 0.06 [ab]	1.08 ± 0.02 [a]
Phenolic acids						
p-Coumaric acid	1.33 ± 0.03 [c,2]	1.27 ± 0.02 [b]	1.23 ± 0.03 [ab]	1.27 ± 0.03 [1]	1.28 ± 0.03 [b]	1.21 ± 0.03 [a]
Lignans						
Pinoresinol	29.52 ± 2.91 [c,2]	19.25 ± 2.15 [b]	11.94 ± 1.61 [a]	9.85 ± 0.44 [1]	9.46 ± 1.23 [a]	8.08 ± 1.20 [a]
DPPH (μg TE/g oil)	83.47 ± 11.66 [ab,1]	77.20 ± 7.60 [a]	114.63 ± 5.91 [c]	119.81 ± 11.59 [2]	117.08 ± 12.03 [c]	102.02 ± 10.35 [bc]
Rancimat (h)	15.43 ± 0.34 [a,1]	15.83 ± 0.15 [a]	18.72 ± 0.29 [d]	17.20 ± 0.17 [2]	16.39 ± 0.05 [b]	16.97 ± 0.25 [c]
Carotenoids, chlorophylls, α-tocopherol, and squalene (mg/kg)						
Lutein	2.98 ± 0.12 [ab,1]	2.94 ± 0.12 [a]	3.44 ± 0.16 [c]	3.20 ± 0.15 [2]	2.87 ± 0.13 [a]	3.16 ± 0.13 [b]
β-Carotene	7.66 ± 0.81 [a,1]	9.55 ± 0.65 [b]	12.02 ± 0.58 [c]	10.88 ± 0.59 [2]	7.08 ± 0.35 [a]	9.92 ± 0.36 [b]
Chlorophylls	3.51 ± 0.63 [c,1]	4.24 ± 0.41 [d]	5.50 ± 0.38 [e]	3.06 ± 0.23 [1]	1.77 ± 0.10 [a]	2.62 ± 0.14 [b]
α-Tocopherol	383.05 ± 10.51 [b,2]	335.74 ± 11.03 [a]	312.97 ± 4.95 [a]	317.86 ± 5.05 [1]	316.49 ± 31.73 [a]	321.29 ± 5.59 [a]
Squalene	3900.06 ± 54.48 [d,2]	3555.40 ± 43.18 [c]	3535.57 ± 41.82 [c]	3451.40 ± 23.71 [1]	3369.34 ± 63.20 [a]	3444.33 ± 21.27 [b]

252 * Elenolic acid was not included in the total phenolic content, as it is not a phenolic compound, but a
253 degradation product.

254 The sum of phenolic compounds was not significantly affected by extracting the oil a day after
255 the olive harvest, even though it was slightly higher in O4 (Table 1). However, most of the
256 individual phenolic compounds decreased significantly, most likely due to the action of oxidative
257 enzymes such as polyphenol oxidase (PPO) and peroxidase (POX). When olives are damaged,
258 the oxygen required for the oxidoreductase reactions can enter the fruit, which also favors the
259 proliferation of microorganisms such as yeasts and bacteria, another possible factor contributing
260 to the phenolic loss [19]. In contrast, secoiridoid levels increased, particularly oleuropein aglycone,
261 oleacein, and oleocanthal (Figure 1). This behavior can be attributed to the action of hydrolytic
262 enzymes such as β-glucosidase and esterases during the 17 h of storage. Another relevant factor
263 is that plant synthesis of phenolic compounds is activated as a defense response to repair damage
264 [20]. For example, oleuropein aglycone has been associated with a response to wounding stress in
265 olives [21]. The decrease in α-tocopherol and squalene could also be due to oxidative reactions [22].
266 In addition, the activity of enzymes involved in sterol biosynthesis could contribute to the
267 depletion of squalene [23].

268 Olive storage affected the content of carotenoids, which increased, whereas chlorophyll levels
269 remained unaltered. Chlorophyll is susceptible to photooxidation, but this process was limited as
270 the 17 h of storage was mainly at night, which could also explain why carotenoids, strong
271 protectors against photosensitized oxidation [10], were not depleted. Additionally, α-tocopherol can
272 contribute to the protective effect of carotenoids, avoiding their loss [24]. The increase in carotenoids
273 in the EVOO could be attributed to the degradation of chloroplast membranes during olive
274 storage, which enhances extractability during malaxation [25].

275 Finally, while olive storage did not alter the total FA content, some individual FAs were affected
276 (Table S1). C15:0, C15:1 and linoleic (C18:2 n-6) acids increased, whereas C20:2 n-6, C22:0,
277 C22:1 n-9, C22:2 n-6, C23:0, and C24:0 decreased. Therefore, the very-long-chain FAs (more
278 than 18C) seem to have been damaged by olive storage. Possible explanations could be related to
279 the inactivation of the elongases involved in their biosynthesis [26], or to FA degradation over time.
280 The activity of specific desaturases has been associated with an increase of linoleic acid [27], which

281 in the present study resulted in a significant reduction of the oleic/linoleic and MUFA/PUFA
282 ratios (Figure 1), an indicator that the oil has lost oxidative stability.

283 However, despite having a lower oleic/linoleic ratio and a reduced concentration of α-tocopherol
284 and squalene, O4 had significantly higher DPPH and Rancimat values (Table 1, Figure 1). These
285 findings reflect that phenolic compounds, especially the secoiridoids oleacein, oleocanthal, and
286 oleuropein aglycone, contributed strongly to both the antioxidant capacity and oxidative stability
287 of the oil. The high antioxidant capacity of secoiridoids has been reported previously [28,29]. In other
288 olive cultivars, Rancimat values have been found to remain unaltered over several days of storage
289 [19]. In the case of 'Corbella' olives, our results show that storing healthy fruit with an RI of 1 to
290 1.5 for 17 h before EVOO production enhances the oxidative stability of the oil.

291 3.3. Effect of malaxation conditions on the EVOO composition and oxidative stability

292 3.3.1. Phenolic compounds

293 Malaxation conditions had variable effects on the different phenolic compounds (Table 1).
294 Although the sum of phenolic compounds was not altered by malaxation, phenolic alcohols and
295 flavonoids were negatively affected by the higher temperature ($p < 0.05$) and showed no
296 significant effects due to malaxation time. The higher temperature also negatively affected the
297 secoiridoids, as previously reported [12,16,30–32], but their content increased with malaxation time.

