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# Biophenolic components of olives

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**Abstract**

The occurrence of biophenolic components in olives provides functional value to the Mediterranean food culture, owing to recognized antioxidant activities of these substances. The concentration of biophenolic compounds in olives are closely linked to texture and organoleptic characteristics of agrifood products, i.e. table olive and olive oil. The concentrations of different biophenolic compounds in olives were investigated in order to develop appropriate procedures for determination of these compounds in fresh and processed table olives and in the olive drupes for olive oil production. Olives from Spain (Hojiblanca cv), Portugal (Douro cv), Greece (Thasos and Conservolia cvs) and Italy (Taggiasca and Cassanese cvs) were analysed. Four different protocols were employed. The first allows for an estimate of the total concentration of simple biophenolic compounds; the second, for soluble compounds and soluble esterified derivatives of these compounds; the third, the qualitative determination of cytoplasmatic soluble biophenolic content; the fourth, determination of soluble, glucosidic, esterified and cell-wall bound biophenols by means of a rapid, though more complex, sequential method, for their accurate evaluation on a structural and quantitative basis. Thus, the experimental procedures yield four different fractions of the biophenolic components of the olive, checked by CC, HPLC and NMR. The experimental results depend on the procedure chosen, the degree of ripeness of the olives, and the environment of the olive cultivars. The composition of each of these fractions can be of valuable information, supporting for table olive growing and olive oil producers in maximising the competitive quality of their products by selecting olive materials whose concentrations of biophenolic compounds can be responsible for beneficial effects on human health. © 2000 Published by Elsevier Science Ltd.

*Keywords:* Biophenol analytical procedure; Table olive; Olive oil; Olea europaea cultivars

## 1. Introduction

Recently, a drastic change in consumer demand has occurred; naturally processed, additive-free, and safe products are requested. Consumers search for variety and convenience and begin to question modern methods of food manufacture and distribution. Mass production has been replaced by priority of safety, more palatable and traditional methods, accepted as natural, without other additives; the latter has always been viewed with suspicion. Organic foods are in great demand, since, as far as the general public is concerned, natural is best. Food competitive quality has thus assumed a whole new meaning, characterised by nutritional, hedonistic and functional aspects (Wiseman, Weisgerber, Tijgurg & Kover, 1999).

An understanding of what determines food quality at the finished end product of the food chain requires the investigations of the composition of the raw food material, with focus on colour, textural, and flavour attributes.

Very recently, considerable interest has arisen in the possible impact, exerted by the daily intake of functional foods, on several diseases, for their antioxidant biomolecular components. Modern pathological prevention has been linked to a number of natural phenolic biomolecules from fruits and vegetables, as associated to the Mediterranean diet (Trichopoulou, Lagiou & Papas, 1999).

Due to their phenolic functionality, typical secondary plant metabolites, constitute a distinctive group of phytochemicals, behaving as phytoprotectants. These possess great structural diversity and wide phylogenetic distribution (Haslam, 1998). For those found in olive fruits (Casuscelli, De Nino, Gallo, Procopio, Romeo & Uccella, 1994), metabolite uniqueness lies in their phenolic character. Deriving from natural origin, olive biophenols (BPs) (Romeo & Uccella, 1996), can be simple substituted hydroxyaromatics, in the range of low

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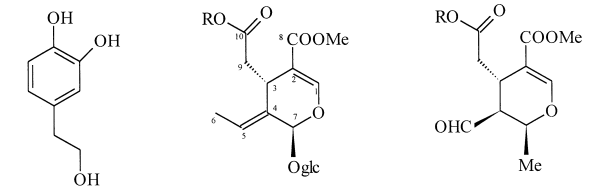
**Nomenclature**

| | |
|---|---|
| BP | biophenols |
| Ty | tyrosol |
| Hty | hydroxytyrosol |
| TBP | total BP |
| sBP | simple BP |
| SAHBP | soluble and alkali-hydrolysable BP |
| CBP | cytoplasmatic BP |
| SBP | soluble BP |
| SEBP | soluble-esterified BP |
| IBBP | insoluble-bound BP |

molecular weight (MW), and complex ones, linked to monoterpenic moiety (Bianco, Mazzei, Melchioni, Romeo & Uccella, 1996). Together with several other functional groups, olive BPs possess a phenolic molecular structure. They are characterised by multifunctional moieties, such as alchene, alcoholic, and carboxylic groups, when simple molecules, e.g. hydroxytyrosol **1**, and by glucosidic and monoterpenoid units, when complex biomolecules, e.g. secoiridoid oleuropein **2** and hydroxytyrosilelenolate **3**, respectively, as shown in Scheme 1. Moreover, the olive BP molecular components, represented as monomer water-soluble compounds (Tsimidou, 1998), range up to 500 MW, with up to eight hydroxyaromatic groups, and to three aromatic rings.

Thus, olive BPs cannot be considered polyphenols; the latter are natural products with MW range 500–4000, and possess some 12–16 hydroxyaromatic groups, and 5–7 aromatic ring, per 1000 relative MW (Haslam, 1996).

Besides conventional phenolic reactivity, olive BP monomers are also distinguished by their ability to associate strongly with primary metabolites such as proteins and carbohydrate models (Vekey, Malorni, Pocsfalvi, Piperno, Romeo & Uccella, 1997), and to give rise to supramolecular moieties, responsible for their bioavailability (Bianco, Chiacchio, Rescifina, Romeo & Uccella, 1997). Olive BPs exert antioxidant activity by free radical quenching and metal chelation (Garrido-Fernandez, Fernandez-Diez & Adams, 1997; Sajia et al., 1998 and 1999), participate in biomolecular defence mechanism against attacking pathogens (Lo Scalzo, Scarpati, Verzegnassi & Vita, 1994) and provide cross-linkage among polysaccharides of plant cell-wall molecular architecture (Appel, 1993), thus affecting the texture characteristics of Mediterranean agrifood product.




**1** Hydroxytyrosol

**2**, R = **1** Oleuropein

**3**, R = **1** Hydroxytyrosilelenolate

Scheme 1.

All the above mentioned phenomena are involved during molecular interactions, substantially influencing the properties of many plant products, from taste, palatability (Acree & Teranishi, 1993) and functional (Goldberg, 1994) values of foodstuffs to the microbial and insect antagonism of vegetable matter (Bisignano, Tomaino, Lo Cascio, Crisafi, Uccella & Saija, 1999).

In particular, the *o*-diphenolic and monoterpenic multifunctionality of olive BP compounds affects the hedonistic-sensorial and functional aspects of fresh and processed agrifoods, derived from *Olea europaea* L., i.e. table olives and olive oil, representing a bitter and pungent substrate in taste perception. BP distribution and stability of table olives and olive oil (Arrigo & Rondinone, 1995), together with their organoleptic features (Vazquez Roncero, Maestro Duran & Graziani Cosante, 1974 and Vazquez Roncero, Janer de Valle, 1977; Visioli & Galli, 1998), i.e. the flavour and fragrance of alimentary product, were proposed to be strictly linked.

