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Biophenolic profile in olives by nuclear magnetic resonance

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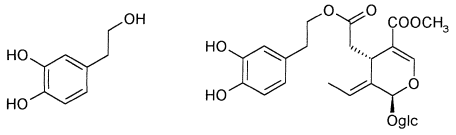
Abstract

The molecular composition of biophenols in olive fruit was investigated in order to experiment with novel procedures for the determination of these microcomponents in fresh and processed table olives. The presence of biophenols in table olives, with recognized antioxidant activity, can be strictly linked to the texture and the organoleptic characteristics of the food product, giving a functional value to this Mediterranean food. Olives from Spain ("Hojiblanca" cv.), Portugal ("Douro" cv.), Greece ("Conservolia" and "Thasos"cv.) and Italy ("Taggiasca" and "Cassanese" cv.) were examined, because the experimental data, checked by high-performance liquid chromatography (HPLC), show molecular composition differences in the tested samples related to the geographic area of analyzed olive fruit cultivars [Bianco, A., & Uccella, N. (2000). Biophenolic components of olives. *Food Research International*, *33*, 475–485]. Three different protocols were utilized: the first one allows the determination of the biophenolic content present as free and esterified compounds; the second affords the total biophenolic content; the third indicates the biophenols present as glycosides. The biophenolic content, which was previously determined by classic HPLC methods (Bianco & Uccella, 2000), was checked by a simple ^{1}H-NMR experiment. The comparison between the data obtained from ^{1}H-NMR with those measured by HPLC, indicates a good agreement and suggests the possibility of employing ^{1}H-NMR for the rapid determination of biophenolic content in olives and also in other foods.

Keywords: Biophenol analytical procedure; ^{1}H-NMR; Table olive; *Olea europaea* cultivars

1. Introduction

Typical secondary plant metabolites, having phenolic functionality, constitute a distinctive group of phytochemicals which posses a great structural diversity and a wide phylogenetic distribution (Bianco, Mazzei, Melchioni, Romeo & Uccella, 1996; Casuscelli, De Nino, Gallo, Procopio, Romeo & Uccella, 1994; Romeo & Ucella, 1996). The uniqueness of this group of metabolites of natural origin (biophenols, BP), found in olive fruits, lies in their phenolic character. The BP olive molecular components are water-soluble monomers. Thus, olive BP cannot be considered polyphenols, since the latter are described as natural products with MW in the range 500–4000 and, per 1000 relative molecular mass, posses some 12–16 hydroxyaromatic groups and 5–7 aromatic ring (Haslam, 1996). Moreover, olive BP are not only simple phenols. They are characterized by multifunctional moieties, such as alcoholic and carboxylic groups, when simple molecules, i.e. hydroxytyrosol 1, and by glycosidic and monoterpenoid units when complex molecules, i.e. oleuropein 2.



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BP monomers, besides the conventional phenolic reactivity, are also distinguished by their ability to associate strongly with primary metabolites such as proteins and carbohydrates (Vekey, Malorni, Pocsfalvi, Piperno, Romeo & Uccella, 1997), and to give rise to supramolecular moieties responsible for their bioavailability (Bianco, Chiacchio, Roscifina, Romeo & Uccella, 1997). Olive BP exert antioxidant activity by quenching free radicals and by metal chelation (Garrido Fernandez, Fernandez Diez & Adams, 1997; Saija et al.,

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1998), participate in the biomolecular defence mechanism against attacking pathogens (Lo Scalzo, Scarpati, Verzegnassi & Vita, 1994) and provide cross-linkage among polysaccharides of the plant cell wall molecular architecture (Appel, 1993), thus affecting the texture characteristics of this Mediterranean food product. All the above mentioned phenomena are of particular interest, since the molecular interactions often substantially influence the properties of many plant products, ranging from the taste, palatability and functional (Goldberg, 1994) value of foodstuffs to the microbial and insect antagonism of the vegetable matter.

In particular, the *o*-diphenolic and monoterpenic multifunctionalities of the natural compounds influences the sensory and functional properties of fresh and processed foods derived from *Olea europaea* L., i.e. table olives and olive oil; the cause a bitter taste. A relationship between the BP distribution and the stability of table olives and olive oils (Arrigo & Rondinone, 1995) as well as its organoleptic features (Vazquez Roncero & Janer del Valle, 1977; Vazquez Roncerno, Maestro Duran & Graziani Costante, 1974; Visioli & Galli, 1998) (flavour and fragrance) was proposed.