298 Among the secoiridoids, which are the major group of phenolic compounds in olive oil,
299 oleuropein aglycone is predominant in 'Corbella' olives and EVOOs [1,12]. The effect of the
300 duration of malaxation on secoiridoids differed with the temperature. At 18 °C the levels of
301 oleuropein aglycone increased slightly with time, whereas at 23 °C they decreased slightly.
302 Similar tendencies were observed for ligstroside aglycone but without significant differences.
303 Both oleocanthal and oleacein increased with time and temperature, as found in the pilot study [12].
304 Finally, hydroxyelenolic acid, oleocanthalic acid, and hydroxyoleuropein aglycone, which are
305 oxidized derivatives of secoiridoids [33,34], showed significant differences only in O1 malaxed at 18
306 °C for 30 min, when their concentration was highest. Although elenolic acid is not a phenolic

307 compound, it forms part of the chemical structure of secoiridoids [35] and is generated by their
308 degradation [36,37]. An increase in both temperature and time of malaxation had a negative effect on
309 the EVOO elenolic acid content, as previously reported [12]. 'Corbella' olives are characterized by
310 a high content of this compound [1].

311 The high concentration of oleuropein aglycone and elenolic acid in 'Corbella' olives suggests this
312 cultivar has a high β-glucosidase activity [36]. Although oleacein and oleocanthal increased with
313 malaxation temperature, presumably due to esterase activity [38], their levels remained low. This
314 indicated that the tested conditions were not optimal for the activity of these enzymes, which is
315 reported to be enhanced at 30 °C [12,39]. Likewise, longer malaxation times significantly increased
316 oleacein and oleocanthal content, as the esterases had more time to develop their activity.
317 Additionally, the difference in oleacein and oleocanthal levels corresponded to the concentration
318 of their precursors, the considerably higher concentration of oleuropein aglycone compared to
319 ligstroside aglycone explaining the higher formation of oleacein versus oleocanthal. The fact that
320 the levels of both aglycones were similar or differed only slightly in the EVOO samples suggests
321 their catabolic and anabolic pathways were balanced. Thus, as well as being transformed by
322 esterases to oleacein and oleocanthal, the aglycones could have been formed from oleuropein and
323 ligstroside by β-glucosidase activity [36]. Three products of secoiridoid oxidation were found,
324 hydroxyelenolic acid, oleocanthalic acid and hydroxyoleuropein aglycone, also detected in other
325 studies [16,40]. Their low and generally constant concentration in all the EVOO samples indicates
326 this oxidation process was not very active. The content of hydroxyelenolic acid was highest and
327 that of oleocanthalic acid lowest, which corresponds with the levels of their respective precursors,
328 elenolic acid and oleocanthal.

329 Two phenomena can contribute to the depletion of phenolic compounds during malaxation: the
330 activity of oxidative and hydrolytic enzymes [41,42], and the transfer of hydrophilic phenols to the
331 water phase [43]. Both phenomena increase with longer malaxation times. According to our results,
332 as the oxidative products did not increase with malaxation time, it seems more likely that the
333 depletion of elenolic acid could be attributed to its transfer to the water phase. This is supported

334 by the observation that hydroxytyrosol, also a degradation product of secoiridoids [37], did not
335 increase with malaxation temperature or time. Additionally, hydroxytyrosol levels were only
336 significantly lower at 23 °C and 40 min, suggesting that its degradation or transfer to the water
337 phase can occur in these malaxation conditions.

338 The flavonoids apigenin and luteolin were negatively affected by increasing the temperature of
339 malaxation, as reported in other studies [12], whereas a longer malaxation time reduced their content
340 only at 18 °C. The same behavior was observed for hydroxytyrosol acetate and the lignan
341 pinoresinol, which were depleted when the malaxation time was increased at 18 °C. Finally, the
342 levels of *p*-coumaric acid decreased when both malaxation parameters were increased, indicating
343 a susceptibility to degradation or transfer to the water phase.

344 According to these results, malaxation at 18 °C for 30 min provides the most favorable conditions
345 to obtain 'Corbella' EVOO with high concentrations of phenolic compounds. However, if the
346 goal is also to obtain EVOOs with a high content of oleocanthal and oleacein, malaxation should
347 be applied at 18 °C for 50 min, as their concentration is enhanced by higher temperatures or longer
348 times.

349 3.3.2. Fatty acid profile

350 The FA profile was the same in all EVOO samples, regardless of the malaxation conditions
351 applied (Table S2). The main FA was oleic acid (C18:1 n-9) (77.75 – 78.89%), followed by
352 palmitic acid (C16:0) (11.68 – 11.86%), linoleic acid (C18:2 n-6) (5.44 – 6.69), stearic acid
353 (C18:0) (1.78 – 1.90%), 9-palmitoleic acid (C16:1 n-7) (0.59 – 0.64), α-linolenic acid (C18:3 n-
354 3) (0.52 – 0.57%), arachidic acid (C20:0) (0.28 – 0.29%), gondoic acid (C20:1 n-9) (0.20 – 0.22%)
355 and behenic acid (C22:0) (0.08 – 0.10%). The percentage of the other FAs was < 0.10%. The FA
356 composition (%) of the samples (Table S2) fell within the limits established for EVOO by the
357 European Commission Nº 2568/91, (2019) [44] and coincides with the FA profile previously
358 reported for 'Corbella' olives [1].

359 ‘Corbella’ EVOO has a higher proportion of oleic acid, and less palmitic, linoleic, 9-palmitoleic,
360 arachidic, and gondoic acids than ‘Arbequina’ EVOO [17], and more palmitic and less oleic, stearic,
361 linoleic, α-linolenic, and arachidic acids than ‘Picual’ EVOO [45]. Variations in the FA composition
362 of olive oils of different cultivars are due to genetic differences [6], such as the variable capacity or
363 expression of desaturase enzymes involved in FA biosynthesis [27].

364 The total FA content was not significantly affected by any of the factors studied, with values
365 ranging between 817.80 mg/g and 866.36 mg/g in all the EVOO samples (Table S1), although it
366 tended to increase with the malaxation temperature. At higher temperatures, viscosity is reduced,
367 and coalescence of oil droplets is enhanced, so the oily phase becomes richer in oil and poorer in
368 other compounds, especially unsaponifiable lipids and water [17].

369 The most abundant FAs, oleic and palmitic acids, did not show any significant differences
370 between samples. Nevertheless, the concentration of relevant FAs such as palmitoleic, linoleic,
371 α-linolenic and gondoic acids increased at the higher temperature, as reported in ‘Arbequina’
372 EVOOs [17]. Linoleic acid was affected by an interaction of both malaxation parameters. At 18 °C,
373 its concentration tended to increase with malaxation time, whereas at 23 °C it tended to decrease,
374 suggesting that prolonging the malaxation at high temperatures promoted its oxidation or
375 lipoxygenase activity [46].

376 Increasing both malaxation parameters reduced the MUFA/PUFA and oleic/linoleic ratios,
377 indicating that higher temperatures and longer times of malaxation produce EVOOs more
378 susceptible to oxidation processes. Accordingly, the most stable EVOO was produced by
379 malaxation at 18 °C for 30 min (MUFA/PUFA = 13.21 ± 0.17, oleic/linoleic = 14.50 ± 0.20),
380 followed by 18 °C for 40 min (MUFA/PUFA = 12.79 ± 0.05, oleic/linoleic = 13.93 ± 0.05) (Table
381 S1).

