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Antioxidant quantification in different portions obtained during olive oil extraction process in an olive oil press mill

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14 **Antioxidant quantification in different portions obtained during olive oil extraction process in**
15 **an olive oil press mill**

16

17 **Short title:** Antioxidants in different portions obtained during olive oil extraction

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25

26 **Abstract**

27 **Background**

28 Different antioxidant compounds are generally transferred from olives to olive oil during the
29 production process. This work characterised the principal total bioactive compounds (tocopherols and
30 phenols) in olives, olive oil and by-products of four cultivars grown in Calabrian areas (Southern
31 Italy), considering the effect of drupe harvesting period. Total antioxidant capacity and individual
32 phenolic compounds were also analysed.

33 **Results**

34 Drupes, olive paste, pomace and olive waste water showed similar phenolic compounds, while
35 different composition appeared in olive oil, indicating that phenols are not only transferred, but also
36 they change during oil production. Tocopherols varied among cultivars and drupe harvesting period:
37 generally, they were more abundant in samples produced in the first harvesting period.

38 Qualitative and quantitative differences in phenolic composition and antioxidant activity were
39 significantly found among cultivars in all the matrices.

40 **Conclusion**

41 The highest amount of total phenolic antioxidants ended up in olive waste water with variability due
42 to drupe origin, while only a small part of them finished in the oil. This work evidenced so the
43 availability in bioactive compounds of different portions from the olive oil extraction belonging to
44 different varietal origins. In particular, new information was acquired on Ottobratica Calipa, a new
45 olive clone, that produced an olive oil interesting for its dotation in antioxidants.

46

47 **KEYWORDS: antioxidant activity; olive; olive oil; olive oil waste, phenolic compounds**

48

49 **1. Introduction**

50 The olive oil production is very important in the Mediterranean countries, with a long tradition in
51 Calabria (Southern Italy) where autochthonous and allochthonous olive cultivars are largely
52 cultivated.¹⁻² Qualitative olive and olive oil productions are related to different factors, as cultivars,
53 geographical origin, agronomical practices, harvesting time and extraction plant.³⁻⁴ Olive drupes and
54 olive oil are potential sources of different bioactive compounds, particularly phenolic compounds and
55 tocopherols, respectively hydrophilic and lipophilic constituents, and other antioxidants.⁵

56 During oil extraction, many of these valuable secondary metabolites can be destroyed, degraded or
57 transferred in olive mill waste. Scientific results have showed that the technological phases of milling
58 and malaxation normally affect quality and antioxidant concentration in olive oil.⁶ The olive oil
59 production generates a considerable amount of olive oil mill waste, rich in organic compounds,
60 mainly phenols. In particular, olive oil contains about 1-2% of the total phenolic content of drupes,
61 so only a small part of this phenolic portion is transferred during the extraction process. The residual
62 amount of antioxidant components is in fact lost in olive waste water (53%) and pomace (45%).⁷

63 Currently, olive oil is obtained mainly through three-phase and two-phase centrifugation systems that
64 produce different wastes. Olive mill waste water (consisting in a mixture of olive vegetation water
65 and additional water, useful to promote a better separation of phases), and olive pomace (stone and

66 pulp residues) are generated in the first system. In the second system a more wet olive pomace is
67 produced as waste. The large amount of these wastes and by-products have so a good potential as a
68 source of bioactive components, also if many of these are still undervalued and wasted.⁸ The phenolic
69 compounds from olive and from their by-products are characterised by antimicrobial, anti-
70 inflammatory, chemo-preventive properties.⁹⁻¹⁰ Their antioxidant activity is attributed to different
71 mechanisms, among which prevention of chain initiation, decomposition of peroxides, prevention of
72 continued hydrogen abstraction, binding of transition metal ion catalysts, reductive capacity, and
73 radical scavenging. Phenolic compounds of olive fruit mainly belong to the class of secoiridoids (e.g.
74 oleuropein group of constituents with high nutraceutical and antioxidant potential, useful for food,
75 pharmaceutical and cosmetic applications).¹¹⁻¹²

76 Oleuropein is the major phenolic compound in olive pulp, with relatively high levels in immature
77 olive fruit that decline during the physiological development of the fruit in correspondence of the
78 green and black maturation phases.¹³ Olive mill wastewaters contain several phenolic compounds
79 among which tyrosol, hydroxytyrosol, and oleuropein are the main components: their extraction has
80 been investigated in literature.¹⁴⁻¹⁵ The recovery of bioactive compounds from wastes could be very
81 important to convert them into a resource of natural antioxidants to reuse for different application
82 such as in food (as preservatives) and in pharmaceutical industries (for the human disease prevention),
83 helping the sustainability of the olive oil production management.

84 The knowledge of the phenols partition during the olive oil processing could be very interesting to
85 evaluate their quantity-in the different waste portions and to improve the final olive oil quality. For
86 these reasons, this work aimed to analyse the transfer of antioxidant compounds from drupes to olive
87 paste, pomace, oil and olive wastewater, considering also harvesting time and olive cultivar as
88 variables.