The quality of table olives is strongly related to both agronomic traits and ripening level of the fruit, as well as to processing phases during production method (Garrido Fernadez et al., 1997). Indeed, all these factors are able to modulate level and spatial relationship of cell wall components, thus influencing texture and taste of the fruit. Hence, details of cell wall composition (Camurati, Rizziolo & Fedeli, 1981) and cellular structure in the olive fruit tissues (Zarrouk, Marzouk & Cherif, 1990) are necessary. The BP content is as relevant for the texture attributes as is appearance. In this context, attention was addressed to BP compounds, which play a role in structural organization of the cell wall skeleton, as cross-linkers between the different polysaccharidic components, strongly influencing the palatability of olive fruit, as well as of oil, by acting as taste molecules and as natural antioxidants (Vinson, Hao, Su & Zubik, 1998).

The determination of the total BP (TBP) profile was done on a series of olive cultivars harvested for the preparation of table olives in the Mediterranean area (Bianco & Uccella, 2000). A different methodology, proton magnetic resonance, was exploted to test the possibility to obtaining a single non-destructive picture of the content of BP in the olive cultivars chosen for the production of table olives.

2. Materials and methods

2.1. Equipment, materials and methods

High-performance liquid chromatography (HPLC) was performed with a pre-packed μ-bondapack C_{18} column, with a Hewlett-Packard A 220 with diode array detector.

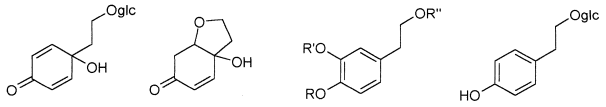
Thin-layer chromatography (TLC) was performed using silica gel SiF_{254} (Merck, Darmstadt, Germany) and RP-8 F_{254} (Merck) prepared plates. Plates were developed with suitable solvents and sprayed with 2N H_2SO_4, followed by heating at 120°C. *o*-Diphenolic compounds, only, were detected by spraying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

Preparative low pressure column chromatography was performed with Silica gel Merck, Kieselgel 60, Korngrosse 0.063–0.20 mm, and with charcoal powder. Preparative medium pressure column chromatography was performed with pre-packed LiChroprep RP-8 (0.040–0.063 nm) Merck columns, (∅ 1 cm).

Nuclear magnetic resonance (NMR) spectra were registered with a 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.

2.2. Reference compounds

Tyrosol (Ty), protocatechuic ac., caffeic ac., hydroxycaffeic ac., vanillic ac, homovanillic ac., 3,4-dihydroxphenyacetic ac., 3-hydroxpheniacetic ac., *p*-hydroxbenzoic, syringic ac., *p*-coumaric ac., ferulic ac., sinapic ac., *m*-coumaric ac., *o*-coumaric ac., cinnamic ac., were very pure products obtained from SIGMA-Chimica (Milano, Italy). 2-(3,4-dihydroxphenyl)ethanol 1 (hydroxytyrosol, HTy) was synthesized according to the method described (Bianco, Passacantilli & Righi, 1998). Oleuropein 2 was prepared according to the procedure described (Panizzi, Scarpati & Oriente, 1960). Oleuropein derivatives were prepared according to the method described (Bianco, Muzzalupo, Uccella, Piperno & Romeo, 1999; Bianco, Iannazzo, Muzzalupo, Piperno, Romeo & Uccella, 1999).



Cornoside 3 and halleridone 4 were isolated according to the procedure described (Bianco, Lo Scalzo & Scarpati, 1993). HTy-1-O-β-D-glucoside 5, HTy-3′-O-β-D-glucoside 6, HTy-4′-O-β-D-glucoside 7 and Ty-1-O-β-D-glucoside 8 were isolated according to the procedure described (Bianco, Mazzei et al., 1998).

