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Microcomponents of olive oil—III. Glucosides of 2(3,4-dihydroxy-phenyl)ethanol

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The molecular structure of the microcomponents of olive fruit was investigated in order to evidence new molecules which could be transferred to the resulting oil and therefore be typical of olive oil. The three glucosides of 2(3,4-dihydroxy-phenyl)ethanol, **1**, **2** and **3** were isolated together with other glucosides previously identified in *Olea europaea*. Glucosides **1–3** were detected in the olive oil, there being always present a small quantity of water as an emulsion, together with the aglycone, the 2(3,4-dihydroxy-phenyl)ethanol. The presence of glucosides **1–3** is closely linked with the organoleptic characteristics and to the recognized anti-oxidant properties of olive oil. © 1998 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Mediterranean foods were recently investigated for their molecular microcomponents which exert several physiological effects in human nutrition and strongly influence the quality and authenticity of regional products. Olive oil is one of the traditional Mediterranean foods and is becoming the vegetable fat of choice in several diets.

We examined the microcomponents of olive oil in order to propose scientific parameters for the determination of the quality of oil. In fact the characteristics of olive oil are strictly linked with the ripening of the fruit. The chemical composition of green olives is different from that of black olives; also the physical features of green olives differ from those of black ones and, therefore, are correlated with the relative amount of the phenolic fraction (Vazquez Roncero and Janer del Valle, 1977), thus determining the harvesting time (Amiot *et al.*, 1989; Donaire *et al.*, 1975).

The microcompontent distribution in olive fruits at harvesting allows prediction of the molecular composition of the resulting oil and rational criteria for its production in relation to quality, authenticity and health effects.

Two galactolipids were isolated and identified (Bianco *et al.*, 1998, in press), both in olive fruits and in olive oil, which could be responsible for the stability of the light emulsion typical of the fresh olive oil and, as consequence, the presence of hydrophilic *o*-diphenolic compounds (the name 'biophenols' is proposed for them) which enhance the antioxidant properties characteristic of this oil, particularly in the first period after the production.

We carefully examined the hydrophilic *o*-diphenolic fraction present in the olive fruit and in the resulting olive oil, because the quality of olive oil strongly depends on the nature and quantity of these derivatives.

MATERIALS AND METHODS

Reagents and analyses

TLC: Silica gel SiF_{254} (Merck) and RP-8 F_{254} (Merck). Spray reagents were 2 N H_2SO_4, heating at 120°C or 2% $FeCl_3$ in water, then exposure to ammonia vapours. NMR: Bruker AM 500, solvent D_2O, internal standard HDO at 4.70 ppm from TMS, coupling constants in Hz. MS: Kratos-80. IR: Shimadzu IR-470. $[\alpha]_D$: Jasco DIP-370. HPLC: Hewlett-Packard A 220 with diode array detector.

Plant material

Olea europaea L. leaves and fruits were hand-collected in Calabria (Italy) in 1995 at the beginning of ripening

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from plants which had been without chemical treatment for 1 year. Voucher specimens were deposited in the ISOL, Istituto Sperimentale per l'Olivicoltura, Rende, Cosenza (Italy) which prepared the olive oil by cold pressing on the same day of the collection. Two cultivars were examined: 'Carolea', cultivated in the neighbourhood of Cropani and 'Ottobratica', cultivated in the neighbourhood of Rende.

Extraction and separation of *o*-diphenolic fraction

Olea europaea *leaves and fruits*

Leaves or olives (sample weight about 500 g) were exhaustively extracted with EtOH at room temperature. The extract was then evaporated to an aqueous suspension. Charcoal (~50 g) was added until negative by $FeCl_3$ test and the resulting suspension stratified on a Gooch funnel. Elution with water, 5 and 10% EtOH, removed salts and simple sugars, whereas 30, 60 and 90% EtOH eluted glycoside-containing fractions. Ninety, 60 and 30% EtOH fractions were separately worked up as follows. After evaporation of volatile materials, the resulting crude glycosidic fraction was chromatographed on Si gel in *n*-BuOH sat. H_2O, performing a first separation of components in relation to their polarity. Successive separations of fractions containing *o*-diphenolic units were performed by chromatography on Si gel in $CHCl_3$/MeOH in ratio from 9:1 to 7:3.

Final purification was obtained by semi-preparative HPLC, using μ-bondapack C_{18} as absorbent and MeOH/H_2O gradient as eluent.

The composition of the olive leaves was as follows. Oleuropein **5** 0.8 g, 0.16%, demethyloeuropein **6** 0.2 g, 0.04%, oleuropein aglycones 0.05 g, 0.01%, biophenol **1** 0.1 g, 0.02%, biophenol **2**, 0.08 g, 0.016%, biophenol **3** 0.1 g, 0.02%, 2(3,4-dihydroxy-phenyl)ethanol **4** 0.15 g, 0.03%.