Table olive and olive oil quality is closely related to both agronomic traits and ripening level of the fruit, including the processing phases of production methods (Garrido-Fernandez et al., 1997; Uccella, 1998). Indeed, all these factors can modulate the level and spatial relationship of cell-wall components, thus influencing the texture and taste of olive fruit products. Hence, detailed information on cell-wall composition (Rovellini & Cortesi, 1998) and on cellular structure in olive fruit tissues (Rangel, Platt & Thomson, 1997) can be investigated in order to improve the knowledge of their effects on food products. The BP content is thus relevant to texture attributes, i.e. appearance and mouthful of agrifoods, as gained in table olive and olive oil production. In this context, attention was addressed to BP compounds, which play a role in the structural organisation of cell-wall skeleton, as cross-linker among different polysaccaridic components, strongly influencing the palatability of processed olive fruit, as well as of oil, acting as taste molecules and as natural antioxidants (Vinson, Mao, Su & Zubik, 1998).

Known experimented procedures for separation, purification, and qualitative and quantitative determination (Romani, Mulinacci, Pinelli, Vincieri & Cimato, 1999) are somewhat inappropriate to provide suitable information on the original molecular structure and on the real content of simple and complex BPs, occurring in the olive fruit material (Esti, Cinquanta & Nolte, 1998). Detailed information of the post-harvest changes, occurring in the drupe, are essential for the relationship between raw material composition and the final Mediterranean

food quality, e.g. for the olive oil and the table olives. Applications of extraction condition could affect the molecular structure of the final products by a chemical transformation of the more reactive biomolecules under enzymatic and catalytic modes (Bianco, Piperno & Uccella, 1999). The interference and formation of artefacts must be carefully considered (Bianco, Muzzalupo, Piperno, Romeo, & Uccella, 1999; Bianco, Piperno, Romeo & Uccella, 1999), in order to acquire an accurate estimation of BPs contained in olive fruits and thus in their products consumed in the Mediterranean food culture, i.e. table olives and olive oil, by experimenting with novel methods for extraction, separation and determination of the biomolecules under investigations.

The estimation of selected BP profiles was, therefore, experimented with in relation to qualitative and quantitative aspects on a series of olive cultivars, harvested for table olive and olive oil production in the Mediterranean area. Different methodologies were tested in order to verify the procedure able to ascertain the precise picture of simple and complex conjugated-glucoside BPs, as far as qualitative and quantitative content in the olive cultivars chosen for table olive and olive oil production. The distribution of BP compounds was investigated in olive fruits, harvested at different development and ripening phases, adopting analytical procedures, whose simple (Bianco, Mazzei, Melchioni, Romeo et al., 1998) and complex (Krygier, Sosulski & Hogge, 1982) protocols can allow the complete qualitative and quantitative pattern determination of BPs contained in olive mesocarp.

The BP composition of the raw olive material can ensure consistency in the selection of olive cultivars and varieties in olive tree breeding programmes, in raw material specification, in oil and table olive manufacture. Thus, the understanding of the BP distribution according to different olive cultivars and pedoclimatic conditions, can explain the biomolecular modifications underlying the development of quality attributes, such as texture, colour, flavour and antioxidant properties.

Some of the most representative olive drupe BPs are complex molecular structures, being *o*-dihydroxyaromatics, esters, monoterpenes, and glucosides, such as **1**,**2** and **3** and can be highly reactive. Thus, four procedures have been designed to separate and identify the natural BP compounds present in the mesocarp of olive drupes.

## 2. Experimental procedure

### 2.1. Plant material

*Olea europaea* L. fruits were collected in Spain (Hojiblanca cv, at three different ripening stages: green, cherry, and black), in Portugal (Douro cv, green, cherry, and black), in Greece (Thasos cv, black and Conservolia cv, black), and in Italy (Taggiasca cv, green, and black, and Cassanese cv, green, and black) in 1997. Voucher specimens were provided by OLITEXT scientific and industrial partners.

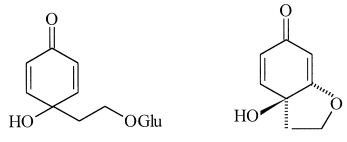
### 2.2. Reference compounds

**1** (HTy) was synthesised as already described (Bianco, Passacantilli & Righi, 1988). BP **2** (Panizzi, Scarpati & Oriente, 1960) and derivatives (Bianco, Muzzalupo et al., 1999), and cornoside **4** and halleridone **5**, see Scheme 2, were obtained as described (Bianco, Lo Scalzo & Scarpati, 1993). Hty-1-*O*-β-D-glucoside, HTy-3′-*O*-β-D-glucoside, HTy-4′-*O*-β-D-glucoside and Ty-1-*O*-β-D-glucoside were isolated according to reported procedures (Bianco, Mazzei, Melchioni, Romeo et al., 1998). BP derivatives **6a–d**, reported in Scheme 3, were previously described (Bianco, Muzzalupo et al., 1999; Bianco, Piperno, et al., 1999). Tyrosol (Ty) **7**, protocatechuic acid **19**, caffeic acid **10**, hydroxycaffeic acid **22**, vanillic acid **30**, homovanillic acid **27**, 3,4-dihydroxyphenylacetic acid **8**, 3-hydroxyphenylacetic acid, p-hydroxybenzoic **9**, syringic acid **11**, p-coumaric acid **12**, clorogenic acid, ferulic acid **31**, sinapic acid **13**, m-coumaric acid, o-coumaric acid **14**, metoxyphenilacetic acid **29**, cinnamic acid **17**, were pure products, from SIGMA-Chimica (Milano, Italy).

### 2.3. Instrumentation

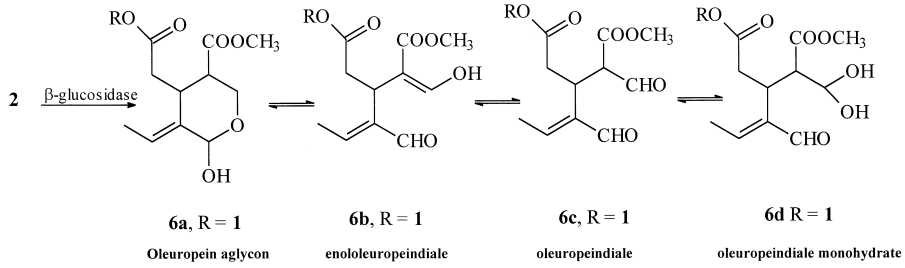
High pressure liquid chromatography (HPLC) was performed with a Hewlet-Packard 1050 with diode array spectrophotometer at 230 and 278 nm or with a Dionex Biolc, with UV photodiode detector; eluates were detected at 278°nm at 25°C. Total BPs (TBPs), determined colorimetrically, used Itaki U-3410 spectrophotometer.