89

90 **2. Materials and methods**

91 **2.1. Samples**

92 The experimental work was performed in Calabria region (Southern Italy), during the olive oil crop
93 season 2019: samples belonged to three olive cultivars: Ottobratica (O), Ciciarello (C) and Tonda di
94 Filogaso (TF), and a new olive clone, named Ottobratica Calipa (OC). This last is not largely
95 cultivated in Calabria, but actually object of research because shows a high olive anthracnose
96 resistance. Fifteen kilograms of drupes per cultivar were harvested in two harvest periods: the second
97 half of October (Oct) at dark green skin colour, and the second half of November (Nov) at black skin
98 colour and submitted to extraction using a small olive oil press mill of the Company Agrimec
99 Valpesana, Calzaiolo, San Casciano (Florence, Italy) at the laboratory of Food Technologies of the
100 Mediterranea University of Reggio Calabria (Italy). The pressure system does not use water and
101 obtains a partition of hydrophilic molecules only by mean of their affinity with solid or fluid phase.
102 Sampling was carried out in triplicate, collecting drupes (D) before processing, paste (OP, at the end
103 of malaxation process); pomace (P); olive waste water (OWW) and extra virgin olive oil (EVO). All
104 the samples were immediately subjected to analyses for the antioxidant compounds evaluation.

105

106 **2.2. Characterization of olive and derivative products (Moisture and fat content)**

107 D (deprived of stone), OP and P were promptly analysed for Moisture content (MC) and fat content.
108 The MC (g kg^{-1}) was determined by gravimetric method (Sartorius Moisture analyzer MA37), drying
109 up to constant mass. Oil content (g kg^{-1}) was extracted following the method reported by Folch et al.
110 (1957).¹⁶

111

112 **2.3 α -tocopherol quantification**

113 D, OP and P fat extracts obtained as reported in 2.2 section, and EVO samples were analysed for
114 tocopherols determination following the method reported by Bakre et al. (2015).¹⁷

115 Sample ($5\mu\text{L}$) was injected into UHPLC system (UHPLC PLATINblue, Knauer, Germany) coupled
116 with fluorescence detector RF-20A/RF-20Axs model (Shimadzu Corporation,) and analysed (flow
117 rate of 0.4 mL min^{-1}) through a mobile phase of methanol/acetonitrile (50:50). The detector was set

118 at 290 nm excitation wavelength and 330 nm emission wavelength. The identification and
119 quantification were performed by calibration curve, using pure α -tocopherol as a standard and
120 concentrations ~~calibration~~ ranging from 10 to 500 mg kg⁻¹. Results were expressed as mg kg⁻¹.

121 **2.4. Polar compounds extraction in samples**

122 The procedure for the extraction of polar compounds was performed for D, OP, P and OWW
123 following the method reported by Othman et al. (2009).¹⁸ 1 g of sample was extracted five times with
124 5 mL of methanol. The extracts were combined, methanol was evaporated under a vacuum (at room
125 temperature) and the residue was dissolved in 5 mL of methanol. Antioxidant phenolic compounds
126 of EVO were instead recovered by liquid–liquid extraction, according to the procedure reported by
127 Baiano et al. (2009).¹⁹ Two millilitres of methanol/water (70:30, v/v) and 2 mL of hexane were added
128 to 5 g of EVO and mixed with a Vortex for 10 min. The hydroalcoholic phase containing phenols
129 was separated from the oil phase by centrifugation (6000 x g, 4 °C, 10 min). Hydroalcoholic phases
130 were collected and submitted to another centrifugation (13000 x g, at room temperature, 4 min) to
131 totally eliminate the residual oil. Finally, hydroalcoholic extracts were recovered and submitted to
132 total phenol and total antioxidant activity determinations and UHPLC analysis.

133

134 **2.4.1 Determination of total phenolic content**

135 The total phenol content was determined following the method described by De Bruno et al. (2018)²⁰
136 with some modifications. An aliquot (0.1 mL) of polar extract (PE), diluted with 20 mL of deionized
137 water, was combined with 0.625 mL of Folin–Ciocalteu reagent and 2.5 mL of saturated solution of
138 Na₂CO₃ (20%) and brought to 25 mL of volume with deionized water. Thereafter, the mixture was
139 incubated for 12 hours at room temperature and dark. The absorbance of the sample was measured at
140 725 nm against a blank using a double-beam ultraviolet-visible spectrophotometer (Agilent 8453
141 UV–Vis, Germany) and compared with a gallic acid calibration curve (1–10 mg L⁻¹). The results were

142 expressed as g of gallic acid (GA) kg⁻¹ for D, OP, P and EVO and g of gallic acid (GA) L⁻¹ for
143 OMWW.