382 A previous analysis of ‘Corbella’ olives with an RI similar to that of the olives used in the present
383 study found lower values for the two ratios [1] compared to the ‘Corbella’ EVOOs, indicating the
384 oxidative stability was enhanced during the production process. Hernández et al. (2021) [6]

385 compiled a list of the oleic/linoleic ratios of olive oils produced from 89 cultivars from the
386 Worldwide Olive Germplasm Bank of Cordoba. According to these values, 'Corbella' EVOOs
387 would be ranked between 10th and 15th. However, the ratios of that study were obtained from
388 EVOOs produced with olives harvested 28–31 weeks after flowering, i.e., with an RI above 2.

389 An 'Arbequina' EVOO produced from olives with an RI between 1.16 and 2.26 and using
390 different malaxation conditions [17] had an oleic/linoleic ratio between 6.21 and 7.82, which is
391 considerably lower than the ratio of 'Corbella' EVOOs (11.62−14.50). The 'Arbequina' ratio
392 reported by Hernández et al. (2021) [6] was even lower (4.17). Linoleic acid is generated by the
393 desaturation of oleic acid, and in some olive cultivars, such as 'Picual', 'Arbequina' and 'Picudo',
394 the content of this PUFA increases with maturation due to a high expression of desaturase genes
395 [27], resulting in a decrease in the oleic/linoleic ratio. However, in 'Corbella' olives the ratio was
396 found to increase with ripeness up to an RI of 2 [1], suggesting this cultivar has a different
397 expression pattern of the desaturases involved in the biosynthesis of both FA. Considering these
398 results, it is likely that 'Corbella' EVOOs produced from olives with an RI of 2 would have a
399 higher oleic/linoleic ratio, and would therefore be more stable than cultivars with a higher linoleic
400 acid content, such as 'Arbequina'. As mentioned, the oleic/linoleic ratio differs between
401 'Corbella' and 'Arbequina' EVOOs because the former has a higher proportion of oleic acid and
402 lower proportion of linoleic acid. Accordingly, 'Corbella' olives seem to be a suitable choice for
403 the production of EVOOs with high oleic/linoleic ratios. However, before reaching a definitive
404 conclusion, the evolution of the ratio should be tracked over the whole maturation process of
405 'Corbella' olives.

406 3.3.3. Carotenoids, chlorophylls, α-tocopherol, and squalene

407 All the pigments (lutein, β-carotene, and chlorophylls) increased with longer malaxation (Table
408 1), as there was more time for their transfer to the oily phase [47], and chlorophylls decreased at the
409 higher temperature. Pigments are also susceptible to degradation when exposed to temperatures
410 above 30 °C [10,48].

411 α-Tocopherol and squalene were negatively affected by the higher malaxation temperature and
412 times; a decrease in levels due to a higher temperature has been reported in other studies [16,49].
413 Tocopherols are strong antioxidants that protect olive oil from lipid oxidation [2], so an oxidation
414 process during malaxation could have caused their depletion in our study. Squalene also has a
415 protective effect, helping to prevent the temperature-dependent autoxidation of PUFAs [50].
416 Additionally, as an unsaturated molecule, squalene is unstable and easily oxidized, which could
417 also explain the depletion observed [23]. As previously discussed, the PUFA content increased
418 slightly with the malaxation temperature. Rastrelli et al., (2002) [22] found that PUFA levels
419 remained constant during 8 months of EVOO storage, and only started to decline when
420 antioxidant levels had decreased considerably. Therefore, the decrease in α-tocopherol and
421 squalene in the EVOO samples could be related to their contribution to protecting PUFAs from
422 thermal-oxidation.

423 3.3.4. Oxidative stability (Rancimat) and antioxidant capacity (DPPH assay) of the EVOO
424 samples

425 Increasing the temperature without changing the malaxation time led to a slight increase in the
426 oxidative stability of the EVOO samples (Table 1). The same pattern was observed when the
427 malaxation time was extended without altering the temperature. The EVOO with the highest
428 oxidative stability was produced by malaxation at 18 °C for 50 min.

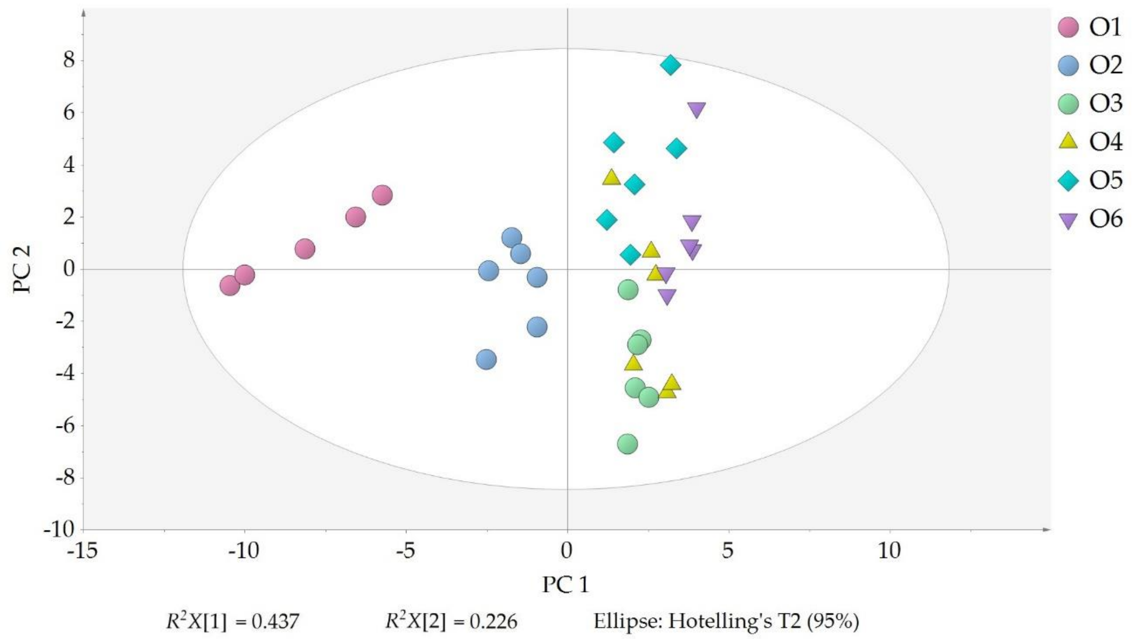
429 When the temperature was increased without changing the malaxation time, the DPPH assay
430 revealed that the resulting EVOOs had a higher antioxidant capacity (Table 1). In correlation with
431 the results for optimum oxidative stability, the best values were obtained with conditions of 18
432 °C/50 min.