Nuclear magnetic resonance (NMR) spectra were recorded with Bruker AM 500, Varian XL300, Varian Gemini 200; solvents were: $D_2O$, internal standard HDO at 4.70 ppm from TMS; $CD_3OD$, internal standard TMS; $CDCl_3$, internal standard TMS. Compound **6a**: $^1$H NMR (δ, $CDC1_3$): 7.55 (s, H-1), 6.80–6.72 (m, aromatic protons), 5.65 (q, H-5, $J = 7.0$ Hz), 4.25 (d, H-1, $J = 5.0$ and 0.7 Hz), 4.30 (m, H-1α and H-1′β), 3.60 (dd, H-3, $J = 8.9$ and 4.3 Hz), 3.58 (s, $OCH_3$), 2.82 (m, H-2″α and H-2″β), 2.75 (m, H-9α), 2.65 (m, H-9β), 1.65 (d, $CH_3$, $J = 7.0$ Hz). $^{13}$C NMR: 169.20 (C-8), 168.5 (C-10),




**4** cornoside **5** halleridone

Scheme 2.




Scheme 3.

155.70 (C-1), 142.80 (C-3′), 141.01(C-1′), 135.70 (C-4′), 132.05 (C-4), 121.25 (C-6′), 116.30 (C-5′), 116.30 (C-2′), 111.37 (C-2), 96.04 (C-5), 94.31 (C-7), 65.65 (C-11), 51.90 (C-13), 41.30 (C-9), 34.38 (C-12), 31.73 (C-3), 14.30 (C-6). Compound **6b**: $^1$H NMR (δ, $CDC1_3$): 2.03 (d, $J=7.1$ Hz, $CH_3$), 2.70 (m, H-12α and H-12β), 2.75 (m, H-9α), 2.82 (m, H-9β), 3.79 (s, $OCH_3$), 4.10 (m, H-11α and H-11β), 4.19 (dd, $J=7.5$, 7.0 Hz, H-3), 6.70 (q, $J=7.1$, H-5), 6.6–6.85 (aromatic protons), 7.40 (bs, OH) 7.35 (d, $J=6.2$ Hz, H-1), 9.20 (s, H-7). $^{13}$C NMR: 195.31 (C-7), 171.81 (C-8), 171.10 (C-10), 156.93 (C-1), 155.08 (C-5), 143.70 (C-4′), 143.38 (C-4), 142.80 (C-3′), 140.02 (C-1′), 130.37 (C-2), 121.25 (C-6′), 116.30 (C-5′), 116.30 (C-2′), 65.65 (C-11), 51.90 (C-13), 37.15 (C-9), 34.38 (C-12), 31.03 (C-3), 15.30 (C-6). Compound **6c**: $^1$H NMR (δ, $CDC1_3$): 2.00 (d, $J=7.0$ Hz, $CH_3$), 2.60 (m, H-9α), 2.78 (m, H-12α and H-12β), 2.80 (m, H-9β), 3.71 (s, $OCH_3$), 3.75 (m, H-3), 4.18 (m, H-11α and H-11β), 6.75 (q, $J=7.0$, H-5), 6.6–6.85 (aromatic protons), 9.20 (s, H-7), 9.75 (d, $J=2.72$ Hz H-1). $^{13}$C NMR: 195.73 (C-1), 195.21 (C-7), 171.50 (C-8), 171.15 (C-10), 155-10 (C-5), 143.33 (C-4′), 142.95 (C-4), 142.74 (C-3′), 142.71 (C-1′), 119.25 (C-6′), 116.30 (C-5′), 115.94 (C-2′), 65.49 (C-2), 56.00 (C-11), 51.95 (C-13), 36.29 (C-9), 35.81 (C-12), 30.60 (C-3), 15.29 (C-6). Compound **6′c**: $^1$H NMR (δ $CDC1_3$): 1.98 (d, $J=7.0$ Hz, $CH_3$), 2.60 (m, H-9α), 2.78 (m, H-12α and H-12β), 2.80 (m, H-9β), 3.78 (m, H-3), 3.85 (s, $OCH_3$), 4.18 (m, H-11α and H-11β), 6.75 (q, $J=7.0$, H-5), 6.6–6.85 (aromatic protons), 9.20 (s, H-7), 9.48 (d, $J=2.70$ Hz, H-1). $^{13}$C NMR: 195.58 (C-1), 195.21 (C-7), 171.30 (C-8), 171.10 (C-10), 155.10 (C-5), 143.32 (C-4′), 142.80 (C-4), 142.74 (C-3′), 142.71 (C-1′), 119.29 (C-6′), 116 30 (C-5′), 116.00 (C-2′), 65.43 (C-2), 56.50 (C-11), 51.98 (C-13), 36.29 (C-9), 35.60 (C-12), 30.65 (C-3), 15.29 (C-6).

Thin layer chromatography (TLC) used silica gel $SiF_{254}$ (Merck, Darmstat, Germany) and RP-8 $F_{254}$ (Merck) prepared plates, developed with suitable solvents and sprayed with 2N $H_2SO_4$, followed by heating at 120°C. *O*-diphenolic compounds were detected only by spraying plates with 2% $FeCl_3$ in water, followed by exposure to ammonia vapours.

Preparative column chromatography (CC) was performed with Silica gel Merck, Kieselgel 60, Korngrosse 0.063–0.20 mm, or with charcoal powder. Preparative medium pressure (six bar) column chromatography was obtained with pre-packed LiChroprep RP-8 (40–63 μm) Merck column for liquid chromatography, size A (240–10 mm) or size B (250–25 mm).

### 2.4. HPLC analysis

Extracted BP fractions (10 μl), injected in HPLC column (25 cm×4.6 mm i.d. column, filled with J.T. Baker-Bakerbound reversed-phase $C_{18}$ 5 μm, with a short column 5 cm×4 mm, i.d. — placed immediately before main column), give BP contents, reported in Tables 1, 2, 4–6, related to sample concentration, calculated on peak areas compared to internal standard (IS). BP detection was carried out by instrumentation equipped with gradient pumps. Elution was run out at a 1 ml/min flow rate by following mobile phases: water/acetic acid, pH 3.1 (98/2 v/v) (solvent A), and methanol (solvent B). Selected gradient started with 95% A 5% B for 3 min, 80% A 20% B in 15 min, 80% A 20% B for 2 min, 60% A 40% B in 10 min, 50% A 50% B in 10 min, 100% B in 10 min. The latter condition was then kept for 10 min. A 15 min re-equilibration time was then required.