2.3. Plant material

Mono-cultivar olive samples were supplied by a partner of EU Project-OLITEXT FAIR 97-3053. *Olea*

europaea L. fruits were collected in Portugal (''Douro'' cultivar at three different stage of ripening: green, cherry and black), in Spain (''Hojiblanca'' cultivar at three different stage of ripening: green, cherry and black), in Greece (''Thasos'' and ''Conservolia'' cultivar, black), and in Italy (''Cassanese'', ''Carolea'' and ''Taggiasca'' cultivar, black) in 1997. Voucher specimens are deposited in the CIRASAIA, Università della Calabria — Cosenza (Italy) and Università di Roma ''La Sapienza''.

2.4. Extraction and separation of BP fraction from olives

2.4.1. Experimental procedure for the isolation of glucosidic and free BP fraction (GFBP)

Olives (sample weight about 350 g) were left in CH_2Cl_2 at room temperature for 24 h. After this period, two phases were present, an aqueous one and an organic one. These two phases were separated and stored at low temperature, and the treatment with CH_2Cl_2 at room temperature for 24 h was repeated until no more separation of an aqueous phase occurred.

The organic phases were collected and washed with water; the aqueous phases were collected and washed with CH_2Cl_2; the washings were added to the respective phases. The CH_2Cl_2 phase contains oil and the other non-polar components of olives, not examined until now.

The aqueous phase, containing water soluble components of olives, was worked-up to isolate the GFBP fraction, utilizing the charcoal method for the isolation of the polar glycosides (Bianco et al., 1998). The water solution was concentrated to eliminate CH_2Cl_2 residues and charcoal powder (~50 g in all) was added until water gave a negative $FeCl_3$ test. The resulting suspension was then stratified on a Gooch funnel. Salts and simple sugars were removed by elution with water, 5 and 10% EtOH (1 l each), whereas the elution with a discontinuous gradient of EtOH from 20 to 90% (fractions of 300 ml each, each time increasing the EtOH content by 10%), eluted glucosidic compounds. EtOH fractions (20–95%) were collected and, after evaporation of volatile materials, the resulting crude GFBP fraction was chromatographed on silica gel in *n*-BuOH saturated with H_2O, performing a first separation of components in relation to their polarity. Successive separation of GFBP fractions was performed on silica gel with $CHCl_3$/MeOH in of ratio from 9:1 to 7:3, depending on the polarity of components. The final purification of each isolated glucosidic compound was obtained by medium pressure chromatography columns with a discontinuous gradient of H_2O/MeOH as the eluent (fractions of 25 ml, increasing MeOH content by 5%).

GFBP components were identified by comparison with authentic samples, registering their ^{1}H-NMR and ^{13}C-NMR spectra.

Quantitative profiling of GFBP fraction was achieved by HPLC on the crude GFBP fraction. Elution was carried out at a flow rate of 1 ml/min by means of a continuous gradient of H_2O/MeOH with 1% acetic acid, as eluent, during 30 min. Data are reported in Table 1.

Table 1
GFBP fraction content of examined olives (mg of compound 350 g of fresh olives, % of total BP content)[a]

Olives/BP	A	B	C	D	E	F	G	H	I	L	M
Hojioblanca green	501	48	280	85	93	280	80	503	50	10	35
	26	2	14	4	5	14	4	26	2	1	2
Hojioblanca cherry	500	50	290	88	92	270	75	520	61	15	50
	25	2	14	4	4	14	4	26	3	1	3
Hojioblanca black	402	120	403	101	98	240	80	530	55	20	120
	19	5	18	4	4	11	4	25	3	1	6
Douro green	628	88	320	90	90	330	90	602	100	50	100
	25	3	13	4	3	14	4	24	4	2	4
Douro cherry	602	90	350	100	95	340	90	600	102	55	120
	24	3	14	4	4	13	3	24	4	2	5
Douro black	545	150	420	120	80	302	80	620	102	45	150
	21	6	16	4	3	11	3	24	4	2	6
Thasos	635	78	370	80	90	280	80	470	60	40	100
	28	3	16	3	4	12	4	21	3	2	4
Conservolia	690	50	302	100	120	420	70	550	80	50	100
	27	2	12	4	5	16	3	22	3	2	4
Taggiasca	780	50	420	120	140	410	75	600	60	50	100
	28	2	15	4	5	15	3	21	2	2	3
Cassanese	770	70	450	110	145	420	70	650	70	40	50
	27	2	16	4	5	15	2	23	3	1	2
Carolea	750	50	420	130	135	440	60	640	60	40	70
	27	2	15	5	5	16	2	23	2	1	3