144

145 **2.4.2 Determination of the total antioxidant activity by DPPH and ABTS assays**

146 The determination of the total antioxidant activity by DPPH assay (TAC-DPPH) was performed using
147 the Brand-Williams (1995) method,²¹ which is based on the reaction mechanism between DPPH·
148 (2,2-diphenyl-1-picrylhydrazyl) and antioxidants in the samples. 10 µL of each extract obtained as
149 reported in 2.4 section were added to 6 x 10⁻⁵ mM of DPPH solution to achieve a final volume of 3
150 mL and left in the dark for 30 min till stabilisation. The decrement of absorbance was determined at
151 515 nm against methanol using a spectrophotometer (Agilent 8453UV–Vis, Germany) at 20°C to
152 eliminate the risk of thermal degradation of the tested molecules.²² The total antioxidant activity by
153 ABTS assays (TAC-ABTS) evaluates the capacity of the studied sample to inhibit ABTS (2,2'-azino-
154 bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical oxidation. The reaction mixture was prepared by
155 mixing of ABTS and 10 µL of each extract and the absorbance was measured after 6 min at 734 nm.
156 ²³ For both the antioxidant assays the results were expressed as TEAC values (µmol Trolox g⁻¹ of
157 sample for D, OP, P and EVO and µmol Trolox mL⁻¹ of sample for OMWW); where the quenching
158 of initial absorbance was plotted against the Trolox concentration.

159

160 **2.4.3 Individual phenolic quantification by UHPLC**

161 An aliquot of antioxidant extract (5 µL) was injected in an Ultra High Performance Liquid
162 Chromatography system (UHPLC PLATINblue, Knauer, Germany), equipped with a binary pump
163 system. The quantification of phenolic compounds was carried out using the method reported by
164 Romeo et al. (2019).²³ Knauer Blue Orchid column C18A (1.8 µm, 100 × 2 mm) coupled with a PDA-
165 1 (Photo Diode Array Detector) PLATINblue (Knauer, Germany) was used; the mobile phases were
166 water acidified with acetic acid (pH 3.1) and acetonitrile. External standards were used for the

167 quantification and the results were expressed as mg kg⁻¹ in D, OP and P samples and mg L⁻¹ in OWW
168 samples.

169

170 **2.5. Statistical analysis**

171 Experimental results were expressed as mean ± SD of three measurements (n = 3). The significant
172 differences (p<0.05) among samples were determined by analysis of variance (Multivariate and
173 ANOVA analysis) and Tukey's *post hoc* test.

174 SPSS Software (Version 15.0, SPSS Inc., Chicago, IL, USA) was used for data processing. In
175 addition, Pearson's correlation coefficients (r) determined the relation between studied variables.

176

177 **3. Results**

178 Principal results of moisture and oil content in the different tested samples were reported in Table 1.

179 The moisture percentage in samples was reduced from D (630-670 g kg⁻¹), to OP (550-580 g kg⁻¹)
180 and P (340-410 g kg⁻¹). Considering each studied portion (D, OP and P samples), this parameter did

181 not appear significantly affected by varietal origin or harvesting period, except for OP samples
182 obtained at October, where some differences were observed, and, in particular, TF-OP sample

183 possessed a higher moisture than the other cultivars. The oil content, expressed as g kg⁻¹ on dry matter,

184 tended to decrease progressively from D (370-480 g kg⁻¹) to OP (230-310 g kg⁻¹) and P (90-120 g kg⁻¹).

185 Oil content variations were significantly observed during the two harvesting periods only in OP of

186 Ciciarello cv and in P of Tonda di Filogaso cv, with higher values in November (304 g kg⁻¹ and 195

187 g kg⁻¹ respectively).

188

189 **Table 1** Moisture and oil content of individual matrices (D, OP, and P) from different cultivars at two harvesting periods. The data
190 are presented as means ± SDs. Means within a column with different letters are significantly different by Tukey's post hoc test.

191 Abbreviation: ns: not significant; **Significance at p<0 .01; *Significance at p<0 .05

M	?	D	OP	P
---	---	---	----	---

	<i>Cv</i>	<i>Oct</i>	<i>Nov</i>	<i>Sign</i>	<i>Oct</i>	<i>Nov</i>	<i>Sign</i>	<i>Oct</i>	<i>Nov</i>	<i>Sign</i>
	O	655±50	612±34	<i>ns</i>	557±4.2b	529±5.9	*	351±7.6	388±12	<i>ns</i>
	OC	647±41	613±13	<i>ns</i>	563±45ab	596±13	<i>ns</i>	363±27	370±19	<i>ns</i>
	TF	630±2	524±29	*	585±9a	476±38	<i>ns</i>	412±0	365±6	**
	C	671±18	649±41	<i>ns</i>	549±24b	570±46	<i>ns</i>	346±3	393±16	<i>ns</i>
	<i>Sign</i>	<i>ns</i>	<i>ns</i>		*	<i>ns</i>		<i>ns</i>	<i>ns</i>	
Oil (g kg dm⁻¹)	O	450±18	450±58	ns	233±2	288±29	ns	145±3	212±34	ns
	OC	43±15	383±65	ns	240±29	291±46	ns	163±1	273±64	ns
	TF	405±13	371±51	ns	234±59	307±59	ns	147±10	187±0	*
	C	449±59	483±50	ns	226±3	304±17	*	136±33	195±16	<i>ns</i>
	<i>Sign</i>	ns	ns		ns	ns		ns	ns	