### 2.5. TBP colorimetric evaluation

After extraction, TBPs, determined colorimetrically using Folin-Ciocalteau reagent (Carlo Erba Reagents),

Table 1
Procedure A, sBP content of examined olives (BP total content%)

| Olive cultivars/BPs | 1[a] | Ty[a] | Ca[a] | pCo[a] | Hca[a] | U1[a] |
|---|---|---|---|---|---|---|
| Green Hojiblanca | 55 | 8 | 25 | 7 | – | 5 |
| Cherry Hojiblanca | 55 | 8 | 25 | 7 | – | 5 |
| Black Hojiblanca | 50 | 5 | 35 | 5 | – | 5 |
| Green Douro | 45 | 40 | 5 | 5 | – | 5 |
| Cherry Douro | 45 | 40 | 5 | 5 | – | 5 |
| Black Douro | 50 | 10 | 25 | 10 | – | 5 |
| Black Thasos | 50 | 20 | 5 | 10 | 10 | 5 |
| Black Conservolia | 50 | 20 | 5 | 10 | 5 | 10 |
| Black Taggiasca | 55 | 20 | 10 | 5 | – | 10 |
| Black Cassanese | 70 | 5 | 10 | 5 | – | 10 |

[a] **1** = Hydroxytyrosol, Ty = tyrosol, Ca = caffeic acid, pCo = *p*-coumaric acid, Hca = hydroxycaffeic acid, U1 = unknown.

are expressed as caffeic acid on olive fruit methanolic extract. BP solution (0.1 ml) was transferred into a 15 ml test-tube and Folin–Ciocalteau reagent (0.5 ml) added. After 5 min, a 20% sodium carbonate solution (3 ml) was added, volume brought up to 10 ml, with distilled water, and centrifuged at 1500*g* for 15 min. After 30 min, the solution 1 ml was transferred into 1 cm cuvette and adsorbance read at 725 nm, as summarised in Table 7.

Table 2
Procedure B, SAHBP content of examined olives (BP total content%)

| Olive cultivars/BPs | 1[a] | Ty[a] | Ca[a] | pCo[a] | Hca[a] | U1[a] |
|---|---|---|---|---|---|---|
| Green Hojiblanca | 45 | 9 | 26 | 9 | – | 11 |
| Cherry Hojiblanca | 45 | 9 | 26 | 9 | – | 11 |
| Black Hojiblanca | 40 | 7 | 40 | 7 | – | 6 |
| Green Douro | 40 | 40 | 5 | 5 | – | 10 |
| Cherry Douro | 40 | 40 | 5 | 5 | – | 10 |
| Black Douro | 45 | 9 | 26 | 9 | – | 11 |
| Black Thasos | 45 | 25 | 5 | 10 | 5 | 10 |
| Black Conservolia | 45 | 20 | 5 | 10 | 10 | 10 |
| Black Taggiasca | 50 | 25 | 10 | 5 | – | 10 |
| Black Cassanese | 70 | 7 | 7 | 7 | – | 9 |

[a] **1** = Hydroxytyrosol, Ty = tyrosol, Ca = caffeic acid, pCo = *p*-coumaric acid, Hca = hydroxycaffeic acid, U1 = unknown.

### 2.6. Extraction and separation of BP olive fraction

#### 2.6.1. Procedure A: isolation of simple BP fraction (sBP)

Olives (sample weight about 100 g) were 6 N HCl added and completely soaked by acid solution, and left for 3 h under reflux. Then, the acid mixture was ethyl acetate exhaustively extracted, until negative $FeCl_3$ and $H_2SO_4$ tests. The resulting organic phase gave a residue (about 350–550 mg, according to examined cvs), treated with acetonitrile:hexane 1:1 mixture, under vigorous stirring. After equilibration, a microcrystalline insoluble material, together with two immiscible organic phases, appeared. By filtration and drying, two organic phases were separated, with under vacuum solvent removal, affording crude sBP fraction, contained in the acetonitrile phase; the qualitative and quantitative profile of crude sBP fraction is reported in Table 1.

#### 2.6.2. Procedure B: isolation of soluble and alkali-hydrolysable BP fraction (SAHBP)

Olives (sample weight about 100 g) were left at room temperature for 24 h, in argon saturated 2 N NaOH, under argon, with treatment repeated until complete BP extraction, revealed by $FeCl_3$ and $H_2SO_4$ tests. Alkaline solutions were separated and stored at 4°C.

Table 3
Procedure C, CBP fraction content of examined olives (BP total content%)

| Olive cultivars/BPs | **1**[a] | Ty[a] | M[a] | **2**[a] | Dol[a] | Hty1G[a] | Hty3G[a] | Hty4G[a] | Ty1G[a] | **4**[a] | **5**[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Green Hojiblanca | 2 | 1 | 14 | 26 | 2 | 4 | 5 | 14 | 4 | 26 | 2 |
| Cherry Hojiblanca | 3 | 1 | 14 | 25 | 2 | 4 | 4 | 14 | 4 | 26 | 3 |
| Black Hojiblanca | 6 | 1 | 18 | 19 | 5 | 4 | 4 | 11 | 4 | 25 | 3 |
| Green Douro | 4 | 2 | 13 | 25 | 3 | 4 | 3 | 14 | 4 | 24 | 4 |
| Cherry Douro | 5 | 2 | 14 | 24 | 3 | 4 | 4 | 13 | 3 | 24 | 4 |
| Black Douro | 6 | 2 | 16 | 21 | 6 | 4 | 3 | 11 | 3 | 24 | 4 |
| Black Thasos | 4 | 2 | 16 | 28 | 3 | 3 | 4 | 12 | 4 | 21 | 3 |
| Black Conservolia | 4 | 2 | 12 | 27 | 2 | 4 | 5 | 16 | 3 | 22 | 3 |
| Black Taggiasca | 3 | 2 | 15 | 28 | 2 | 4 | 5 | 15 | 3 | 21 | 2 |
| Black Cassanese | 2 | 1 | 16 | 27 | 2 | 4 | 5 | 15 | 2 | 23 | 3 |

[a] **1** = Hydroxytyrosol, Ty = tyrosol, M = oleuropein derivative **6a–d** mixtures, **2** = oleuropein, Dol = demethyloleuropein, Hty1G = Hty-1-*O*-β-D-glucoside, Hty3G = HTy-3′-*O*-β-D-glucoside, Hty4G = Hty-4′-*O*-β-D-glucoside, Ty1G = Ty-1-*O*-β-D-glucoside, **4** = cornoside, **5** = halleridone.