The composition of the olive fruits was Oleuropein **5** 0.3 g, 0.06%, demethyloeuropein **6** 0.7 g, 0.14%, oleuropein aglycones 0.03 g, 0.006%, biophenol **1** 0.1 g, 0.02%, biophenol **2**, 0.08 g, 0.016%, biophenol **3** 0.1 g, 0.02%, 2(3,4-dihydroxy-phenyl)ethanol **4** 0.15 g, 0.03%.

Olea europaea *oil*

Olive oil (10 ml) was emulsified with water (1 ml) and left to stand at 4°C for 3 h. After centrifugation, the aqueous phase was analysed by HPLC with µ-bondapack C_{18} in MeOH/H_2O gradient using compounds **1–6** as references together with oleuropein aglycone. This last compound was prepared from oleuropein (50 mg) by hydrolysis in citrate buffer at pH 6.5 (5 ml) with β-glucosidase (EC 3.2.1.21., 10 mg) for 2 h at room temperature. Aglycone was extracted with EtOAC (3×10 ml) and used without purification.

Olive oil composition (10 ml): oleuropein aglycones 0.15 mg, 0.0015%, biophenol **1** 0.05 mg, 0.0005%, biophenol **2** 0.05 mg, 0.0005%, biophenol **3** 0.02 mg, 0.0002%, 2(3,4-dihydroxy-phenyl)ethanol **4** 0.1 mg, 0.001%.

Biophenol **1**-$[\alpha]_D^{25} = -31$ (MeOH c = 0.2 g/ml). UV (MeOH): $\lambda_{max} = 221$ nm (log $\epsilon = 1.5$) IR (KBr): $\nu_{max} = 3450$, 1680, 1640, 1420, 1230, 850 cm^{-1}. ^{1}H-NMR: 2.62 (2H-α, t, J = 7.0), 3.60 (2H-β, t, J = 7.0), 4.88 (H-1′, d, J = 7.5), 3.3–3.5 (H-2′, H-3′, H-4′, H-5′), 3.60 and 3.80 (2H-6′, AB system, J = 12.5, 2.2, 4.4), 6.98 (H-5, d, J = 8.0), 6.68 (H-6, dd, J = 8.0, 2.0), 6.74 (H-2, d, J = 8.0). ^{13}C-NMR: 40.0 (C-1α), 63.3 (C-6′), 65.3 (C-1β), 72.2 (C-4′), 75.7 (C-2′), 78.8 (C-5′), 78.9 (C-3′), 104.2 (C-1′), 119.7 (C-5), 119.8 (C-6), 137.9 (C-1), 124.1 (C-2), 145.9 (C-3), 148.2 (C-4).

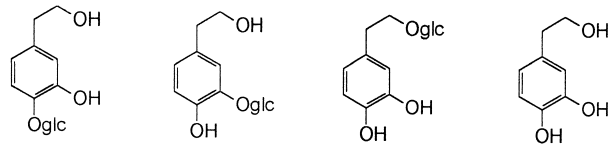
Biophenol **2**: ^{1}H-NMR: 2.63 (2H-α, t, J = 7.0), 3.62 (2H-β, t, J = 7.0), 4.92 (H-1′, d, J = 7.5), 6.90–6.70 (Phenyl), 3.3–3.5 (H-2′, H-3′, H-4′, H-5′), 3.60 and 3.80 (2H-6′, AB system, J = 12.5, 2.2, 4.4). ^{13}CNMR: 40.0 (C-1α), 65.3 (C-1β), 104.0 (C-4′), 75.6 (C-2′), 78.2 (C-5′), 78.8 (C-3′), 104.0 (C-1′), 146.7 (C-3), 147.3 (C-4), 134.6 (C-1), 127.2 (C-2), 120.0 (C-5), 119.2 (C-6).

Biophenol **3**: ^{1}H-NMR: 2.67 (2H-α, t, J = 7.0), 3.74 (2H β, t, J = 7.0), 4.30 (H-1′, d, J = 7.5), 3.1–3.4 (H-2′, H-3′, H-4′, H-5′), 6.74 (H-2, d, J = 8.0), 6.64 (H-6, dd, J = 8.0, 1.0), 6.77 (H-5, d, J = 8.0). ^{13}CNMR: 146.8 (C-4), 145.3 (C-3), 134.3 (C-1), 124.1 (C-2), 119.6 (C-5), 119.1 (C-6), 105.0 (C-1′), 78.7 (C-5′), 78.6 (C-3′), 75.8 (C-2′), 73.7 (C-1β), 72.3 (C-4′), 37.1 (C-1α), 63.5 (C-6′).

RESULTS AND DISCUSSION

The microcomponent of leaves, fruits and oil of two cultivars of *Olea europaea* L. (ottobratica and carolea, see Materials and methods) were investigated, focusing, in particular, on the hydrophilic *o*-diphenolic fraction. Separation of this fraction of olive fruits and leaves was performed using the 'charcoal method' (Bianco, 1990) which completely separated this polar fraction from the predominant fat material constituted by glycerides of saturated and unsaturated fatty acids.