433 The increase in antioxidant activity correlates with the higher levels of the strongly antioxidant
434 phenolics hydroxytyrosol, oleuropein aglycone, oleocanthal and oleacein, as well as the
435 carotenoids lutein and β-carotene. A high contribution of phenolic compounds to the oxidative
436 stability measured by Rancimat has been previously reported [2,3,5,8]. Thus, in agreement with the

437 results obtained when analyzing the effect of olive storage, the highest antioxidant capacity and
438 oxidative stability were observed in EVOOs with the highest content of phenolic compounds,
439 especially oleacein, oleocanthal, and oleuropein aglycone.

440 3.4. Principal Component Analysis

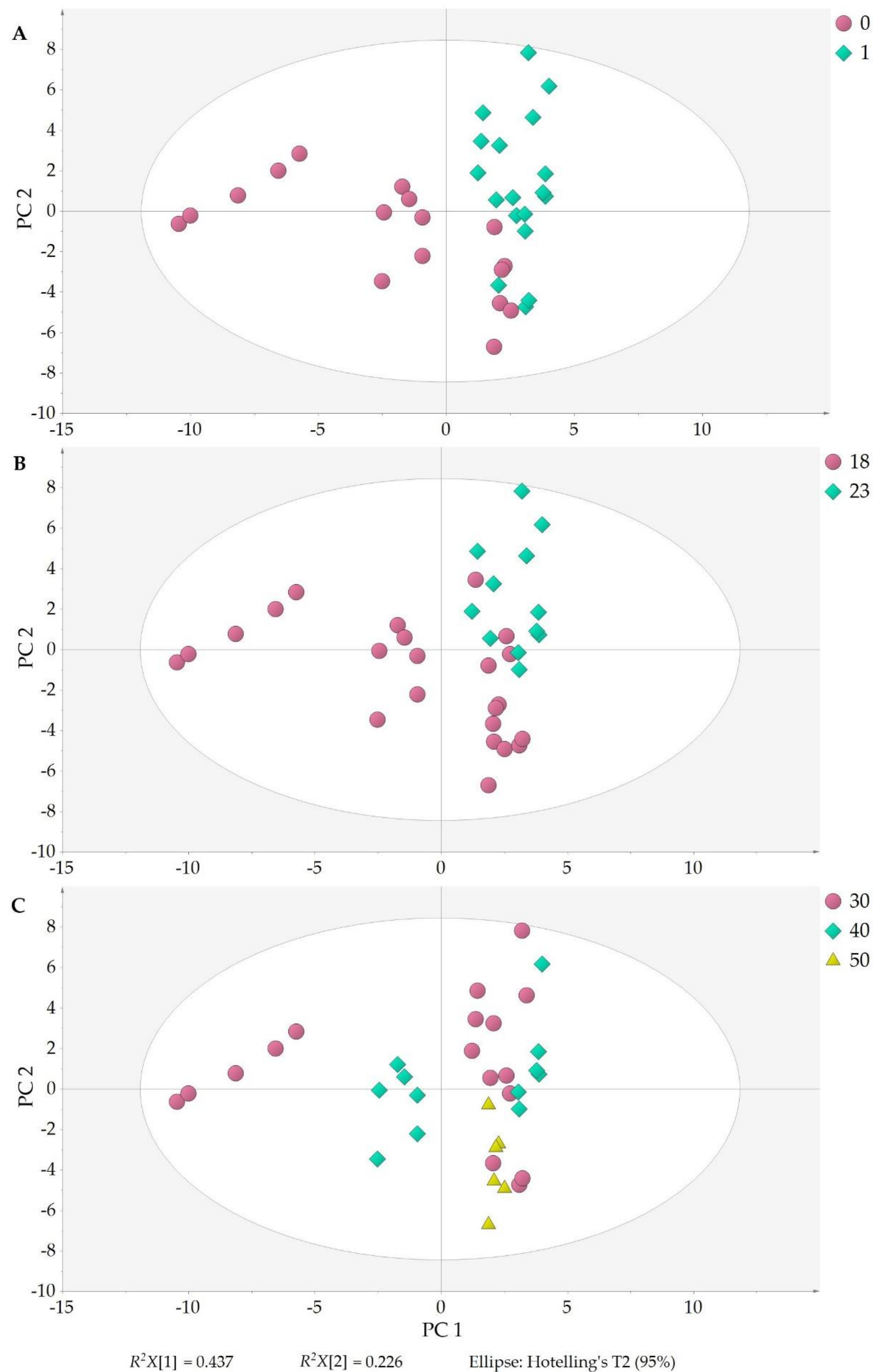
441 The PCA model with five PC had an explained variation (R^2X) of 0.850 and a predicted variation
442 (Q^2X) of 0.675. In the score scatter plot (Figure 2), O1 (18 °C, 30 min) is clearly separated from
443 the other samples and located on the left side. O2 (18 °C, 40 min) is clustered in the middle of the
444 plot, but closer to the remaining samples. Finally, the other samples (O3, O4, O5 and O6) are on
445 the right side of the plot. Although all three factors evaluated (malaxation temperature and time,
446 and olive storage) seem to contribute to the separation of the samples (Figures 3A, 3B and 3C),
447 olive storage appears to be the most influential, as samples produced on the day of harvesting are
448 distributed on the left side, appearing on the right side when produced the following day (Figure
449 3A). O3 samples are an exception, as they appear on the right side of the plot, despite being
450 produced on the day of harvesting, indicating that the malaxation conditions (18 °C, 50 min)
451 resulted in EVOOs with a similar composition to those produced with stored olives. Nevertheless,
452 O3 samples are positioned toward the upper right of the plot, while O4, O5 and O6 are more in
453 the bottom right, indicating that the malaxation conditions still have an influence on the
454 separation.



455

456 **Figure 2.** Score scatter plot of the Principal Component Analysis (PCA). EVOO samples are colored and
457 shaped according to their production conditions: **O1** (no storage, 18 °C/30 min), **O2** (no storage, 18 °C/40
458 min), **O3** (no storage, 18 °C/50 min), **O4** (17 h storage, 18 °C/30 min), **O5** (17 h storage, 23 °C/30 min),
459 and **O6** (17 h storage, 23 °C/40 min). $R^2X[1]$ and $R^2X[2]$ in the PCA are the variations explained by the first
460 PC and the second PC, respectively, together explaining 66.3% of the variation. All samples were inside
461 the Ellipse Hotelling's T2, indicating there were no strong outliers.

462



463

464 **Figure 3.** Score scatter plot of the Principal Component Analysis (PCA). EVOO samples are colored and
465 shaped according to the olive storage (**A**) (0: no storage, 1: 17 h of storage), (**B**) malaxation temperature
466 (18 °C, 23 °C), and malaxation time (**C**) (30 min, 40 min, 50 min). $R^2X[1]$ and $R^2X[2]$ in the PCA are the

467 variations explained by the first PC and the second PC, respectively, together explaining 66.3% of the
468 variation. All samples were inside the Ellipse Hotelling's T2, indicating there were no strong outliers.