Table 4
SBP (mg/100 g pulp of fresh olives)*

| Olive cultivars/BPs | Pr | **1** | Ty | Dpa | Hbe | Va | Ca | Ho | Sy | pCo | Fe | Si | mCo | oCo | 6a | 6b | **2** | U1 | Dol | U2 | Ci | **3** | U3 | U4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Black Hojiblanca | 21 | 52 | 3 | – | 7 | – | 9 | 3 | – | 4 | 1 | 40 | 25 | – | 14 | 129 | 17 | – | 43 | – | – | 17 | – | 9 |
| Green Douro | – | 116 | 12 | – | trc | 4 | – | 4 | 12 | 17 | 4 | 5 | trc | trc | 81 | 145 | 51 | trc | 12 | 27 | 26 | 22 | – | – |
| Green Cassanese | – | 93 | – | – | 14 | 12 | – | – | – | – | 5 | 83 | 16 | 20 | 36 | 135 | 325 | 50 | 14 | 61 | 13 | – | – | – |
| Black Cassanese | trc | 13 | 1 | 1 | 1 | – | 1 | – | 1 | 2 | trc | 14 | trc | 1 | 2 | 2 | 62 | 4 | 20 | trc | 1 | trc | 1 | – |

Pr = Protocatechuic acid., **1** = hydroxytyrosol, Ty = tyrosol, Dpa = 3,4-dihydroxphenilacetic acid, Hbe = *p*-hydroxbenzoic acid, Va = vanillic acid, Ca = affeic acid, Ho = homovanillic acid, Sy = syringic acid, pCo = *p*-coumaric acid, Fe = ferulic acid, Si = sinapic acid, mCo = *m*-coumaric acid, oCo = *o*-coumaric acid, **6a** = oleuropein aglycon, **6b** = enololeuropeindiale, **2** = oleuropein, U1 = unknown RT 40.5, Dol = demethyloleuropein, U2 = unknown RT 42.5, Ci = cinnammic acid, **3** = hydroxytyrosilelenolate, U3 = unknown RT 47.5, U4 = unknown RT 48.2. trc = traces, RT = retention time. *Relative to gallic acid, IS.

Table 5
SEBP (mg/100 g pulp of fresh olives)*

| Olive cultivars/BPs | Pr | **1** | U5 | U6 | Ty | Dpa | Hbe | Hca | Hpa | Cl | Va | Ca | U7 | Ho | U8 | Sy | pCo | Mpa | Fe | Si | oCo | **6a** | **6b** | U1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Black Hojiblanca | 7 | 6 | – | – | – | – | – | – | – | 40 | – | – | 12 | 33 | – | – | 1 | – | 1 | – | 1 | – | – | – |
| Green Douro | trc | 7 | – | 3 | trc | 3 | – | 12 | trc | – | 2 | – | 4 | 13 | 6 | 2 | 1 | trc | trc | 2 | trc | 1 | 1 | 1 |
| Green Cassanese | – | 10 | – | – | 1 | trc | – | – | trc | – | – | – | 12 | 21 | – | – | – | – | 1 | – | 1 | – | trc | 10 |
| Black Cassanese | 37 | 3 | 7 | 3 | 2 | 3 | 9 | 3 | 7 | 2 | 3 | 2 | 9 | 74 | 3 | 39 | 1 | 9 | – | – | 2 | 3 | 11 | – |

Pr = Protocatechuic acid, **1** = hydroxytyrosol, U5 = unknown RT 12.0, U6 = unknown RT 13.7, Ty = tyrosol, Dpa = 3,4-dihydroxphenilacetic acid, Hbe = *p*-hydroxbenzoic acid, Hca = hydroxcaffeic acid, Hpa = 4 hydroxphenilacetic acid, Cl = clorogenic acid, Va = vanillic acid, Ca = caffeic acid, U7 = unknown RT 26.5, Ho = homovanillic acid, U8 = unknown RT 29.0, Sy = syringic acid, pCo = *p*-coumaric acid, Mpa = metoxyphenilacetic acid, Fe = ferulic acid, Si = sinapic acid, oCo = *o*-coumaric acid, **6a** = oleuropein aglycon, **6b** = enololeuropeindiale, U1 = unknown RT 40.5. trc = traces; RT = retention time. *Relative to gallic acid, IS.

Table 6
IBBP (mg/100 g pulp of fresh olives)*

| Olive cultivars/BPs | Pr | **1** | Ty | Va | Ca | U7 | Ho | U8 | Sy | pCo | Fe | oCo | **6b** | U1 | U2 | Ci | **3** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Black Hojiblanca | 46 | – | 4 | 32 | – | – | – | 29 | – | 9 | 3 | – | 16 | 4 | – | 10 | 9 |
| Green Douro | – | 13 | 2 | – | – | – | – | 1 | 1 | – | – | 45 | 32 | – | – | 3 | 10 |
| Green Cassanese | – | – | 12 | 2 | 28 | – | 22 | – | – | 3 | 34 | 10 | 11 | 10 | – | – | – |
| Black Cassanese | 6 | – | 1 | 14 | 2 | 7 | 37 | – | 8 | 9 | 11 | 14 | 8 | – | 3 | 8 | 2 |

Pr = Protocatechuic acid, **1** = hydroxytyrosol, Ty = tyrosol, Va = vanillic acid, Ca = caffeic acid, U7 = unknown RT 26.5, Ho = homovanillic acid, U8 = unknown RT 29.0, Sy = syringic acid, pCo = *p*-coumaric acid, Fe = ferulic acid, oCo = *o*-coumaric acid, **6b** = enololeuropeindiale, U1 = unknown RT 40.5, U2 = unknown RT 42.5, Ci = cinnammic acid, **3** = hydroxytyrosilelenolate. RT = retention time. *Relative to gallic acid, IS.

Collected aqueous phases, acidified at pH∼2 (conc. HCl), at solution temperature below 4°C, were ethylacetate extracted, washed with brine, dried with anhydrous sodium sulphate, and evaporated to dryness under vacuum at room temperature. Residue (about 100–170 mg, according to cvs) was treated as described above for protocol A.

The resulting precipitate was essentially high molecular weight compounds only, as check by the $^1$H-NMR spectra, and not further investigated, while the hexane phase mostly contained fatty acids. After solvent removal, the BP compounds in the acetonitrile phase, afforded crude SAHBP fraction.

Simple BPs were identified by comparison with authentic samples, according to their $^1$H-NMR spectra. The SAHBP quantitative profile, see Table 2, was performed by HPLC on crude SAHBP fraction, using the above reported experimental procedure.

### *2.6.3. Procedure C: isolation of cytoplasmatic BP fraction (CBP)*

The olives (sample weight of about 100 g) were left at room temperature for 24 h in $CH_2Cl_2$. After this treatment, aqueous and organic phases were separated and stored at 4°C; the procedure was then repeated until complete aqueous phase expulsion.

Collected organic and aqueous phases were washed with water and $CH_2Cl_2$, respectively, with washings recombined to their relative phases.