192

193 Vitamin E (α -tocopherol) is the major lipophilic antioxidant, and an efficient scavenger of alkoxyyl
194 and peroxy radicals. Its content was detected in D, OP and EVO, as reported in Table 2: in drupes
195 it ranged from 0.1 to 36 mg kg⁻¹, with higher amount in Oct-D than in Nov-D, except for Ciciarello
196 drupes. α -tocopherol concentration ranged from 0.3 to 57 mg kg⁻¹ in OP samples, with significant
197 differences among cultivars and harvesting times: samples obtained at November showed major
198 quantities, with the only exception of Tonda di Filogaso cv. EVO samples possessed 216–312 mg kg⁻¹
199 of α -tocopherol: these results are similar than those reported in literature.²⁴⁻²⁵ Between harvesting
200 periods, higher amounts were quantified in EVO of October: only TF-EVO did not show significant
201 differences (p>0.05). Also among studied cultivar significant differences were observed, and in
202 particular EVO of Ottobratica Calipa clone stood out for the absolute highest concentration of this
203 important antioxidant at both harvesting periods.

204 Regarding the determination of TPC in samples, a decrease of total phenols was observed during
205 drupe harvesting time in all the studied cultivars, as expected: the order for TPC in olives collected
206 at October was TF>O>C>OC with the range of 5.76-8,70 g GA kg⁻¹.

207 Only a small quantity of the total phenols was transferred from drupes to the oils: EVO in fact
208 possessed 0.03-1.15 g GA kg⁻¹, with higher results generally in the first production period. EVO of

209 Ottobratica cv extracted in October were the samples with the highest TPC (1.15 g GA kg⁻¹) among
210 studied samples and respect other observations for the same cultivar and similar extraction
211 processing.²⁶⁻²⁸

212 Despite the varietal differences, this parameter can indeed vary among crop years, linked to the plant
213 response toward environmental conditions.²⁹⁻³⁰ Concerning the produced wastes by the oil extraction,
214 OWW showed higher TPC (4.3-7.5 g GA L⁻¹) than P samples (0.8-4.4 g GA kg⁻¹) with a general
215 reduction during the harvesting time. The variation of total antioxidant capacity of vegetables may
216 depend on the growing season, geographical origin, and agricultural practices.³¹ Figure 1 shows the
217 partition results of total phenolic compounds in the different portions (P, OWW, EVO) obtained after
218 the olive oil production. A large amount of TPC possessed by D ended up in OWW. This portion
219 showed in fact the highest percentage, ranging from 57 to 84% among samples. These data confirmed
220 the high water affinity of phenols. In a pressure system, such that used in this research where adding
221 water was not used, the substances showed the real affinity for the aqueous phase without additional
222 dilution. TPC varied from 11 to 40% in P samples and they were in the range of 0-10% in EVO.

223 Concerning TAC-DPPH, a trend similar to TPC determinations was observed, with the highest value
224 expressed by D of Tonda di Filogaso cv (189.71 $\mu\text{mol Trolox g}^{-1}$). A high and positive correlation
225 was calculated between TPC and DPPH in D of Ottobratica, Ottobratica Calipa and Tonda di Filogaso
226 harvested at October (respectively $r=0.863$, 0.777 and 0.969), whereas lower Pearson coefficients
227 were observed at November ($r=0.802$, 0.527 , 0.544 and 0.697 , for O, OC, TF and C). Antioxidant
228 capacity evaluated through ABTS assay showed significantly higher results in all the studied samples
229 ($194\text{-}4795 \mu\text{mol Trolox g}^{-1}$) compared to DPPH assay ($1\text{-}190 \mu\text{mol Trolox g}^{-1}$), as also confirmed by
230 literature.³² This is probably due to the different composition of analysed samples containing
231 hydrophilic and lipophilic antioxidant compounds. Indeed, the ABTS assay is more applicable to both
232 hydrophilic and lipophilic antioxidant systems; whereas DPPH assay is more related to hydrophobic
233 system response (Kim et al., 2002).³³ Among OWW, samples of Ottobratica Calipa cv processed in
234 November expressed the highest TAC ($4795 \mu\text{mol Trolox mL}^{-1}$). Moreover, higher positive

235 correlation between TPC and ABTS was found in all portions for O, OC, TF and C obtained in the
 236 second harvest period ($r= 0.985, 0.995, 0.982, 0.950$) than results of previous sampling ($r= 0.498,$
 237 $0.824, 0.771, -0.650$).

