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HPLC-MS SCREENING OF THE ANTIOXIDANT PROFILE OF ITALIAN OLIVE CULTIVARS

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Freeze-dried olive fruits from Italian cultivars such as Carolea, Cassanese, *and* Coratina *were analyzed by HPLC-MS. During different stages of maturation of olive fruits, the biophenolic (antioxidant) composition was examined. Twelve biophenolic compounds, viz. hydroxytyrosol, tyrosol, oleoside 11-methyl ester, demethyloleuropein, verbascoside, demethylligstoside, oleacin, oleuropeindiale, demethyloleuropeindiale, oleuropein, ligstroside, and elenolate were identified by HPLC-MS. Oleuropein, the major olive fruit soluble biophenolic fraction, decreased significantly during fruit maturation and showed significant differences between olive cultivars. Coratina drupes extracted from October month contained a higher amount of biophenols than other mono-cultivar extracted drupes.*

Key words: antioxidant, beta glucosidase, demethyloleuropein, oleuropein, olive.

The olive tree (*Olea europaea* L.) provides one of the most characteristic food product of the Mediterranean Basin, i.e., olive oil and table olives containing functional secoiridoid biophenol glucosides, shown to be protective against the risk of breast, colorectal, pharyngeal, and esophageal cancer [1]. Olive biophenols in general, with their catechol functional groups, exert radical scavenging activity [2] as well as chelating action onto iron, with loss of the potential to generate free radicals, thus preventing autoxidation of olive oil and table olives, and therefore influencing the agri-food product quality [3]. Biophenols are also responsible for the taste of olive oil products, since their metabolites are determinants of the characteristic perception response of some *cuvee* products. Oleuropein, one of the major biophenols in olive fruits, while conferring the bitter taste to olives, is responsible for the bitterness and pungency of olive oil through its metabolites as shown in Fig. 1 [4]. Moreover, biophenol molecules play a role in the structural organization of the cell wall skeleton by acting as a cross-linker between the different polysaccharidic components [5], which in turn strongly influence fruit texture.

The aim of our present study was to investigate the soluble biophenolic profile [6] of different-maturation olive fruits by comparing three olive cultivars, *Carolea*, *Cassanese*, and *Coratina*, which are commercially important olive cultivars growing in an area of south Italy, since it is known that biophenolic molecules in olive drupes are responsible for the antioxidant effect of olive oil, which plays an important role as nutraceuticals.

The mass spectra of each soluble biophenol peak from three different olive fruit cultivars showed the presence of abundant $[M–H]^–$ ions at the appropriate *m/z* values, along with a unique HPLC retention time for each of the soluble biophenols. These chromatograms at *m/z* 153, 137, 403, 525, 623, 509, 319, 377, 377, 539, 539 and 377 show the corresponding mass numbers of hydroxytyrosol, tyrosol, oleoside 11-methyl ester, demethyloleuropein, verbascoside, demethylligstroside, oleacin, oleuropeindiale, demethyloleuropeindiale, oleuropein, ligstroside, and elenolate (Fig. 2). Table 1 shows the contents (mg g^{-1}DW) for biophenols of olive drupe samples at different maturation stages from August to December. The main compounds were oleuropein and ligstroside in all cultivars. *Coratina* drupes extracted in September showed the highest oleuropein and ligstroside values (14.6 mg g^{-1} and 11.4 mg g^{-1}, respectively), whereas in *Carolea* they are 12.7 mg g^{-1} and 11.0 mg g^{-1}, and in *Cassanese* they are 10.1 mg g^{-1} and 9.3 mg g^{-1}.