The aqueous phase, containing olive water soluble components, was worked-up to isolate BP fraction, according to the charcoal method (Bianco, Mazzei, Melchioni, Scarpati, Romeo & Uccella, 1998a). Charcoal powder (∼50 g whole) was added to $CH_2Cl_2$ free water solution, until $FeCl_3$ negative test. The resulting suspension, stratified on a Gooch funnel, was salt and simple sugar removed by 5% water and 10% EtOH (1L each) elution, whereas the discontinuous gradient elution, with EtOH from 20 to 90% (300 ml fractions each, were

Table 7
Protocol D, total BP content (mg/100 g pulp of fresh olives)*

| Olive cultivars | BPs | mg/100 g |
|---|---|---|
| Black Hojiblanca | SBP | 394 |
| | SEBP | 101 |
| | IBBP | 162 |
| Green Douro | SBP | 540 |
| | SEBP | 59 |
| | IBBP | 107 |
| Green Cassanese | SBP | 876 |
| | SEBP | 57 |
| | IBBP | 132 |
| Black Cassanese | SBP | 129 |
| | SEBP | 232 |
| | IBBP | 130 |

SBP = soluble BP; SEBP soluble-esterified BP; IBBP insoluble-bound BP. *Relative to caffeic acid.

increased each time 10% EtOH content), separated glucosidic BP compounds. Collected EtOH fractions and volatile material evaporation resulted in crude CBPs (about 150–210 mg), chromatographed on Si gel in *n*-BuOH saturated with $H_2O$, performing BP component separation in relation to polarity. Successive CBP separation was obtained on Si gel with $CHCl_3$:MeOH in a 9:1–7:3 ratio, depending on component polarity. Isolated glucosidic BP compound purification was obtained by medium pressure chromatography columns with discontinuous gradient of $H_2O$/MeOH as eluent (25 ml fractions, increasing 5% MeOH content).

CBPs were identified by comparison with authentic samples, according to their HPLC, $^1$H NMR and $^{13}$C-NMR spectra.

The CBP quantitative profile together with **4** and **5** derivatives, as performed by CC on crude CBPs, is reported in Table 3.

*2.6.4. Procedure D: isolation of soluble (SBP), soluble-esterified (SEBP) and insoluble-bound BP fraction (IBBP)*

Olive samples (100 g randomly chosen), selected from different table olive cultivars, see Tables 4–6, frozen under liquid nitrogen, and freeze dried, were pitted by blending and homogenised in methanol:acetone (1:1, 80 ml), saturated with sodium disulphite, at top 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. The supernatant was separated and the pellet resuspended four times in methanol:acetone (1:1, 80 ml), saturated with sodium disulphite, until a colourless solution was obtained. The combined supernatants were analysed for SBP and SEBP, and the residue was stored for IBBP determination. The combined supernatants were evaporated under vacuum at 45°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 hexane to water phase 1:1 ratio to remove free fatty acids and other lipidic contaminants. The SBPs were then ether/ethylacetate (1:1) extracted six times at a solvent to water phase 1:1 ratio. The ether/ethylacetate extracts were dehydrated with anhydrous sodium sulfate, filtered, evaporated to dryness under vacuum at 30°C, and the dry residue transferred by methanol (5 ml) into vials containing an IS known amount, i.e. gallic acid as IS, and HPLC analysed.

SEBPs, recovered by washing the moist sodium sulphate, and recombining it with the water extract and the cloudy precipitate, hydrolysed with 1 N NaOH (10 ml) for 24 h, under nitrogen and at room temperature, acidified to pH 2 before removing lipidic contaminants with hexane, were then extracted with ether/ethylacetate, and analysed as described above.

The IBBP residue from methanol:acetone (1:1) extraction, hydrolysed directly with 1 N NaOH (80 ml) for 24 h under similar conditions as soluble-esters, after acidification and centrifugation, was extracted as clear supernatants with hexane, then with ether/ethylacetate as described above, and thus HPLC analysed.

Each extraction and separation procedure was repeated three times, and the data presented are the means ± standard error.

## 3. Results and discussion

Four classes of BP derivatives can be considered to occur in olive fruits at different maturity stages: some are soluble in the cytoplasmatic medium of the olive drupe, such as simple-, complex-cytoplasmatic, and esterified biomolecules, and one contains insoluble BPs, since they are cell-wall bound derivatives.

In the olive oil and table olive industry, the first three classes can be relevant for olive oil production, because of their physicochemical, antioxidative, and hedonistic-sensorial properties.

The same classes appear to be crucial during the debittering process of table olive products, while the fourth contributes to the textural aspects of olive foodstuffs.

For consumer acceptance, all can be functional to specific health benefits, via antioxidant or other more peculiar biological activities.

The extent of the four BP class extractions was evaluated by experimenting several methods, i.e. protocols A–D, to investigate olive fruits from Spain (Hojiblanca cv), Portugal (Douro cv), Greece (Conservolia and Thasos cvs), and Italy (Taggiasca and Cassanese cvs).

Finally, four different protocols were set up for isolation, qualitative and quantitative determinations of olive BP contents from the Mediterranean cultivars under investigation. Every protocol was focalised to one or more different classes of biomolecules, all having the characteristic of BP functional groups. Each procedure employed intact olive drupes in order to avoid fast β-glucosidase hydrolytic effect or boiling methanol causing methanolysis of ester linkages (La Londe, Wong & Inn-Mei Tsai, 1976).

The extraction procedures required different experimental conditions in order to ascertain the general BP profile of olive fruits. The four protocols, A–D, were performed by acid hydrolysis for the total simple BPs, by alkali treatment giving information on soluble and esterified BP molecules, by $CH_2Cl_2$ extractions, which provided the qualitative identification of cytoplasmatic BPs, and by solvent (methanol/acetone), alkaline hydrolysis, separation and purification procedures onto freeze-dried olive fruits for soluble, soluble-esterified and insoluble-bound BPs, as shown in the HPLC chromatogram of black Cassanese cv extract, reported in Fig. 1.

Protocol A, characterised by an acid work up, gives information regarding the TBP fraction, consisting of