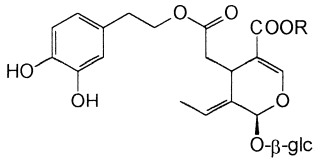
Examination of this fraction allowed the isolation of the three glucosides of the 2(3,4-dihydroxy-phenyl)-ethanol, the biophenols **1–3**, together with the free alcohol **4**.



1 **2** **3** **4**

We also verify the presence of oleuropein **5** (Panizzi *et al.*, 1958, 1960, 1965; Inouye *et al.*, 1970; Tsukamoto *et al.*, 1984) and of demethyl-oleuropein **6** (Gariboldi *et al.*, 1986; Kuwajima *et al.*, 1988) which are the main secoiridodic constituents together with the aglycones of

oleuropein and their derivatives (Gariboldi *et al.*, 1986; Kuwajima *et al.*, 1988; Bianco *et al.*, 1996). Ligstroside (Gariboldi *et al.*, 1986; Kuwajima *et al.*, 1988), oleoside (Gariboldi *et al.*, 1986; Kuwajima *et al.*, 1988) and oleuroside (Kuwajima *et al.*, 1988) appeared not to be present in significant quantities in the examined cultivars.



5 R=CH_3
6 R=H

Compound **2** has already been identified in *Osmanthus asiaticus*, a plant of the Oleaceae family (Sugiyama and Kikuchi, 1992), while **3** has already been identified in *Prunus grayana*, Rosaceae (Shimomura *et al.*, 1987) and in *Ricciocarpus natans*, Ricciaceae (Kunz and Becker, 1992), but neither has been detected in *O. europaea*.

Other polyphenolic glycosidic compounds have been isolated from plants of the Oleaceae family (Thomas La Londe *et al.*, 1976; Kitagawa *et al.*, 1984; Kobayashi *et al.*, 1984; Molgaard and Ravn, 1988; Sugiyama and Kikuchi, 1990, 1991), while compound **1** has not been isolated until now. Its presence in *O. europaea* has only been assumed on the basis of chromatographic considerations (Vazquez Roncero *et al.*, 1974*a*,*b*).

Structures of **2** and **3** were confirmed by comparison of their ^{1}H NMR data (see Materials and methods) with those reported.

The structure of compound **1** was assigned by spectroscopic methods, which demonstrated it to be 2(3-hydroxy-4-*O*-D-β-glucopyranosyl)phenyl-ethanol. ^{1}H- and ^{13}C-NMR data unambiguously determined the nature of the aglyconic and glucosidic part of the molecule and the relative position of the glucose moiety.

In particular, the ^{1}H NMR spectrum revealed, besides the aromatic resonances in the range 6.65–7.05 ppm showing the typical pattern of the 1,3,4-trisubstitution, the $-CH_2-CH_2-$ methylene part as triplets centred at 2.65 and 3.65. The glycosidic moiety of the molecule gave rise to resonances at 4.88 (the anomeric proton) and in the region 3.2–3.8 ppm.

NOE experiments assigned the position of the glucose unit as the phenolic hydroxyl in the 4 position. In fact, on irradiating the anomeric proton at 4.88δ, a significant positive NOE effect was evidenced for the doublet relative to the aromatic proton at C-5; likewise irradiation of the hydrogen atom at C-5 gave a NOE effect on the anomeric proton.

Since the natural glycosylation of hydroxytyrosol, occurring in derivatives **1–3**, seems to show a non-regioselective enzymatic process, this may indicate that the plant organism requires the phenolic moiety to be highly mobile, through its hydrosolubility characteristics, in order to exert the known antioxidant activity.

Glucosides **1–3** of 2(3,4-dihydroxy-phenyl)-ethanol are present in the leaves and in the fruits of olive. As a consequence of the process oil production, they are partially hydrolysed by β-glucosidase which is released in the paste, affording glucose and the 2(3,4-dihydroxy-phenyl)-ethanol **4**, but significant quantities remain unchanged in the aqueous phase which is always emulsified with the fat phase of olive oil. In fact we identified biophenols **1–3** in the light emulsion typical of fresh olive oil, together with oleuropein **5** and demethyloleuropein **6** (see Materials and methods).

The presence of 2(3,4-dihydroxy-phenyl)-ethanol, **4**, together with its glucosides **1–3**, in olive oil constitutes a natural protection for the oil against the auto-oxidation process; it contributes to the organoleptic characteristics of olive oil, is a guarantee that the oil derives from the simple cold pressing of olives, and constitutes a very useful dietary microcomponent, exerting a well-documented anti-free radical and in general antioxidant activity in man.

On the basis of these considerations it is proposed to use the name 'biophenols' for ortho-diphenolic compounds such as **1–3** and oleuropein **5** and its derivatives.

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