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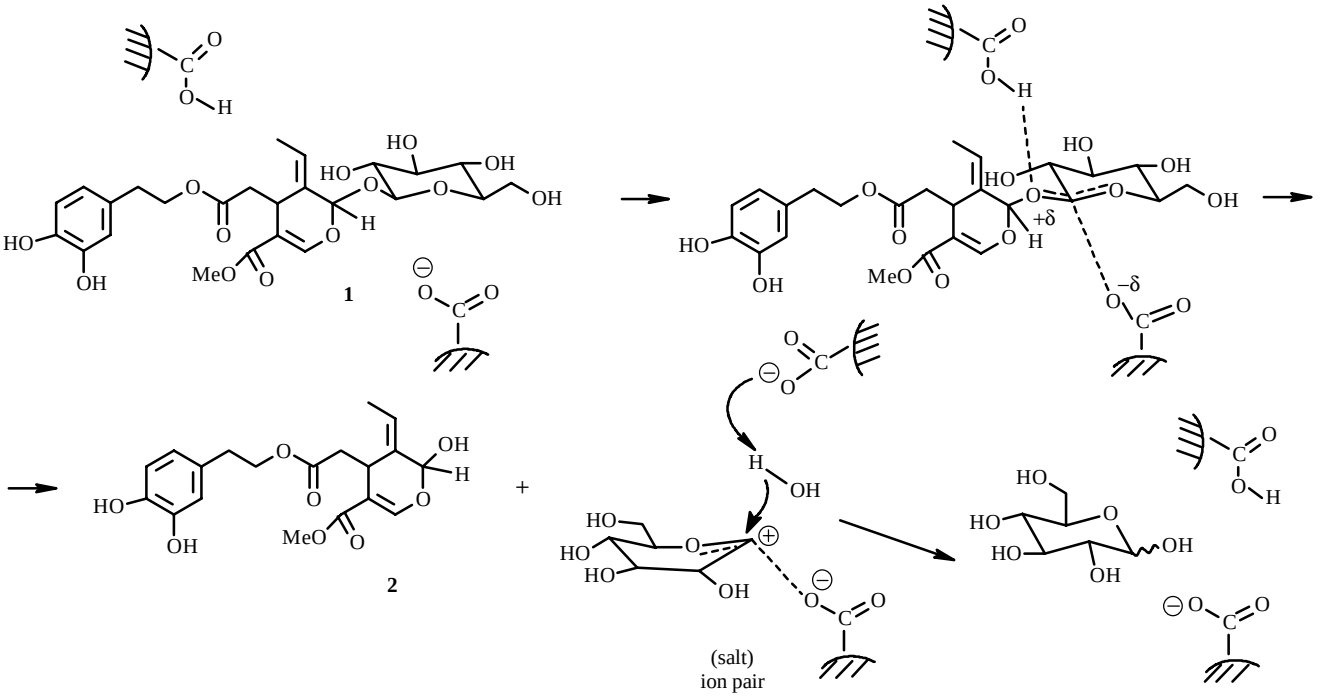

Fig. 1. Molecular mechanism of β-glucosidase hydrolysis exerted on the secobiophenol oleuropein (1-oleuropein; 2-oleuropeindiale).

These two major biophenols were strongly and positively correlated, and there was a clear differentiation into three groups of cultivars. We found that demethyloleuropein, oleacin, and demethyloleuropeindiale were only present in *Coratina* cultivars (Table 1), showing that they are cultivar dependent [8]. Fruits extracted from December recorded the lowest biophenol content in *Coratina* and *Carolea*, and it is absent in *Cassanese*. Oleuropein and ligstroside contents started to decrease in October, but the hydroxytyrosol, tyrosol, oleoside 11-methyl ester, verbascoside, demethylligstroside, and elenolate contents increased in all cultivars. This was understood as being due to the higher synthesis of oleuropein in the early stages of development of the fruit in September, which became degraded with endogenous β-glucosidase hydrolysis, leading to the oleuropeindiale and to the elenolate (Fig. 1) as ripening progressed. With respect to maturation stage, there was a tendency for the oleuropein content to decline. This had also been noted by other workers [9, 10]. It could be a powerful disease resistant mechanism against olive pathogens. When comparing *Carolea* and *Cassanese*, the oleuropeindiale content was higher in *Carolea* (Table 1) whereas it was absent in *Coratina* (Table 1). A remarkable difference between the three varieties is that *Coratina* drupes from October showed the highest contents of all biophenols with demethyloleuropein and demethyloleuropeindiale when compared to *Carolea* and *Cassanese*.