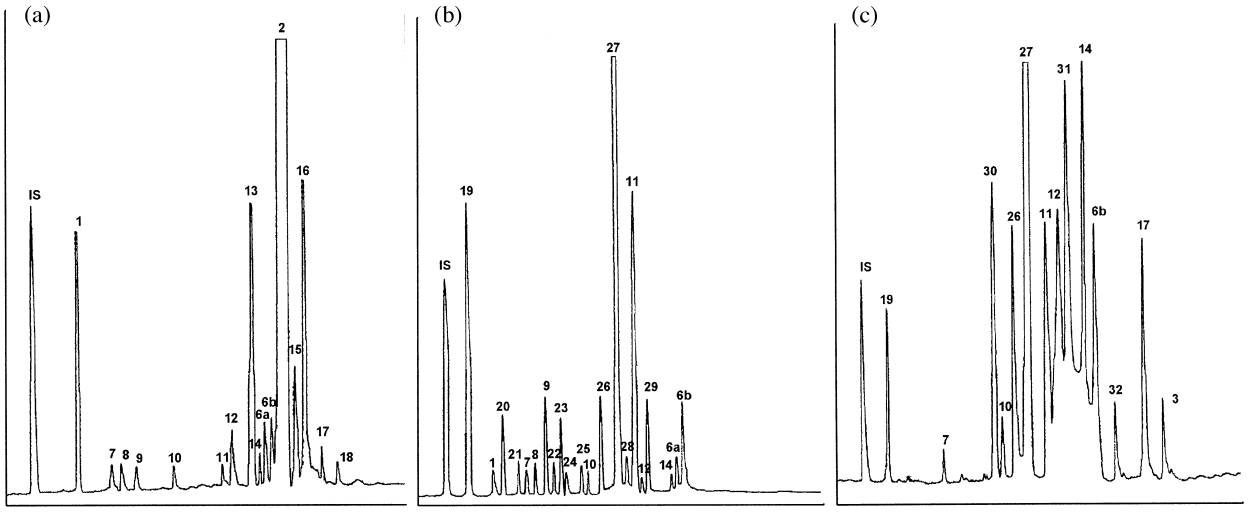



Fig.1. HPLC profile of black Cassanese cv extract. (a). **SBP** = soluble BP: **1** = hydroxytyrosol, **2** = oleuropein, **6a** = oleuropein aglycon, **6b** = enololeuropeindiale, **7** = tyrosol, **8** = 3,4-dihydroxyphenilacetic acid, **9** = *p*-hydroxbenzoic acid, **10** = caffeic acid, **11** = syringic acid, **12** = *p*-coumaric acid, **13** = sinapic acid, **14** = *o*-coumaric acid, **15** = unknown RT 40.5, **16** = demethyloleuropein, **17** = cinnammic acid, **18** = unknown RT 48.2. (b). **SEBP** = soluble-esterified **BP: 1** = hydroxytyrosol, **6a** = oleuropein aglycon, **6b** = enololeuropeindiale, **7** = tyrosol, **8** = 3,4-dihydroxphenilacetic acid, **9** = *p*-hydroxbenzoic acid, **10** = caffeic acid, **11** = syringic acid, **12** = *p*-coumaric acid, **14** = *o*-coumaric acid, **19** = protocatechuic acid., **20** = unknown RT 12.0, **21** = unknown RT 13.7, **22** = hydroxcaffeic acid, **23** = 4-hydroxphenilacetic acid, **24** = clorogenic acid, **25** = vanillic acid, **26** = unknown RT 26.5, **27** = homovanillic acid, **28** = unknown RT 29.0, **29** = metoxyphenilacetic acid. (c). **IBBP** = insoluble-bound BP: **3** = hydroxytyrosilelenolate, **6b** = enololeuropeindiale, **7** = tyrosol, **10** = caffeic acid, **11** = syringic acid, **12** = *p*-coumaric acid, **14** = *o*-coumaric acid, **17** = cinnammic acid, **19** = protocatechuic acid, **26** = unknown RT 26.5, **27** = homovanillic acid, **30** = vanillic acid, **31** = ferulic acid, **32** = unknown RT 42.5. RT = retention time. Relative to gallic acid, IS.

simple molecular structures, free in the cytoplasmatic continuum and formed from both ester and glycosidic functional groups, affected by acid hydrolysis, which releases compounds **1**, Ty, *p*-coumaric, and hydroxycaffeic acids together with an unidentified product. The above experimental conditions were adopted, in order to prevent BP oxidation. Estimated loss could be a negligible result, with **1** appearing to prevail over all the analysed olive samples; consistent differences occur among the other BPs, contained in Hojiblanca and Cassanese cvs, where **1** was revealed to be noticeably higher than Ty.

The general trend of BP distribution, obtained by protocol A in relation to cultivars, summarised in Table 1, was shown to be analogous to that described in Table 2. Therefore, simple and bounded BP complete hydrolysis maintains almost unaltered the relative amounts of simple BP components found in table olives under investigation.

Protocol B is characterised by an alkaline work up, which selectively extracts the mesocarp fraction of soluble and alkali-hydrolyzable BPs, with some resulting from β-eliminative degradation (Parr, Ng & Waldron, 1997). Cold alkali-releasable BPs have thus been detected from intact olive fruits, using a high concentration of alkali, under controlled experimental conditions, i.e. argon saturated atmosphere, to avoid basic catalysed radical autoxidation, since certain BP components are known to be quite strongly bound through acetal linkage, i.e. the glycosidic bond to the cell-wall structure of vegetable tissue matrix, and only slowly released by alkaline hydrolysis (Hartley & Morrison, 1991). Information about the ratio between the latter two BP classes contained in olive mesocarp, is lost, while **1** and Ty glucosides cannot be detected, because they remain soluble in aqueous phase under the experimental mode of protocol B.

Results, reported in Table 2, indicate **1**, the major BP component, always found in the olive samples examined by protocol B. The Hojiblanca cultivar shows caffeic acid to be more abundant in green and cherry olives (26%), becoming identical to **1** in black olives (40%). A Douro cv characteristic feature appears to be the amount of Ty in green and cherry olives (40%, identical to **1**), decreasing to 9% in black ones with the increase of caffeic acid. Typical Greek cultivars are characterised by the presence of hydroxycaffeic acid (5–10%), and by significant quantities of Ty (20–25%) and p-coumaric acid (10%). Italian cultivars are characterised by higher **1** content (50% in Taggiasca and 70% in Cassanese), than other cultivars, comprising between 40 and 45%.

Alkaline degradation, resulting during protocol B treatment, can be related to the industrial debittering of table olives (Brenes, Rejano, Garcia, Sanchez & Garrido, 1995). As shown by the experiments carried out, this process influences the BP content in qualitative and quantitative terms, converting original biomolecular structures into more simple ones, which are somewhat

retained in the olive fruit after processing, thus partially preserving the functional BP properties of starting agri-food matter.

Protocol C, a modification of the charcoal method, used for BP isolation from plant material (Bianco et al., 1998a), offers information regarding BP fractions spread out in the cytoplasmatic medium of olive cellular systems. Similarly to the above, the adopted procedure relays the development of extraction procedure which avoids initial pitting, blending and homogenizing, and the separation of the aqueous-alcoholic solution to be defatted again with hexane, after volatile solvent removal. The latter extraction step may cause artefacts by enzymatic degradation, vide infra, occurring on the original molecular structure of olive BPs, e.g. glucosides **2** and related derivatives **6**, oleuropein aglycon **6a**, enololeuropeindiale **6b**, oleuropeindiale **6c**, and oleuropeindiale hydrate **6d**, (Scheme 3). Experimenting with protocol C, involves $CH_2Cl_2$ treatment of selected intact olive fruits for several hours at room temperature. Under these conditions, the polar solvent penetrates throughout the epidermis zone into the olive mesocarp, thus disturbing the supramolecular interactions between phospholipidic bilayers, which disrupts the cellular and vacuolar membranes of olive tissues. Three layers are then usually observed: a lower organic layer containing the fatty components, an interfacial layer of precipitated proteins, and an upper aqueous layer, $CH_2Cl_2$ saturated. After the olive tissue structure completely collapses and the aqueous content is made accessible to diffusion, protocol C allows the extraction and separation of particularly hydrophilic species, soluble in olive cytoplasmatic medium, such as simple and complex BPs, without substantial enzymatic intervention.