469 The loading plot (Figure 4) shows that the variables most associated with O1 samples are the
470 majority of the phenolic compounds, α-tocopherol, squalene, the oleic/linoleic ratio, and the very-
471 long-chain FAs (C22:0, C22:1 n-9, C22:2 n-6, C23:0 and C24:0), and that the samples produced
472 the day after harvesting had a higher content of the other FAs and secoiridoids. The proximity of
473 Rancimat values to secoiridoids, particularly oleacein and oleocanthal, corroborates the strong
474 positive correlation between these variables. DPPH values and oleuropein aglycone are also
475 situated quite closely to these variables, as are lutein and β-carotene, indicating a positive
476 correlation. These positive correlations demonstrate the contribution of these compounds to the
477 oil oxidative stability: the closer to Rancimat, the greater the contribution. Therefore, oleacein
478 and oleocanthal are the major contributors. All these variables are associated with O3 and O4
479 samples.

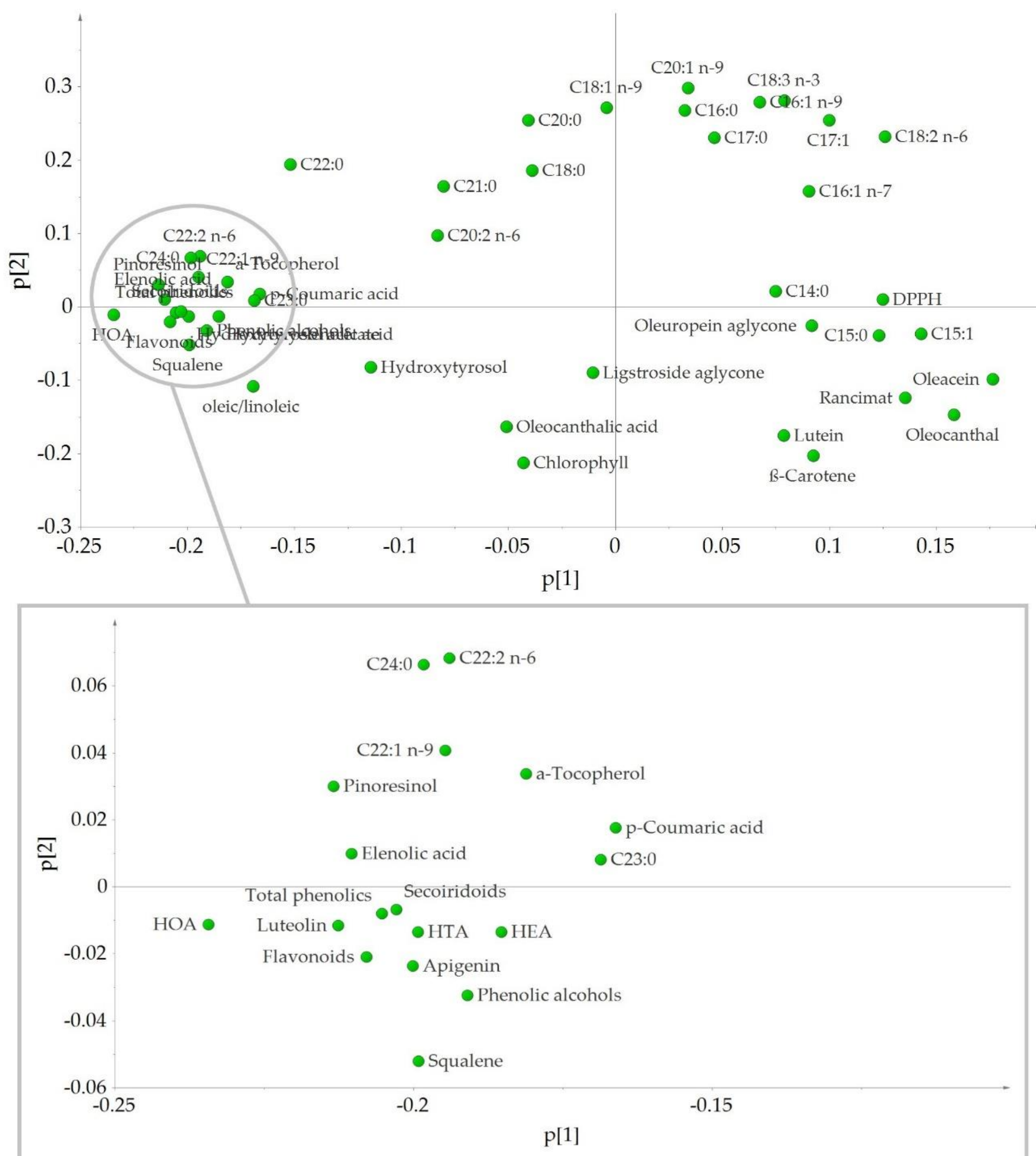


Figure 4. Loading scatter plot of the first and second principal components of the PCA showing the distribution and correlation of the different variables analyzed in the 'Corbella' EVOO samples. HOA: Hydroxyoleuropein aglycone; HTA: Hydroxytyrosol acetate; HEA: Hydroxyelenolic acid.

This study of 'Corbella' EVOO, which was aimed at improving its oxidative stability, revealed that the content of phenolic compounds, α-tocopherol and squalene was negatively affected when malaxation temperature and time were increased and olives were stored before production. However, the three factors increased the levels of the secoiridoids oleacein, and oleocanthal. The pigments lutein and β-carotene increased with malaxation time and after olive storage, whereas chlorophylls decreased after malaxation at a higher temperature and increased when the process was extended. Although the FA profile was not altered by any of the tested factors, linoleic acid

was favored by olive storage and a higher malaxation temperature. Consequently, the oleic/linoleic ratio was higher at the lower malaxation temperature and time (18 °C and 30 min), and when the oil was produced on the same day of olive harvest. Accordingly, the 'Corbella' cultivar seems to be a promising candidate for the production of EVOOs with a high oleic/linoleic ratio.

Although producing the EVOOs on the day of the olive harvest with malaxation at 18 °C for 30 min resulted in a better composition in terms of phenolic content, α-tocopherol, squalene, and oleic/linoleic ratio, these conditions did not produce the best values of antioxidant activity, and oxidative stability. In fact, the EVOOs with the optimum antioxidant capacity and oxidative stability were obtained by malaxation at the higher temperature and times, and after storing the olives. These desirable attributes were positively correlated with the content of oleacein and oleocanthal.

The results of this study therefore indicate that oleacein and oleocanthal contribute strongly to the antioxidant capacity and oxidative stability of 'Corbella' EVOOs, and that oils with a high content of these two secoiridoids will be more stable and have a longer shelf life. Therefore, for the production of 'Corbella' EVOO, it can be recommended that (a) olives are stored at environmental temperature during the night and (b) the malaxation temperature is at least 23 °C with a malaxation time of 40-50 min (depending on the temperature), conditions that increase the oleacein and oleocanthal content and thus the oxidative stability.