238

239 **Table 2** α -tocopherol, TPC and total antioxidant capacity (DPPH and ABTS) values for the samples (D, OP, P: Pomace; OWW:
 240 olive waste water; EVO: oil). The data are presented as means. Means within a column with different letters are significantly
 241 different by Tukey's post hoc test. Abbreviation: ns: not significant; **Significance at $p<0.01$; *Significance at $p<0.05$

Portion	Cv	Harvesting	α -tocopherol (mg kg ⁻¹)	TPC (g GA kg ⁻¹ g GA L ⁻¹)	TAC –DPPH (μ mol Trolox g ⁻¹ μ mol Trolox mL ⁻¹)	TAC – ABTS (μ mol Trolox g ⁻¹ μ mol Trolox mL ⁻¹)	
D	O	<i>Oct</i>	32.55 ^{ab}	8.18 ^{ab}	90.51 ^b	711.26 ^d	
		<i>Nov</i>	3.45 ^e	3.85 ^d	3.07 ^e	654.69 ^d	
	OC	<i>Oct</i>	6.95 ^{de}	5.76 ^c	31.87 ^d	787.05 ^d	
		<i>Nov</i>	0.19 ^e	3.48 ^d	29.13 ^d	4272.42 ^a	
	TF	<i>Oct</i>	23.05 ^{bc}	8.70 ^a	189.71 ^a	2093.48 ^c	
		<i>Nov</i>	19.09 ^{cd}	5.06 ^c	16.18 ^{de}	2162.40 ^c	
	C	<i>Oct</i>	0.11 ^e	7.56 ^b	64.39 ^c	655.03 ^d	
		<i>Nov</i>	36.05 ^a	4.94 ^c	27.15 ^d	3387.22 ^b	
	Sign.		**	**	**	**	
	OP	O	<i>Oct</i>	8.13 ^e	6.86 ^a	58.08 ^b	590.93 ^c
			<i>Nov</i>	12.95 ^d	4.02 ^{cd}	6.71 ^e	600.38 ^c
		OC	<i>Oct</i>	0.33 ^f	5.13 ^b	25.41 ^{cd}	528.14 ^c
			<i>Nov</i>	33.20 ^c	3.43 ^{de}	21.09 ^d	3903.21 ^a
		TF	<i>Oct</i>	57.20 ^a	6.76 ^a	134.55 ^a	1684.46 ^b
<i>Nov</i>			37.50 ^b	3.15 ^e	8.95 ^e	1981.02 ^b	
C		<i>Oct</i>	0.36 ^f	5.70 ^b	33.36 ^c	537.98 ^c	
		<i>Nov</i>	33.20 ^c	4.42 ^c	20.61 ^d	4083.87 ^a	
Sign.			**	**	**	**	
P		O	<i>Oct</i>	/	3.16 ^b	1.67 ^b	281.70 ^e
			<i>Nov</i>	/	1.27 ^{de}	2.14 ^b	302.75 ^{de}
		OC	<i>Oct</i>	/	1.01 ^{de}	0.49 ^b	291.01 ^{de}
			<i>Nov</i>	/	2.00 ^{bed}	59.37 ^a	2409.06 ^b

	TF	<i>Oct</i>	/	2.54 ^{bc}	2.42 ^b	753.69 ^c
		<i>Nov</i>	/	0.81 ^e	3.81 ^b	650.01 ^c
	C	<i>Oct</i>	/	4.44 ^a	2.48 ^b	421.85 ^d
		<i>Nov</i>	/	1.89 ^{cde}	53.10 ^a	2591.66 ^a
	Sign.			**	**	**
OWW	O	<i>Oct</i>	/	7.15 ^a	71.32 ^c	1444.70 ^d
		<i>Nov</i>	/	6.66 ^{ab}	34.17 ^e	779.55 ^d
	OC	<i>Oct</i>	/	6.82 ^{ab}	27.43 ^e	1412.09 ^d
		<i>Nov</i>	/	4.41 ^c	143.53 ^{ab}	4795.45 ^a
	TF	<i>Oct</i>	/	7.56 ^a	155.29 ^a	3953.15 ^b
		<i>Nov</i>	/	4.39 ^c	53.30 ^d	2262.03 ^c
	C	<i>Oct</i>	/	5.98 ^b	35.25 ^e	1125.01 ^d
		<i>Nov</i>	/	7.00 ^a	130.27 ^b	4708.11 ^{ab}
	Sign.			**	**	**
EVO	O	<i>Oct</i>	258.69 ^b	1.15 ^a	28.45 ^b	590.66 ^c
		<i>Nov</i>	216.32 ^c	0.03 ^d	1.02 ^f	193.61 ^f
	OC	<i>Oct</i>	333.72 ^a	1.10 ^a	20.53 ^c	341.60 ^e
		<i>Nov</i>	301.57 ^a	0.33 ^c	26.34 ^b	465.03 ^d
	TF	<i>Oct</i>	224.99 ^c	0.57 ^b	11.99 ^d	974.39 ^b
		<i>Nov</i>	231.64 ^c	0.44 ^{bc}	8.03 ^e	553.47 ^c
	C	<i>Oct</i>	311.96 ^a	0.39 ^{bc}	55.31 ^a	1636.13 ^a
		<i>Nov</i>	221.30 ^c	0.42 ^{bc}	7.82 ^e	553.73 ^c
	Sign.		**	**	**	**

242

243

244 Figure 2 reports as example the obtained chromatograms by UHPLC for samples from Ottobratica
 245 cv. with the identification of the major compounds.