A further rapid work-up of the aqueous phase can again avoid structural modification of the original BP glucosides, like **2**, by pouring charcoal powder into the BP solution. Most of the BP components can be extracted by absorption/desorption partitioning from charcoal, providing free cytoplasmatic BP content with some underestimation, due to organic material loss, because of retention onto the powder.

Experimental results, reported in Table 3, show significant differences among typical Iberian cultivars, and Greek and Italian ones. The results obtained from Hojiblanca and Douro cultivars, examined at different ripening stages, green, cherry and black, confirm the trends observed previously (Amiot, Fleuriet & Macheix, 1989). BP **2** decreases during olive maturation and its derivatives increase conversely. BP **2** appears to be more consistent in black olives from Italy and Greece (27–28%) compared with amounts (18–21%) detected in black olives from Spain and Portugal.

Sample NMR analysis showed hydrolytic derivatives of **2**, i.e. **6a–d**, mixtures of **6a** and **6c–d**, **6d** 90% in $D_2O$, and **6a** and **6c** 15 and 85%, respectively, in $CDCl_3$, with **6c,** i.e. **6c** and **6′c** and **6d** revealing the occurrence of two epimers at C-2 (Bianco, Piperno, Romeo et al., 1999).

Black olive components **4** and **5**, as shown in Scheme 2, are lacking BP molecular structure and thus are not considered further; the most abundant is found to be glucoside **4** (25.5% in Hojiblanca and 24.5% in Douro cvs), in Spain and Portugal samples. This glucoside, detected in Olea europaea and in several oleaceae, and biogenetically related to BP **1**, Ty and **5** (Bianco et al., 1993), shows a percentage similar to **2**, when the above mentioned cultivars are considered at green and cherry maturity stages. Greek and Italian black olives, i.e. Thasos, Conservolia, Taggiasca and Cassanese cvs, reveal **2** to be the main BP component. The latter BP derivatives are also present in olive pulp, as simple biomolecules in unbounded form. Owing to the polar character of simple BPs **1** and Ty, the charcoal method could reveal their relative occurrence, together with the complex forms, i.e. BP **2**, **4**, and so on (Bianco, 1990)

The protocol D work up provides a general picture of the BP whole content, allowing for the determination of three different BP classes, contained in table olive pulp: soluble, soluble-esterified and insoluble cell-wall bound BPs, as shown in Tables 4–6. Although complex, the experimental procedure D gave a satisfactory estimation of the TBP content as reported in Table 7, which in relation to qualitative and quantitative responses, could be higher than the above reported A–C results, but not directly comparable. TBPs obtained by protocol D, show similar trends as found for those from protocols A–C, thus confirming the large variance of BPs contained in olive drupes, according to cultivars and ripeness stages.

The BP total amount and composition, as determined by colorimetric and HPLC analysis, yielded a satisfactory well-resolved profile of each class of biomolecules under investigation contained in the olive drupes. The HPLC procedure adopted for protocol D afforded separation of most of the relevant peaks in the extracts, thus allowing for identification of up to 94, 79 and 91% of the total area at 278 nm, for the three BP classes respectively, while unidentified peaks made up $<6$, 21, and 9% of the same area respectively. The amount of structural assignment to soluble-esterified BPs from olive fruits depends on the unknown composition and on the molecular complexity of these mixtures which must be further investigated.

The extraction by protocol D required careful optimisation, still in progress for soluble-esterified BPs, because of their potential effects on the antioxidant activity of the extra virgin olive oil products. Biomimetic experiments in identical solvent, time and temperature conditions indicated a partial enzymatic conversion, exerted during the solvent removal (Bianco, Piperno & Uccella, 1999), when direct olive pulp was manipulated, as revealed by $^1$H NMR test. This influenced the final BP profile. In fact, the β-glucosidase activity on BP **2**,

being in MeOH, remained ineffective. Solvent removal and water addition revealed the formation of oleuropeindiale **6c**, and aldehydic derivative **6b** and **6d**, reported in Scheme 3. This was shown by the presence of appropriate NMR resonances assigned to these compounds (Bianco, Piperno, Romeo et al., 1999b), demonstrating enzyme activity during BP extraction from olive pulp. The relative ratio among hydrolytes of **2**, i.e. **6a–d**, has been acquired by NMR biomimetic experiments, which provides estimation on the equilibrium established at HPLC conditions, furnishing information on peak assignments reported in the Fig. 1.

Furthermore, experimental results from protocols C and D indicate that BP **2** and its metabolites **6a–d** are dependent on the state of the olive raw materials, mainly its phytosanitary one.

## 4. Conclusion

The BP profile determination, performed on different appropriately selected olive cultivar samples, was achieved by various methodologies applied to olive fruits, chosen to explore the qualitative and quantitative BP content in the olive matrix, suitable for table olive and olive oil production. The adopted protocols, thus investigated, can be considered as implementations to general BP extraction procedures from olive drupes, constituting a whole methodology for examining olive BP contents, related to the nature of BP molecular structures.

The procedure of protocol D for the accurate quantification of soluble, soluble-esterified, and insoluble-bound BP in olive fruits can thus be applied to freeze dried tissues, before any initial manipulation, such as peeling and pitting, because the β-glucosidase fast reacting enzyme activity must be avoided (Barnes & Williams, 1995). The three classes of BPs, extracted with methanol/acetone, and the esterified and cell-wall bound BPs released by alkaline hydrolysis, can be rapidly separated and then purified, to be HLPC analysed, and NMR structurally identified.

On the basis of the above considerations and similar procedures applied to different agrifood products (Guyot, Marnet, Labara, Sanoner & Drillearm, 1998), protocol D was adopted to give a qualitative and quantitative profile of BP components, reported in Tables 4–7 and found in intact olive drupes, after freeze drying and low temperature treatments only. The comparison of BP profiles, obtained from the same sample in different periods, confirmed the results reported in Tables 1–3.

The protocols A–D, thus described, could be applied to raise differences in molecular composition related to BPs found in olive samples harvested from several environments and cultivars at different ripeness stages. The experiments may constitute a new contribution to procedure for the qualitative and quantitative determination of BPs in olive raw materials for table olive and olive oil industrial processing.

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