Supporting information

Table S1. Concentration of fatty acids (mg/g); **Table S2.** Fatty acid composition (%).

Author contributions

Alexandra Olmo-Cunillera: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Maria Pérez:** Writing – review & editing, Supervision. **Anallely López-Yerena:** Investigation,

Writing – review & editing. **Mohamed M. Abuhabib:** Investigation. **Antònia Ninot:** Conceptualization, Methodology, Investigation, Resources, Data curation, Writing – review & editing. **Agustí Romero-Aroca:** Conceptualization, Methodology, Investigation, Resources, Data curation, Writing – review & editing. **Anna Vallverdú-Queralt:** Methodology, Writing – review & editing. **Rosa Maria Lamuela-Raventós:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

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

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References

(1) Olmo-Cunillera, A.; Pérez, M.; López-Yerena, A.; Abuhabib, M. M.; Ninot, A.; Romero-Aroca, A.; Vallverdú-Queralt, A.; Lamuela-Raventós, R. M. Targeted Metabolic Profiling of the Revived Ancient 'Corbella' Olive Cultivar during Early Maturation. *Food Chem* **2023** [Submitted for publication].

(2) Velasco, J.; Dobarganes, C. Oxidative Stability of Virgin Olive Oils. *European Journal of Lipid Science and Technology* **2002**, *104* (9–10), 661–676. https://doi.org/https://doi.org/10.1002/1438-9312(200210)104:9/10<661::AID-EJLT661>3.0.CO;2-D.

(3) Aparicio, R.; Roda, L.; Albi, M. A.; Gutiérrez, F. Effect of Various Compounds on Virgin Olive Oil Stability Measured by Rancimat. *J Agric Food Chem* **1999**, *47* (10), 4150–4155. https://doi.org/10.1021/jf9812230.

554 (4) Gutiérrez, F.; Jímenez, B.; Ruíz, A.; Albi, M. A. Effect of Olive Ripeness on the Oxidative
555 Stability of Virgin Olive Oil Extracted from the Varieties Picual and Hojiblanca and on
556 the Different Components Involved. *J Agric Food Chem* **1999**, *47* (1), 121–127.
557 https://doi.org/10.1021/jf980684i.

558 (5) Nieto, L. M.; Hodaifa, G.; Lozano Peña, J. L. Changes in Phenolic Compounds and
559 Rancimat Stability of Olive Oils from Varieties of Olives at Different Stages of Ripeness. *J*
560 *Sci Food Agric* **2010**, *90* (14), 2393–2398. https://doi.org/10.1002/jsfa.4097.

561 (6) Hernández, M. L.; Sicardo, M. D.; Belaj, A.; Martínez-Rivas, J. M. The Oleic/Linoleic Acid
562 Ratio in Olive (Olea Europaea L.) Fruit Mesocarp Is Mainly Controlled by OeFAD2-2 and
563 OeFAD2-5 Genes Together With the Different Specificity of Extraplastidial
564 Acyltransferase Enzymes. *Front Plant Sci* **2021**, *12*, 345.
565 https://doi.org/10.3389/fpls.2021.653997.

566 (7) O'Keefe, S. F.; Wiley, V. A.; Knauft, D. A. Comparison of Oxidative Stability of High- and
567 Normal-Oleic Peanut Oils. *J Am Oil Chem Soc* **1993**, *70* (5), 489–492.
568 https://doi.org/10.1007/BF02542581.

569 (8) Baldioli, M.; Servili, M.; Perretti, G.; Montedoro, G. F. Antioxidant Activity of
570 Tocopherols and Phenolic Compounds of Virgin Olive Oil. *J Am Oil Chem Soc* **1996**, *73*
571 (11), 1589–1593. https://doi.org/10.1007/BF02523530.

572 (9) Criado, M.-N.; Romero, M.-P.; Casanovas, M.; Motilva, M.-J. Pigment Profile and Colour
573 of Monovarietal Virgin Olive Oils from Arbequina Cultivar Obtained during Two
574 Consecutive Crop Seasons. *Food Chem* **2008**, *110* (4), 873–880.
575 https://doi.org/10.1016/j.foodchem.2008.02.075.

576 (10) Rahmani, M.; Csallany, A. S. Role of Minor Constituents in the Photooxidation of Virgin
577 Olive Oil. *J Am Oil Chem Soc* **1998**, *75* (7), 837–843. https://doi.org/10.1007/s11746-
578 998-0234-1.

579 (11) Gutiérrez-Rosales, F.; Garrido-Fernández, J.; Gallardo-Guerrero, L.; Gandul-Rojas, B.;
580 Minguez-Mosquera, M. I. Action of Chlorophylls on the Stability of Virgin Olive Oil. *J Am*
581 *Oil Chem Soc* **1992**, *69* (9), 866–871. https://doi.org/10.1007/BF02636334.

582 (12) López-Yerena, A.; Ninot, A.; Jiménez-Ruiz, N.; Lozano-Castellón, J.; Pérez, M.; Escribano-
583 Ferrer, E.; Romero-Aroca, A.; Lamuela-Raventós, R. M.; Vallverdú-Queralt, A. Influence
584 of the Ripening Stage and Extraction Conditions on the Phenolic Fingerprint of
585 'Corbella' Extra-Virgin Olive Oil. *Antioxidants* **2021**, *10* (6), 877.
586 https://doi.org/10.3390/antiox10060877.

587 (13) Kafkaletou, M.; Ouzounidou, G.; Tsantili, E. Fruit Ripening, Antioxidants and Oil
588 Composition in Koroneiki Olives (Olea Europea L.) at Different Maturity Indices.
589 *Agronomy* **2021**, *11* (1), 122. https://doi.org/10.3390/agronomy11010122.

590 (14) Beltrán, G.; Aguilera, M. P.; Rio, C. Del; Sanchez, S.; Martinez, L. Influence of Fruit
591 Ripening Process on the Natural Antioxidant Content of Hojiblanca Virgin Olive Oils.
592 *Food Chem* **2005**, *89* (2), 207–215. https://doi.org/10.1016/j.foodchem.2004.02.027.

593 (15) Rotondi, A.; Morrone, L.; Bertazza, G.; Neri, L. Effect of Duration of Olive Storage on
594 Chemical and Sensory Quality of Extra Virgin Olive Oils. *Foods* **2021**, *10* (10), 2296.
595 https://doi.org/10.3390/FOODS10102296/S1.

596 (16) Olmo-Cunillera, A.; Lozano-Castellón, J.; Pérez, M.; Miliarakis, E.; Tresserra-Rimbau, A.;
597 Ninot, A.; Romero-Aroca, A.; Lamuela-Raventós, R. M.; Vallverdú-Queralt, A. Optimizing
598 the Malaxation Conditions to Produce an Arbequina EVOO with High Content of
599 Bioactive Compounds. *Antioxidants 2021, Vol. 10, Page 1819* **2021**, *10* (11), 1819.
600 https://doi.org/10.3390/ANTIOX10111819.

