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

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

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Abstract

Background

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

Results

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

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

Conclusion

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

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

1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1. Samples

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

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

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

2.3 α-tocopherol quantification

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

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

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

2.4. Polar compounds extraction in samples

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

2.4.1 Determination of total phenolic content

The total phenol content was determined following the method described by De Bruno et al. (2018)[20] with some modifications. An aliquot (0.1 mL) of polar extract (PE), diluted with 20 mL of deionized water, was combined with 0.625 mL of Folin–Ciocalteau reagent and 2.5 mL of saturated solution of Na_2CO_3 (20%) and brought to 25 mL of volume with deionized water. Thereafter, the mixture was incubated for 12 hours at room temperature and dark. The absorbance of the sample was measured at 725 nm against a blank using a double-beam ultraviolet-visible spectrophotometer (Agilent 8453 UV–Vis, Germany) and compared with a gallic acid calibration curve (1-10 mg L^{-1}). The results were

expressed as g of gallic acid (GA) kg^{-1} for D, OP, P and EVO and g of gallic acid (GA) L^{-1} for OMWW.

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

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

2.4.3 Individual phenolic quantification by UHPLC

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

quantification and the results were expressed as mg kg^{-1} in D, OP and P samples and mg L^{-1} in OWW samples.

2.5. Statistical analysis

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

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

3. Results

Principal results of moisture and oil content in the different tested samples were reported in Table 1. The moisture percentage in samples was reduced from D (630-670 g kg^{-1}), to OP (550-580 g kg^{-1}) and P (340-410 g kg^{-1}). Considering each studied portion (D, OP and P samples), this parameter did not appear significantly affected by varietal origin or harvesting period, except for OP samples obtained at October, where some differences were observed, and, in particular, TF-OP sample possessed a higher moisture than the other cultivars. The oil content, expressed as g kg^{-1} on dry matter, 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}). Oil content variations were significantly observed during the two harvesting periods only in OP of Ciciarello cv and in P of Tonda di Filogaso cv, with higher values in November (304 g kg^{-1} and 195 g kg^{-1} respectively).

Table 1 Moisture and oil content of individual matrices (D, OP, and P) from different cultivars at two harvesting periods. The data are presented as means ± SDs. Means within a column with different letters are significantly different by Tukey's post hoc test. Abbreviation: ns: not significant; **Significance at p<0 .01; *Significance at p<0 .05

M	D	OP	P

	Cv	*Oct*	*Nov*	*Sign*	*Oct*	*Nov*	*Sign*	*Oct*	*Nov*	*Sign*
	O	655±50	612±34	***ns***	557±4.2b	529±5.9	*****	351±7.6	388±12	***ns***
	OC	647±41	613±13	***ns***	563±45ab	596±13	***ns***	363±27	370±19	***ns***
	TF	630±2	524±29	*****	585±9a	476±38	***ns***	412±0	365±6	******
	C	671±18	649±41	***ns***	549±24b	570±46	***ns***	346±3	393±16	***ns***
	Sign	***ns***	***ns***		*****	***ns***		***ns***	***ns***	
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	***ns***
	Sign	**ns**	**ns**		**ns**	**ns**		**ns**	**ns**	

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

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

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

Ottobratica cv extracted in October were the samples with the highest TPC (1.15 g GA kg^{-1}) among studied samples and respect other observations for the same cultivar and similar extraction processing. [26-28]