246 As reported in Table 3 a and b the phenol composition varied significantly among cultivars and among
 247 the different portions, despite the same oil extraction system was used. For this reason, the effect of
 248 the technological process may not be considered. The varietal effect on these components was
 249 confirmed by literature,³⁴⁻³⁵ proving the genetic and agronomical effect on phenolic compounds of
 250 olive fruits and oil.

251 Oleuropein is bitter-tasting secoiridoid glycoside and the main phenolic compound present in olives,
252 that tends to notably decrease during fruit ripening and processing. In our study the highest amount
253 of this compound was instead detected in D of Ciciarello cv harvested at November (448.20 mg kg⁻¹)
254 ¹), followed by D of Ottobratica cv harvested at October (409.02 mg kg⁻¹). In general, oleuropein
255 tended to decrease during malaxation process, as evidenced by comparison between D and OP. In our
256 study, OWW samples of O, OC and TF possessed high content of hydroxytyrosol, particularly those
257 produced in November (TF>O>OC, respectively 304.25, 263.36, 116.88 mg L⁻¹): this high amount
258 in OWW can be justified by the hydrolysis of oleuropein.³⁶ Hydroxytyrosol is in fact obtained by
259 chemical and/or enzymatic hydrolysis of oleuropein during olive mill extraction and is the most active
260 phenol in olive oil and by-products.³⁷ Ciciarello samples showed instead a different trend of this
261 compound, that was more abundant in P of both harvesting periods, denoting a different varietal
262 response linked to the different initial oleuropein concentration in drupes, varying in parallel with the
263 hydrolysis of that compound of higher molecular weight. Tyrosol is another important antioxidant,
264 derived by oleuropein metabolism with essential health properties for humans.¹⁵ Among the
265 considered olive by-products that can be valorised for biophenols recovery, the highest quantities
266 were quantified in OWW of Tonda di Filogaso and Ottobratica collected at November (36.31 mg L⁻¹
267 and 34.97 mg L⁻¹, respectively). Around 10 mg L⁻¹ of tyrosol were instead quantified in OWW of
268 Ottobratica Calipa and in P of Ciciarello, as the major content. With the only exception of Ottobratica
269 Calipa clone, tyrosol was more abundant in OWW obtained at November than in October, ranging
270 from 8.66 to 36.31 mg L⁻¹.

271 Rutin and luteolin 7-*O*-glucoside are the major flavonoids found in olive drupes with some
272 differences among origin and harvesting variables. The highest amounts were observed in D-Nov of
273 Tonda di Filogaso cv for rutin (139.08 mg kg⁻¹) and in D-Oct of Ciciarello cv (46.25 mg kg⁻¹) for
274 luteolin 7-*O*-glucoside. Health beneficial effects of flavonoids and lignans have appeared, such those
275 related to cancer and coronary heart diseases.³⁸⁻³⁹ Among lignans, the highest concentration of
276 pinosresinol was possessed by D of Ottobratica Calipa clone (81.95 mg kg⁻¹) and tended to decrease

277 generally in samples obtained at later time. Pinoresinol varied in concentration depending on all three
278 variables (varietal origin, harvesting period and portions): the reasons probably being also related to
279 differences among the production zones and in the climate. ⁴⁰EVO analysed in our studies possessed
280 also higher pinoresinol concentration than those reported in literature: ⁴⁰⁻⁴¹ in Tonda di Filogaso olive
281 oils it was more than 100 mg kg⁻¹.

Table 3 (a): Changes in the amount of individual phenolic compounds (mg kg⁻¹ and mg L⁻¹) in the analysed samples of Ottobratica and Ottobratica Calipa cvs (D. OP. P. OWW. O). The data are presented as means. Means within a column with different letters are significantly different by Tukey's post hoc test. Abbr.: ns: not significant; **Significance at p<0 .01; *Significance at p<0 .05