In conclusion, the soluble biophenol profiles in olive drupes from the three Italian cultivars studied showed the same trend during maturation except demethyloleuropein accumulation in *Coratina*. Oleuropein, the main secoiridoid identified in the olive fruit soluble biophenolic fraction, decreased significantly during fruit maturation and showed significant differences between olive cultivars. *Coratina* drupes extracted from October contained higher amount of biophenols than other cultivar-extracted drupes. These olive biophenols could be transformed into olive oil, thus becoming a naturally enriched functional food for its nutraceutical constituents, novel ingredients that reduce the levels of free radical reaction in humans.

TABLE 1. Soluble Biophenol Content (mg g^{-1} DW) of *Carolea* (1), *Coratina* (2), *and Cassanese* (3) Drupes from Different Maturation Periods

Biophenols	August			September			October			November			December		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Hydroxytyrosol	0.4 d	0.2 d	-	1.4 c	0.7 d	0.8 d	1.7 c	0.9 e	1.9 d	-	-	-	-	-	-
Tyrosol	0.3 d	-	0.3 c	0.3 d	0.1 e	0.3 e	0.7	0.4 e	0.7 e	-	-	-	-	-	-
Oleoside 11-methyl ester	0.3 d	0.1 d	0.5 c	1.3 c	0.5 d	1.6 c	1.7 c	1.3 d	2.8 c	1.0 b	0.3 c	1.4 b	0.2 a	0.3 b	-
Demethyloleuropein	-	-	-	-	0.9 d	-	-	4.4 c	-	-	2.1 a	-	-	0.7 a	-
Verbascoside	0.9 c	1.6 c	-	1.1 c	2.2 c	0.9 d	1.9 c	3.2 c	0.9 e	1.1 b	-	-	-	-	-
Demethylligstroside	-	-	-	-	-	-	1.2 d	2.5 d	1.4 d	0.9 b	0.8 b	1.0 b	-	0.3 b	-
Oleacin	-	-	-	-	-	-	-	1.6 d	-	-	0.2	-	-	-	-
Oleuropeindiale	-	-	-	0.2 d	-	0.1 e	1.4 c	-	0.7 e	1.4 b	-	1.2 b	-	-	-
Demethyloleuropeindiale	-	-	-	-	-	-	-	1.7 d	-	-	1.1 b	-	-	-	-
Oleuropein	11.9 a	13.3 a	9.7 a	12.7 a	14.6 a	10.1 a	6.1 b	10.6 a	4.2 b	0.8 b	2.1 a	-	-	0.1 b	-
Ligstroside	9.2 b	10.1 b	7.4 b	11.0 b	11.4 b	9.3 b	8.4 a	8.3 b	5.7 a	2.7 a	1.6 b	2.4 a	0.9 a	0.9 a	-
Elenoate	-	-	-	0.2 d	0.6 d	-	0.7 d	1.4 d	0.6 e	0.2 c	0.4 c	-	-	0.1 b	-

(-) Not detected. Values are expressed as mg g^{-1} of dry weight of olive drupe. Different letters within the same row indicate a significant difference ($P \leq 0.05$).

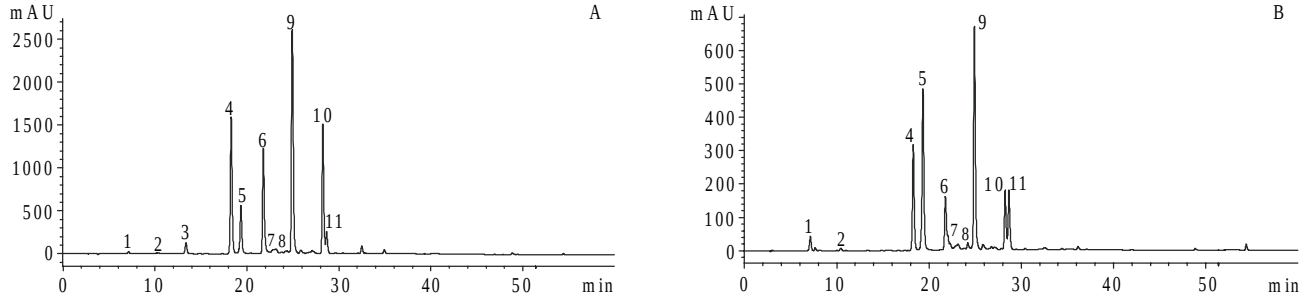

Fig. 2. HPLC soluble biophenolic profile from October – harvested *Coratina* olive cultivar (A = λ 240; B = λ 280): 1) hydroxytyrosol; 2) tyrosol; 3) oleoside 11-methyl ester; 4) demethyloleuropein; 5) verbascoside; 6) demethylligstroside; 7) oleacin; 8) demethyloleuropeindiale; 9) oleuropein; 10) ligstroside; 11) elenolate.