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

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

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

Table 2 α-tocopherol, TPC and total antioxidant capacity (DPPH and ABTS) values for the samples (D, OP, P: Pomace; OWW: olive waste water; EVO: oil). The data are presented as means. Means within a column with different letters are significantly 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 (µmol Trolox g^{-1} µmol Trolox mL^{-1})	TAC – ABTS (µmol Trolox g^{-1} µmol Trolox mL^{-1})
D	**O**	*Oct*	32.55^{ab}	8,18^{ab}	90.51^{b}	711.26^{d}
		Nov	3.45^{e}	3.85^{d}	3.07^{e}	654.69^{d}
	OC	*Oct*	6.95^{de}	5.76^{c}	31.87^{d}	787.05^{d}
		Nov	0.19^{e}	3.48^{d}	29.13^{d}	4272.42^{a}
	TF	*Oct*	23.05^{bc}	8.70^{a}	189.71^{a}	2093.48^{c}
		Nov	19.09^{cd}	5.06^{c}	16.18^{de}	2162.40^{c}
	C	*Oct*	0.11^{e}	7.56^{b}	64.39^{c}	655.03^{d}
		Nov	36.05^{a}	4.94^{c}	27.15^{d}	3387.22^{b}
	Sign.		**	**	**	**
OP	**O**	*Oct*	8.13^{e}	6.86^{a}	58.08^{b}	590.93^{c}
		Nov	12.95^{d}	4.02^{cd}	6.71^{e}	600.38^{c}
	OC	*Oct*	0.33^{f}	5.13^{b}	25.41^{cd}	528.14^{c}
		Nov	33.20^{c}	3.43^{de}	21.09^{d}	3903.21^{a}
	TF	*Oct*	57.20^{a}	6.76^{a}	134.55^{a}	1684.46^{b}
		Nov	37.50^{b}	3.15^{e}	8.95^{e}	1981.02^{b}
	C	*Oct*	0.36^{f}	5.70^{b}	33.36^{c}	537.98^{c}
		Nov	33.20^{c}	4.42^{c}	20.61^{d}	4083.87^{a}
	Sign.		**	**	**	**
P	**O**	*Oct*	/	3.16^{b}	1.67^{b}	281.70^{e}
		Nov	/	1.27^{de}	2.14^{b}	302.75^{de}
	OC	*Oct*	/	1.01^{de}	0.49^{b}	291.01^{de}
		Nov	/	2.00^{bcd}	59.37^{a}	2409.06^{b}

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

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

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

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

Rutin and luteolin 7-*O*-glucoside are the major flavonoids found in olive drupes with some differences among origin and harvesting variables. The highest amounts were observed in D-Nov of Tonda di Filogaso cv for rutin (139.08 mg kg^{-1}) and in D-Oct of Ciciarello cv (46.25 mg kg^{-1}) for luteolin 7-*O*-glucoside. Health beneficial effects of flavonoids and lignans ~~have~~ appeared, such those related to cancer and coronary heart diseases.[38-39] Among lignans, the highest concentration of pinoresinol 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. [40] EVO 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		*Ottobratica cv*						*Ottobratica Calipa cv*					
		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-*o*-G	**Oct**	24.67^{a}	7.95c	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		***Tonda di Filogaso cv***						***Ciciarello cv***					
		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}	1.18b^{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.

References

1 Mafrica R, Piscopo A, De Bruno A, Pellegrino P, Zappia A, Zappia R and Poiana M, Integrated Study of Qualitative Olive and Oil Production from Three Important Varieties Grown in Calabria (Southern Italy). *Eur J Lipid Sci Technol* 1900147(2019).

2 Piscopo A, De Bruno A, Zappia A, Ventre C and Poiana M, Characterization of monovarietal olive oils obtained from mills of Calabria region (Southern Italy). *Food Chem* **213**: 313-318 (2016).

3 Conte P, Squeo G, Difonzo G, Caponio F, Fadda C, Del Caro A, Urgeghe PP, Montanari L, Montinaro A and Piga A, Change in quality during ripening of olive fruits and related oils extracted from three minor autochthonous Sardinian cultivars. *Emir J Food Agr* **31**(3): 196-205 (2019).

4 Stillitano T, Falcone G, De Luca AI, Piga A, Conte P, Strano A and Gulisano G, A Life Cycle Perspective to Assess the Environmental and Economic Impacts of Innovative Technologies in Extra Virgin Olive Oil Extraction. *Foods* **8**: 209 (2019).

5 Basumatary B, Bhattacharya S and Baran Das A, Olive (Elaeagnus latifolia) pulp and leather: Characterization after thermal treatment and interrelations among quality attributes. *J Food Eng* **278**: 109948 (2020).

6 Fadda C, Del Caro A, Sanguinetti AM, Urgeghe PP, Vacca V, Arca PP and Piga A, Changes during storage of quality parameters and in vitro antioxidant activity of extra virgin monovarietal oils obtained with two extraction technologies. *Food Chem* **134**: 1542–1548 (2012).