Phenolic Compound		<i>Ottobratica cv</i>						<i>Ottobratica Calipa cv</i>					
		D	OP	P	OWW	EVO	Sign.	D	OP	P	OWW	EVO	Sign.
Hydroxytyrosol	Oct	164.81 ^b	139.29 ^c	41.71 ^d	215.54 ^a	5.20 ^e	**	81.17 ^b	86.99 ^b	22.22 ^c	110.24 ^a	5.45 ^c	**
	Nov	64.77 ^c	132.11 ^b	45.20 ^{cd}	263.36 ^a	3.84 ^d	**	92.87 ^b	89.80 ^b	35.94 ^c	116.88 ^a	2.03 ^d	**
Tyrosol	Oct	21.45 ^b	29.79 ^a	21.49 ^b	29.94 ^a	8.66 ^c	**	5.45 ^b	9.28 ^a	6.83 ^{ab}	9.72 ^a	5.65 ^b	**
	Nov	7.35 ^b	28.09 ^a	6.35 ^b	34.97 ^a	9.30 ^b	**	5.71 ^{bc}	8.50 ^{ab}	7.98 ^{ab}	9.55 ^a	4.04 ^c	**
Vanillic Acid	Oct	0.65 ^d	4.54 ^b	2.10 ^c	5.83 ^a	0.66 ^d	**	2.05 ^b	3.18 ^a	2.18 ^b	1.36 ^c	0.78 ^d	**
	Nov	0.15 ^b	0.62 ^b	0.38 ^b	1.54 ^a	tr	**	0.58 ^c	4.16 ^a	3.08 ^b	4.03 ^a	0.34 ^c	**
Homovanillic Acid	Oct	9.79 ^b	13.50 ^{ab}	tr	18.11 ^a	nd	**	tr	2.05 ^b	tr	3.20 ^a	nd	**
	Nov	tr	91.67 ^b	tr	174.23 ^a	nd	**	tr	0.92	tr	tr	nd	**
Vanillin	Oct	5.10 ^a	3.65 ^b	2.24 ^c	4.14 ^b	0.60 ^d	**	2.62 ^a	2.92 ^a	1.58 ^{ab}	2.26 ^a	0.56 ^c	**
	Nov	1.53 ^c	3.49 ^b	1.07 ^{cd}	4.38 ^a	0.56 ^d	**	3.31 ^a	2.99 ^a	2.02 ^b	2.53 ^{ab}	0.55 ^c	**
Rutin	Oct	78.14 ^a	7.78 ^b	4.01 ^b	8.36 ^b	3.64 ^b	**	25.78 ^a	7.55 ^{bc}	2.25 ^c	11.74 ^b	3.21 ^c	**
	Nov	46.88 ^a	4.46 ^c	2.27 ^c	32.44 ^b	2.86 ^c	**	12.95 ^a	5.33 ^c	2.86 ^d	7.21 ^b	3.05 ^d	**
Luteolin-7- <i>o</i> -G	Oct	24.67 ^a	7.95 ^c	7.91 ^c	11.89 ^b	11.61 ^b	**	26.46 ^a	13.94 ^b	8.28 ^b	22.02 ^a	11.27 ^b	**
	Nov	8.66 ^a	7.74 ^b	7.39 ^c	8.66 ^a	7.64 ^b	**	20.10 ^a	13.67 ^{bc}	7.81 ^d	16.63 ^{ab}	11.04 ^{cd}	**
Oleuropein	Oct	409.02 ^a	22.47 ^b	4.44 ^b	9.67 ^b	3.10 ^b	**	230.15 ^a	8.73 ^b	2.60 ^b	16.11 ^b	5.71 ^b	**
	Nov	128.10 ^a	3.56 ^b	0.89 ^b	1.48 ^b	tr	**	222.25 ^a	8.89 ^{bc}	3.15 ^d	11.74 ^b	3.61 ^{cd}	**
Pinoresinol	Oct	40.78 ^b	26.09 ^c	21.38 ^c	45.04 ^b	78.66 ^a	**	81.95 ^a	50.19 ^b	14.47 ^c	73.42 ^a	72.39 ^a	**
	Nov	3.46 ^b	20.07 ^a	5.94 ^b	19.23 ^a	6.87 ^b	**	60.17 ^{ab}	18.42 ^c	22.62 ^c	53.81 ^b	68.1 ^a	**

Apigenin	Oct	13.39 ^a	1.45 ^b	1.72 ^b	tr	tr	**	0.66 ^b	1.16 ^a	0.53 ^b	tr	tr	**
	Nov	tr	3.59 ^b	tr	4.62 ^a	tr	**	1.88 ^a	tr	0.83 ^b	tr	tr	**

Table 3 (b): Changes in the amount of individual phenolic compounds (mg kg⁻¹ and mg L⁻¹) in the analysed samples of Tonda di Filogaso and Ciciarello cvs (D. OP. P. OWW. O). The data are presented as means. Means within a column with different letters are significantly different by Tukey's post hoc test. Abbr.: ns: not significant; **Significance at p<0 .01; *Significance at p<0 .05

Phenolic Compound		<i>Tonda di Filogaso cv</i>					<i>Ciciarello cv</i>						
		D	OP	P	OWW	EVO	D	OP	P	OWW	EVO		
Hydroxytyrosol	Oct	84.62 ^b	65.30 ^c	12.07 ^d	115.86 ^a	3.02 ^d	**	44.59 ^b	34.31 ^c	53.82 ^a	5.20 ^d	5.45 ^d	**
	Nov	204.20 ^c	227.26 ^b	31.11 ^{cd}	304.25 ^a	8.71 ^e	**	29.06 ^b	25.26 ^c	35.44 ^a	3.84 ^d	2.03 ^d	**
Tyrosol	Oct	13.63 ^{bc}	12.37 ^c	20.02 ^a	15.85 ^b	7.55 ^d	**	11.78 ^{ab}	10.33 ^{bc}	13.51 ^a	8.66 ^c	5.65 ^d	**
	Nov	9.52 ^b	38.33 ^a	15.24 ^b	36.31 ^a	11.18 ^b	**	8.63 ^a	5.44 ^b	10.26 ^a	9.30 ^a	5.26 ^b	**
Vanillic Acid	Oct	0.09 ^c	1.58 ^a	tr	1.07 ^b	tr	**	2.17 ^{ab}	1.51 ^b	3.44 ^a	0.66 ^b	0.78 ^b	**
	Nov	0.37 ^c	1.21 ^b	tr	1.96 ^a	tr	**	0.64 ^b	0.02 ^c	1.46 ^a	tr	0.35 ^{bc}	**
Homovanillic Acid	Oct	5.86 ^c	19.76 ^a	tr	14.49 ^b	nd	**	11.80 ^b	3.76 ^c	21.49 ^a	tr	nd	**