[a] A, Oleuropein 2; B, Demethyloeuropein; C, not separated oleuropein derivatives; D, HTy-1-O-β-D-glucoside; E, HTy-3′-O-β-D-glucoside; F, HTy-4′-O-β-D-glucoside; G, Ty-1-O-β-D-glucoside; H, cornoside; I, halleridone; L, Ty; M, HTy.

2.4.2. Experimental procedure for the isolation of the free and alkali-hydrolyzable BP fraction (FAHBP)

Olives (sample weight about 350 g) were left in an argon saturated 2 N NaOH at room temperature for 24 h, under an argon atmosphere. After this period, the alkaline solution was separated and stored at low temperature, and the treatment with 2 N NaOH was repeated until no more extraction of BP material was revealed ($FeCl_3$ and H_2SO_4 tests).

The aqueous phases were collected and acidified at pH~2 with conc. HCl, keeping the temperature of the solution not higher than 4°C. The resulting solution was extracted with ethyl acetate until no more organic material was passed in the organic phase which was then washed with brine and dried with anhydrous Na_2SO_4. After elimination of volatile material under reduced pressure, the residue (about 500 mg, in relation with the examined olives) was treated, under vigorous stirring, with a mixture of acetonitrile/hexane 1:1. After equilibration, a microcrystalline not insoluble material, together with the two immiscible organic phases, appeared to be present. The solid was separated by filtration and dried and the two organic phases were separated, dried and the solvent evaporated under reduced pressure.

The precipitate appeared to be constituted, by check with ^{1}H-NMR spectra, only of high molecular weight terpenoid compounds and was not further examined, while the hexane phase appeared to contain, essentially, fatty acids. The BP compounds remained in the acetonitrile phase which, after evaporation of volatile materials, afforded the crude FAHBP fraction.

Quantitative and qualitative profiling of the FAHBP fraction was achieved by registering ^{1}H-NMR spectra on the crude FAHBP fraction in CD_3OD and comparing the obtained data with those obtained from a standard solution of reference compounds. In Table 2 the content of the isolated BP fraction is reported, in relation to the samples examined and in comparison with HPLC data.

2.4.3. Experimental procedure for the isolation of total BP fraction (TBP)

Olives (sample weight about 100 g) were rapidly chopped and a suitable quantity of 6N HCl was added (olive material must be completely covered by acid). Temperature was raised until reflux started and the mixture was left under reflux for about 3 h. After this period, the acid mixture was exhaustively extracted with ethyl acetate, until no more extraction of organic material was revealed ($FeCl_3$ and H_2SO_4 tests).

The resulting organic phase was then washed with brine and dried with anhydrous Na_2SO_4. After elimination of volatile material under reduced pressure, the residue (about 450 mg) was treated, under vigorous stirring, with a mixture of acetonitrile/hexane 1:1 as described in procedure B, affording the crude TBP fraction.

Quantitative and qualitative profiling of the TBP fraction was achieved by registering ^{1}H-NMR spectra

Table 2
FAHBP content of examined olives (% of total BP content)[a]

Olives/BPs	A	B	C	D	E	F
Hojiblanca green (HPLC)	46	8	26	9		11
idem (^{1}H-NMR)	45	10	25	10		10
Hojiblanca cherry (HPLC)	46	8	26	9		11
idem (^{1}H-NMR)	45	10	25	10		10
Hojiblanca black (HPLC)	39	7	39	7		8
idem (^{1}H-NMR)	40	5	40	5		10
Douro green (HPLC)	41	41	6	4		8
idem (^{1}H-NMR)	40	40	5	5		10
Douro cherry (HPLC)	41	41	6	4		8
idem (^{1}H-NMR)	40	40	5	5		10
Douro black (HPLC)	46	9	26	8		11
idem (^{1}H-NMR)	50	10	25	10		5
Thasos (HPLC)	44	24	6	11	11	4
idem (^{1}H-NMR)	45	25	5	10	10	5
Conservolia (HPLC)	47	21	6	12	9	5
idem (^{1}H-NMR)	50	20	5	10	10	5
Taggiasca (HPLC)	51	24	11	6		8
idem (^{1}H-NMR)	55	25	10	5		5
Cassanese (HPLC)	71	7	7	7		8
idem (^{1}H-NMR)	70	5	5	5		15
Carolea (HPLC)	73	7	7	7		6
idem (^{1}H-NMR)	75	5	5	5		10