7 Clodoveo ML, Malaxation: Influence on virgin olive oil quality. Past. present and future e an overview. *Trends Food Sci Technol* **25**: 13e23 (2012).

8 Flamminii F, Di Mattia CD, Difonzo G, Neri L, Faieta M, Caponio F and Pittia P, From by-product to food ingredient: evaluation of compositional and technological properties of olive-leaf phenolic extracts. *J Sci Food Agric*. **99**: 6620–6627 (2019).

9 De Bruno A, Zappia A, Piscopo A and Poiana M, Qualitative evaluation of fermented olives grown in Southern Italy (cvs. Carolea. Grossa of Gerace and Nocellara Messinese). *Emir J Food Agr* **31**(8): 587-596 (2019).

10 De Toffoli A, Monteleone E, Bucalossi G, Veneziani G, Fia G, Servili M, Zanoni B, Pagliarini E, Gallina Toschi T and Dinnella C, Sensory and chemical profile of a phenolic extract from olive mill waste waters in plant-base food with varied macro-composition. *Food Res Int* **119:** 236–243. (2019)

11 Soberón LF, Carelli AA, González MT and Ceci LN, Method for phenol recovery from "alperujo": numerical optimization and predictive model. *Eur Food Res Technol* **245**:1641–1650 (2019).

12 Difonzo G, Pasqualone A, Silletti R, Cosmai L, Summo C, Paradiso VM and Caponio F, Use of olive leaf extract to reduce lipid oxidation of baked snacks *Food Res Int* **108**: 48–56 (2018).

13 Artajo LS, Romero MP and Motilva MJ, Transfer of phenolic compounds during olive oil extraction in relation to ripening stage of the fruit. *J Sci Food Agric* **86**: 518 – 527 (2006).

14 Abu-Lafi S, Al-Natsheh MS, Yaghmoor R and Al-Rimawi F, Enrichment of phenolic compounds from olive mill wastewater and in vitro evaluation of their antimicrobial activities. *Evid-Based Compl Alt* **3706915**: 9 (2017).

15 Jiménez-Herrera S, Ochando-Pulidoa JM and Martínez-Fereza A, Comparison between different liquid-liquid and solid phase methods of extraction prior to the identification of the phenolic fraction present in olive oil washing wastewater from the two-phase olive oil extraction system. *Grasas Aceites* **68** (3): e208 (2017).

16 Folch J, Lees M and Soane GH, A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* **226**: 497–50 (1957).

17 Bakre SM, Gadmale DK, Toche RB and Gaikwad VB, Rapid determination of alpha tocopherol in olive oil adulterated with sunflower oil by reversed phase high-performance liquid chromatography. *J Food Sci Technol* **52**(5): 3093–3098 (2015).

18 Othman NB, Roblain D, Chammen N, Thonart P and Hamdi M, Antioxidant phenolic compounds loss during the fermentation of Chétoui olives. *Food Chem* **116**: 662–669 (2009).

19 Baiano A, Gambacorta G, Terracone C, Previtali MA, Lamacchia C and La Notte E, Changes in phenolic content and antioxidant activity of italian extra-virgin olive oils during storage. *J Food Sci* **74**: 2 (2009).

20 De Bruno A, Romeo R, Fedele FL, Sicari A, Piscopo A and Poiana M, Antioxidant activity shown by olive pomace extracts. *J Environ Sci Heal B* **53**: 526–533 (2018).

21 Brand-Williams W, Cuvelier ME and Berset C, Use of free radical method to evaluate antioxidant activity. *Lebensm Wiss Technol* **28**. 25–30 (1995).

22 Bondet V, Brand-Williams W and Berset C, Kinetics and mechanism of antioxidant activity using the DPPH free radical method. *Lebensm Wiss Technol* **30**. 609–615 (1997).

23 Romeo R, De Bruno A, Imeneo V, Piscopo A and Poiana M, Evaluation of enrichment with antioxidants from olive oil mill wastes in hydrophilic model system. *J Food Process Preserv* **43** (11): e14211 (2019).

24 Piscopo A, De Bruno A, Zappia A, Gioffre G, Grillone N, Mafrica R and Poiana M, Effect of olive storage temperature on the quality of Carolea and Ottobratica oils. *Emir J Food Agric* **30**: 563-572 (2018).