	Nov	9.66	34.99	12.96	56.96	nd	ns	7.27 ^b	2.03 ^c	15.84 ^a	tr	nd	**
Vanillin	Oct	1.72 ^{bc}	2.85 ^a	1.11 ^{cd}	2.57 ^{ab}	0.56 ^d	**	4.45 ^{ab}	2.88 ^b	5.85 ^a	0.60 ^c	0.56 ^c	**
	Nov	4.29 ^b	1.85 ^c	1.31 ^c	7.06 ^a	0.57 ^c	**	3.72 ^a	2.59 ^b	4.62 ^a	0.56 ^c	0.55 ^c	**
Rutin	Oct	17.82 ^a	6.18 ^c	3.14 ^d	9.76 ^b	5.48 ^c	**	4.35 ^b	2.40 ^d	5.25 ^a	3.64 ^{bc}	3.21 ^c	**
	Nov	139.08 ^a	9.53 ^b	3.36 ^b	10.21 ^b	4.67 ^b	**	4.33 ^b	3.07 ^c	6.34 ^a	2.86 ^c	3.05 ^c	**
Luteolin-7-O-glucoside	Oct	21.50 ^b	13.83 ^c	9.21 ^d	26.61 ^a	15.34 ^c	**	46.25 ^a	10.78 ^c	9.46 ^c	16.11 ^b	12.19 ^{bc}	**
	Nov	14.65 ^a	12.06 ^b	7.59 ^c	14.25 ^{ab}	12.94 ^{ab}	**	12.17	10.19	7.62	13.96	10.20	ns
Oleuropein	Oct	306.85 ^a	25.97 ^b	7.00 ^d	12.29 ^c	tr	**	475.50 ^b	23.11 ^a	16.18 ^b	3.10 ^c	5.71 ^c	**
	Nov	249.76 ^a	14.27 ^b	0.69 ^c	16.88 ^b	tr	**	448.20 ^{ab}	1.18 ^b ^c	4.20 ^a	tr	2.92 ^{abc}	**
Pinoresinol	Oct	33.06 ^c	60.29 ^b	59.49 ^b	41.97 ^c	133.25 ^a	**	32.17 ^c	17.07 ^d	66.32 ^b	78.66 ^a	72.39 ^{ab}	**
	Nov	19.70 ^d	50.46 ^c	83.52 ^b	21.82 ^d	102.57 ^a	**	13.48 ^d	38.88 ^b	28.80 ^c	6.87 ^d	68.19 ^a	**
Apigenin	Oct	12.24 ^a	3.08 ^b	tr	1.97 ^b	tr	**	9.72 ^a	tr	3.30 ^b	tr	tr	**
	Nov	1.54 ^b	0.48 ^c	4.13 ^a	tr	tr	**	4.17 ^a	tr	0.35 ^b	tr	tr	**

Conclusions

In the present study antioxidant compounds displayed clear qualitative and quantitative differences among studied cultivars; in particular, their partition during oil extraction process and their related radical scavenging activity are specific for the olive characters defined by cultivar and harvesting time. As well known, harvesting time and climate conditions influence the phenol composition as ratios of phenol compounds and their total amount in drupes. Also, the enzyme activity influences phenols and its partition in oil, wastewater and pomace. The hydrolysis of complex glycoside phenols produces more reactive molecules that act as antioxidants; in the same way they quickly disappear from the olive paste and derived fractions. The driving of the extraction process is an important factor to obtain high quality oil but nowadays also to recover high quality-antioxidant fraction that could be exploited for other uses. This work confirms the high specificity of the olive varietal characters, that impacts on the applied process: probably the best results could be obtained by a customization of the malaxation and separation steps during olive oil extraction. Finally, this work evidences the possibility to promote the cultivation of Ottobratica Calipa clone in the areas considered in this research for the good quality of olive productions, in particular concerning α -tocopherol and phenol content in EVO.

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Conflict of interest statement

All authors confirmed no conflicts of interest.

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Figure legends

Figure 1. Distribution of TPC among the different portions (P, OWW and EVO)

Figure 2. UHPLC chromatograms of the different portions (D, OP, EVO, P and OWW) of Ottobratica cv, Identified compounds: (1) hydroxytyrosol; (2) tyrosol; (3) vanillic acid; (4) homovanillic acid; (5) vanillin; (6) rutin; (7) luteolin-7-*O*-glucoside (8) oleuropein; (9) pinoresinol; (10) apigenin.