601 (17) Olmo-Cunillera, A.; Casadei, E.; Valli, E.; Lozano-Castellón, J.; Miliarakis, E.; Domínguez-
602 López, I.; Ninot, A.; Romero-Aroca, A.; Lamuela-Raventós, R. M.; Pérez, M.; Vallverdú-
603 Queralt, A.; Bendini, A. Aromatic, Sensory, and Fatty Acid Profiles of Arbequina Extra
604 Virgin Olive Oils Produced Using Different Malaxation Conditions. *Foods* **2022**, *11* (21),
605 3446. https://doi.org/10.3390/foods11213446.

606 (18) Gutiérrez-Rosales, F. Determinación de La Estabilidad Oxidativa de Aceites de Oliva
607 Vírgenes: Comparación Entre El Método Del Oxigeno Activo (A.O.M.) y El Método
608 Rancimat. *Grasas y aceites* **1989**, *40* (1), 1–5.

609 (19) Plasquy, E.; García Martos, J. M.; Florido Fernández, M. D. C.; Sola-Guirado, R. R.; García
610 Martín, J. F. Cold Storage and Temperature Management of Olive Fruit: The Impact on
611 Fruit Physiology and Olive Oil Quality—A Review. *Processes 2021, Vol. 9, Page 1543*
612 **2021**, *9* (9), 1543. https://doi.org/10.3390/PR9091543.

613 (20) Hu, W.; Sarengaowa, W.; Guan, Y.; Feng, K. Biosynthesis of Phenolic Compounds and
614 Antioxidant Activity in Fresh-Cut Fruits and Vegetables. *Front Microbiol* **2022**, *13*, 1782.
615 https://doi.org/10.3389/FMICB.2022.906069/BIBTEX.

616 (21) Koudounas, K.; Thomopoulou, M.; Rigakou, A.; Angeli, E.; Melliou, E.; Magiatis, P.;
617 Hatzopoulos, P. Silencing of Oleuropein β-Glucosidase Abolishes the Biosynthetic
618 Capacity of Secoiridoids in Olives. *Front Plant Sci* **2021**, *12*.
619 https://doi.org/10.3389/fpls.2021.671487.

620 (22) Rastrelli, L.; Passi, S.; Ippolito, F.; Vacca, G.; de Simone, F. Rate of Degradation of α-
621 Tocopherol, Squalene, Phenolics, and Polyunsaturated Fatty Acids in Olive Oil during
622 Different Storage Conditions. *J Agric Food Chem* **2002**, *50* (20), 5566–5570.
623 https://doi.org/10.1021/JF011063J.

624 (23) Spanova, M.; Daum, G. Squalene – Biochemistry, Molecular Biology, Process
625 Biotechnology, and Applications. *European Journal of Lipid Science and Technology*
626 **2011**, *113* (11), 1299–1320. https://doi.org/10.1002/ejlt.201100203.

627 (24) Psomiadou, E.; Tsimidou, M. Simultaneous HPLC Determination of Tocopherols,
628 Carotenoids, and Chlorophylls for Monitoring Their Effect on Virgin Olive Oil Oxidation.
629 *J Agric Food Chem* **1998**, *46* (12), 5132–5138.
630 https://doi.org/10.1021/JF980250N/ASSET/IMAGES/LARGE/JF980250NF00005.JPEG.

631 (25) Yousfi, K.; Weiland, C. M.; García, J. M. Effect of Harvesting System and Fruit Cold
632 Storage on Virgin Olive Oil Chemical Composition and Quality of Superintensive
633 Cultivated "Arbequina" Olives. *J Agric Food Chem* **2012**, *60* (18), 4743–4750.
634 https://doi.org/10.1021/JF300331Q/ASSET/IMAGES/JF-2012-00331Q_M001.GIF.

635 (26) Batsale, M.; Bahammou, D.; Fouillen, L.; Mongrand, S.; Joubès, J.; Domergue, F.
636 Biosynthesis and Functions of Very-Long-Chain Fatty Acids in the Responses of Plants to

Abiotic and Biotic Stresses. *Cells* **2021**, *10* (6), 1284. https://doi.org/10.3390/cells10061284.

(27) Hernández, M. L.; Padilla, M. N.; Mancha, M.; Martínez-Rivas, J. M. Expression Analysis Identifies FAD2-2 as the Olive Oleate Desaturase Gene Mainly Responsible for the Linoleic Acid Content in Virgin Olive Oil. *J Agric Food Chem* **2009**, *57* (14), 6199–6206. https://doi.org/10.1021/jf900678z.

(28) Presti, G.; Guarrasi, V.; Gulotta, E.; Provenzano, F.; Provenzano, A.; Giuliano, S.; Monfreda, M.; Mangione, M. R.; Passantino, R.; San Biagio, P. L.; Costa, M. A.; Giacomazza, D. Bioactive Compounds from Extra Virgin Olive Oils: Correlation between Phenolic Content and Oxidative Stress Cell Protection. *Biophys Chem* **2017**, *230*, 109–116. https://doi.org/10.1016/J.BPC.2017.09.002.

(29) Vidal, A. M.; Alcalá, S.; Ocaña, M. T.; De Torres, A.; Espínola, F.; Moya, M. Elaboration of Extra-Virgin Olive Oils Rich in Oleocanthal and Oleacein: Pilot Plant's Proposal. *European Food Research and Technology* **2020**, *246* (7), 1459–1468. https://doi.org/10.1007/S00217-020-03503-1/FIGURES/5.

(30) Jolayemi, O. S.; Tokatli, F.; Ozen, B. Effects of Malaxation Temperature and Harvest Time on the Chemical Characteristics of Olive Oils. *Food Chem* **2016**, *211*, 776–783. https://doi.org/10.1016/j.foodchem.2016.05.134.

(31) Marx, Í. M. G.; Rodrigues, N.; Veloso, A. C. A.; Casal, S.; Pereira, J. A.; Peres, A. M. Effect of Malaxation Temperature on the Physicochemical and Sensory Quality of Cv. Cobrançosa Olive Oil and Its Evaluation Using an Electronic Tongue. *LWT - Food Science and Technology* **2021**, *137*. https://doi.org/10.1016/j.lwt.2020.110426.

(32) Taticchi, A.; Esposto, S.; Veneziani, G.; Urbani, S.; Selvaggini, R.; Servili, M. The Influence of the Malaxation Temperature on the Activity of Polyphenoloxidase and Peroxidase and on the Phenolic Composition of Virgin Olive Oil. *Food Chem* **2013**, *136* (2), 975–983. https://doi.org/10.1016/j.foodchem.2012.08.071.

(33) Lozano-Castellón, J.; Vallverdú-Queralt, A.; Rinaldi de Alvarenga, J. F.; Illán, M.; Torrado-Prat, X.; Lamuela-Raventós, R. M. Domestic Sautéing with EVOO: Change in the Phenolic Profile. *Antioxidants* **2020**, *9* (1), 77. https://doi.org/10.3390/antiox9010077.