25 Gliszczyńska-Świgło A, Sikorska E, Khmelinskii I and Sikorski M, Tocopherol content in edible plant oils. *Pol J Food Nutr Sci* **57** (4 A):157–161 (2007).

26 Fontana A, Piscopo A, De Bruno A, Tiberini A, Muzzalupo I and Albanese G, Impact of Olive Leaf Yellowing Associated Virus on Olive (Olea europaea L.). *Oil Eur J Lipid Sci Technol* 1800472(2019).

27 Sicari V, Giuffrè AM, Piscopo A and Poiana M, Effect of ‘‘Ottobratica” variety ripening stage on the phenolic profile of the obtained olive oil. *Riv Ital Sostanze Gr* **86**: 215–219 (2009).

28 Piscopo A, Zappia A, De Bruno A and Poiana M, Effect of the harvesting time on the quality of olive oils produced in Calabria. *Eur J Lipid Sci Technol* **120**: 1700304 (2018a).

29 Borges TH, López LC, Pereira JA, Cabrera–Vique C and Seiquer I, Comparative analysis of minor bioactive constituents (CoQ10, tocopherols and phenolic compounds) in Arbequina extra virgin olive oils from Brazil and Spain. *J Food Comp Anal* **63**: 47-54 (2017).

30 Di Vaio C, Nocerino S, Paduano A and Sacchi R, Influence of some environmental factors on drupe maturation and olive oil composition. *J Sci Food Agric* **93**: 1134-1139 (2013).

31 Chun OK, Kim DO, Smith N, Schroeder D, Han JT and Lee CY, Daily consumption of phenolics and total antioxidant capacity from fruits and vegetables in the American diet. *J Sci Food Agric* **85**: 1715–1724 (2005).

32 Floegel A, Kim DO, Chung SJ, Koo SI and Chun OK, Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. *J Food Compos Anal* **24**: 1043–1048 (2011).

33 Kim DO, Lee KW, Lee HJ and Lee CY, Vitamin C equivalent antioxidant capacity (VCEAC) of phenolic phytochemicals. *J Agric Food Chem* **50**: 3713–3717 (2002).

34 Talhaoui N, Gómez-Caravaca AM, León L, De la Rosa R, Fernández-Gutiérrez A and Segura-Carretero A, From olive fruits to olive oil: phenolic compound transfer in six different olive cultivars grown under the same agronomical conditions. *Int J Mol Sci* **17**: 337 (2016).

35 Cioffi G, Pesca MS, Caprariis P, Braca A, Severino L and De Tommasi N, Phenolic compounds in olive oil and olive pomace from Cilento (Campania, Italy) and their antioxidant activity. *Food Chem* **121**: 105–111 (2010).

36 Di Donna L, Benabdelkamel H, Mazzotti F, Napoli A, Nardi M and Sindona G, High-throughput assay of oleopentanedialdheydes in extra virgin olive oil by the UHPLC-ESI-MS/MS and isotope 148 dilution methods. *Anal Chem* **83**:1990-1995 (2011).

37 Ozdemir Y, Guven E and Ozturk A, Understanding the characteristics of oleuropein for table olive processing. *J Food Process Technol* **5**: 328 (2014).

38, Kanadaswami C, Lee LT, Lee PPH, Hwang JJ, Ke FC, Huanh YT and Lee MT, The antitumour activities of flavonoids. *In vivo:* **19**: 895–909 (2005).

39 MacRae WD and Towers GHN, Biological Activities of Lignans. *Phytochemistry* **23**:1207-1220 (1984),

40 Tripoli E, Giammanco M, Tabacchi G, Di Majo D, Giammanco S and La Guardia M, The phenolic compounds of olive oil: Structure, biological activity and beneficial effects on human health. *Nutr Res Rev*: **18**(1), 98-112 (2005).

41 Brenes M, Hidalgo FJ, García A, Rios JJ, García P, Zamora R and Garrido A, Pinoresinol and 1-acetoxypinoresinol, two new phenolic compounds identified in olive oil. *J Am Oil Chem Soc* **77:** 715–720 (2000).

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.