EXPERIMENTAL

HPLC. The instrument used was a 1100 LC system (Hewlett-Packard, Waldbronn, Germany) reversed-phase C_{18} 25 cm × 4.6 mm i.d.) column, with UV/VIS multiwavelength detector; eluates were detected at 280 and 240 nm. Extracted BP fractions (20 μL), were injected into HPLC. Elution was run out at a 0.5 ml/min flow rate by following mobile phases: methanol (solvent A) and water/acetic acid, pH 3.3 (0.1%) (solvent B). Selected gradients started with 10% A 90% B; then solvent A was raised to 90% in 60 min and acquisition was stopped. The column was washed 10 min with 100% A and then kept at the initial conditions for another 10 min. BP detection was performed simultaneously at 280 nm and 240 nm. HPLC eluate was introduced into the ESI-MS interface.

Mass Spectrometry – Electrospray Ionization (MS-ESI). MS detection was carried out using a Micromass, Quattro LC (Germany). The system was monitored by a Philips Power PC computer equipped with the software's MassLynx for instrument control and data acquisition, data reprocessing, and solute quantification. Nitrogen was employed as the nebulizing gas at a pressure of 60 p.s.i. The operative parameters were set as follows: ionspray voltage: 4.0 kV (in negative ion mode) applied to the sprayer during all experiments. Ions generated in the ion source were sampled into the mass analyzer by passing through a 25 μm I.D. orifice (voltage from 0 to 30 V) at the rear end of the atmospheric chamber.

Plant Material. Plants of *Olea europea* L. *Carolea*, *Cassanese* and *Coratina* cvs. grown in the ISOL orchard, Rende (CS), Italy were selected for our experiments. These olive trees had a characteristically low-spreading growth habit, reaching about 5 to 10 m in height at maturity. They were grown in good fertile soil and with spring nitrogen fertilization and summer mowing. All the steps in cultivation were recorded as well as phenomenological stages. The parasite incidence was monitored from the month of June and, in particular, the population of the olive-fly *Bactrocera oleae* Gmel. was followed by chromotropic cards (data not shown). The olive fruits from each cultivar were selected randomly for sampling during the different maturation stages of the fruit (August–December). Three representative subsamples were picked in each sampling and taken to the laboratory for analysis.

Extraction and Characterisation of BP Fraction. Freeze-dried fruits from each cultivar weighing 15 g, worked up as described [6], were separately pitted by blending and homogenized in methanol–acetone (1:1, 80 ml) saturated with sodium metadisulfite at medium speed in an Ultraturrax homogenizer (Janke & Kunkel, IKA-Labortechnik, Germany) at 0°C for 3 min, and centrifuged at 5000 g for 20 min at 4°C [7]. The supernatant was separated and the pellet resuspended four times in the same solvent until a colorless solution was obtained. The combined supernatants were used for soluble biophenols (SBP) and evaporated under vacuum at 35°C. The dry residue was resuspended with pH 2 water solution and centrifuged to separate a cloudy precipitate. The clear supernatant was extracted five times with hexane at a 1:1 hexane to water phase ratio to remove free fatty acids and other lipid contaminants. The SBPs were then ether–ethylacetate (1:1) extracted six times at a 1:1 solvent to water phase ratio. The ether–ethylacetate extracts were dehydrated with anhydrous sodium sulfate, filtered, and evaporated to dryness under vacuum at 30°C. After evaporation to dryness, the residue was redissolved in LC-MS CHROMASOLV® grade methanol in vials containing a known amount of an internal strandard (IS), i.e., gallic acid, and filtered through a 0.45 μm millipore filter.

Statistical analysis was performed according to the SAS System (Version 6.21, SAS Institute Inc. Cary, NC 27513, USA). Statistical significance of the differences observed among mean values was assessed using Duncan's multiple range test. A probability of $P \leq 0.05$ was considered significant.

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