(34) Tsolakou, A.; Diamantakos, P.; Kalaboki, I.; Mena-Bravo, A.; Priego-Capote, F.; Abdallah, I. M.; Kaddoumi, A.; Melliou, E.; Magiatis, P. Oleocanthalic Acid, a Chemical Marker of Olive Oil Aging and Exposure to a High Storage Temperature with Potential Neuroprotective Activity. *J Agric Food Chem* **2018**, *66* (28), 7337–7346. https://doi.org/10.1021/acs.jafc.8b00561.

(35) Soler-Rivas, C.; Espín, J. C.; Wichers, H. J. Oleuropein and Related Compounds. *J Sci Food Agric* **2000**, *80* (7), 1013–1023. https://doi.org/10.1002/(SICI)1097-0010(20000515)80:7<1013::AID-JSFA571>3.0.CO;2-C.

(36) Gutierrez-Rosales, F.; Romero, M. P.; Casanovas, M.; Motilva, M. J.; Mínguez-Mosquera, M. I. Metabolites Involved in Oleuropein Accumulation and Degradation in Fruits of Olea Europaea L.: Hojiblanca and Arbequina Varieties. *J Agric Food Chem* **2010**, *58* (24), 12924–12933. https://doi.org/10.1021/jf103083u.

678 (37) Domínguez-López, I.; Maria Pérez, M.; López-Yerena, A.; Lozano-Castellón, J.; Olmo-
679 Cunillera, A.; Vallverdú-Queralt, A.; Lamuela-Raventós, R. M. Human Health and the
680 Consumption of Fat-Associated Compounds: Tyrosol, Hydroxytyrosol, Oleuropein,
681 Oleacein and Oleocanthal. In *Fats and associated compounds. Consumption and human*
682 *health*; Mirando Lopez, J. M., Cepeda Sáez, A., Eds.; Royal Society of Chemistry: London,
683 United Kingdom, 2021.

684 (38) Volk, J.; Sarafeddinov, A.; Unver, T.; Marx, S.; Tretzel, J.; Zotzel, J.; Warzecha, H. Two
685 Novel Methylesterases from Olea Europaea Contribute to the Catabolism of Oleoside-
686 Type Secoiridoid Esters. *Planta* **2019**, *250* (6), 2083–2097.
687 https://doi.org/10.1007/s00425-019-03286-0.

688 (39) Diamantakos, P.; Giannara, T.; Skarkou, M.; Melliou, E.; Magiatis, P. Influence of Harvest
689 Time and Malaxation Conditions on the Concentration of Individual Phenols in Extra
690 Virgin Olive Oil Related to Its Healthy Properties. *Molecules* **2020**, *25* (10).
691 https://doi.org/10.3390/MOLECULES25102449.

692 (40) López-Yerena, A.; Lozano-Castellón, J.; Olmo-Cunillera, A.; Tresserra-Rimbau, A.; Quifer-
693 Rada, P.; Jiménez, B.; Pérez, M.; Vallverdú-Queralt, A. Effects of Organic and
694 Conventional Growing Systems on the Phenolic Profile of Extra-Virgin Olive Oil.
695 *Molecules* **2019**, *24* (10), 1986–1999. https://doi.org/10.3390/molecules24101986.

696 (41) Servili, M.; Selvaggini, R.; Taticchi, A.; Esposto, S.; Montedoro, G. Volatile Compounds
697 and Phenolic Composition of Virgin Olive Oil: Optimization of Temperature and Time of
698 Exposure of Olive Pastes to Air Contact during the Mechanical Extraction Process. *J*
699 *Agric Food Chem* **2003**, *51* (27), 7980–7988. https://doi.org/10.1021/jf034804k.

700 (42) Polari, J. J.; Garcí-Aguirre, D.; Olmo-García, L.; Carrasco-Pancorbo, A.; Wang, S. C.
701 Interactions between Hammer Mill Crushing Variables and Malaxation Time during
702 Continuous Olive Oil Extraction. *European Journal of Lipid Science and Technology* **2018**,
703 *120* (8). https://doi.org/10.1002/EJLT.201800097.

704 (43) Rodis, P. S.; Karathanos, V. T.; Mantzavinou, A. Partitioning of Olive Oil Antioxidants
705 between Oil and Water Phases. *J Agric Food Chem* **2002**, *50* (3), 596–601.
706 https://doi.org/10.1021/JF010864J.

707 (44) European Commission. Commission Regulation (EEC) N° 2568/91 of 11 July 1991 on the
708 Characteristics of Olive Oil and Olive-Residue Oil and on the Relevant Methods of
709 Analysis. *Official Journal of the European Communities* **2019**, No. L248, 83.

710 (45) Reboredo-Rodríguez, P.; González-Barreiro, C.; Cancho-Grande, B.; Fregapane, G.;
711 Salvador, M. D.; Simal-Gándara, J. Characterisation of Extra Virgin Olive Oils from
712 Galician Autochthonous Varieties and Their Co-Crushings with Arbequina and Picual Cv.
713 *Food Chem* **2015**, *176*, 493–503. https://doi.org/10.1016/J.FOODCHEM.2014.12.078.

714 (46) Feussner, I.; Wasternack, C. The Lipoxygenase Pathway. *Annu Rev Plant Biol* **2002**, *53*,
715 275–297. https://doi.org/10.1146/ANNUREV.ARPLANT.53.100301.135248.

716 (47) Ranalli, A.; Pollastri, L.; Contento, S.; Iannucci, E.; Lucera, L. Effect of Olive Paste
717 Kneading Process Time on the Overall Quality of Virgin Olive Oil. *European Journal of*
718 *Lipid Science and Technology* **2003**, *105* (2), 57–67.
719 https://doi.org/10.1002/EJLT.200390018.

(48) Ranalli, A.; Malfatti, A.; Lucera, L.; Contento, S.; Sotiriou, E. Effects of Processing Techniques on the Natural Colourings and the Other Functional Constituents in Virgin Olive Oil. *Food Research International* **2005**, *38* (8–9), 873–878. https://doi.org/10.1016/j.foodres.2005.02.011.

(49) Seçmeler, Ö.; Üstündağ, Ö. G. Partitioning of Predominant Lipophilic Bioactives (Squalene, α-Tocopherol and β-Sitosterol) during Olive Oil Processing. *Int J Food Sci Technol* **2019**, *54* (5), 1609–1616. https://doi.org/10.1111/IJFS.14029.

(50) Dessì, M. A.; Deiana, M.; Day, B. W.; Rosa, A.; Banni, S.; Corongiu, F. P. Oxidative Stability of Polyunsaturated Fatty Acids: Effect of Squalene. *European Journal of Lipid Science and Technology* **2002**, *104* (8), 506–512. https://doi.org/10.1002/1438-9312(200208)104:8<506::AID-EJLT506>3.0.CO;2-1.