[a] A, HTy; B, Ty; C, caffeic acid; D, *p*-coumaric acid; E, hydroxycaffeic acid; F, other not identified biophenols.

on the crude TBP fraction in CD_3OD and comparing the obtained data with those obtained from a standard solution of reference compounds, as for the protocol B. In Table 3 the content of the isolated BP fraction is reported, in relation to the samples examined and in comparison with HPLC data.

3. Results and discussion

The determination of the BP profile, by protomagnetic resonance, was on different table olive cultivar samples, appropriately selected. The three different described protocols ascertain the precise contents of simple and conjugated-glucoside-BP contained in the olive cultivar chosen to produce table olives (Bianco & Uccella, 2000). Each protocol focusseson one or more class of compounds, all having the characteristic of the presence of a biophenolic function.

The proposed NMR determination is not an alternative to HPLC procedures, but constitutes a rapid, alternative methodology to examine the BP contents, in relation to the main components. In fact, the sensitivity of the NMR technique does not allow determination of components that are present in quantities less than 5% of the total.

The tables show comparisons between the data obtained by NMR and by HPLC. There is very good agreement, obviously as regards the main biophenolic components.

Isolation of the glycosidic and free biophenolic fraction (protocol A), shows that the amount of biophenolic compounds present in olives is quite large and generally about 1%. Protocol A quantifies the GFBP fraction. The protocol is a modification of the charcoal method used for the isolation of glycosidic fraction from plant material (Bianco, Mazzei, Melchioni, Scarpati, Romeo & Uccella, 1998). With this procedure, which is absorption/desorption chromatography, the total content of GFBP is underestimated by 2–4%, typical for organic material on charcoal.

Oleuropein **2** appears to be present in larger quantities in olives originating in Italy and Greece (27–28%) than that (18.5–21%) detected in olives of Spain and Portugal. In addition, 2 appears to be the main glucosidic component of olives originating in Italy (''Taggiasca'' ''Carolea'' and ''Cassanese'' cultivars) and Greece (''Thasos'' and ''Conservolia'' cultivars). The main glucosidic component of olives, originating in Spain and Portugal, appears to be, on the other hand, cornoside 3 (24.5% in ''Hojiblanca'' and 23.5% in ''Douro'' cultivars), a glucoside detected in *O. europaea* and in several *oleaceae* (Bianco et al., 1993) and biogenetically related to hydroxytyrosol **1**, tyrosol and halleridone **4** (Bianco, Iannazzo et al., 1999). These last BP derivatives are also present in the olive pulp as simple molecules, not in the bound form, and, owing to their polar character, they are isolated with the charcoal method, revealing their quantities relative to bound forms (oleuropein **2**, cornoside, etc.)

Table 3
Total BP content of examined olives (% of total BP content)[a]

Olives/BPs	A	B	C	D	E	F
Hojiblanca green (HPLC)	54	8	26	7		5
idem (^{1}H-NMR)	55	10	25	5		5
Hojiblanca cherry (HPLC)	53	8	26	8		5
idem (^{1}H-NMR)	55	10	25	5		5
Hojiblanca black (HPLC)	48	6	36	6		4
idem (^{1}H-NMR)	50	5	35	5		5
Douro green (HPLC)	47	39	6	4		4
idem (^{1}H-NMR)	45	40	5	5		5
Douro cherry (HPLC)	48	40	4	4		4
idem (^{1}H-NMR)	45	40	5	5		5
Douro black (HPLC)	52	9	24	9		6
idem (^{1}H-NMR)	50	10	25	10		5
Thasos (HPLC)	51	20	6	9	9	5
idem (^{1}H-NMR)	50	20	5	10	10	5
Conservolia (HPLC)	50	21	6	10	8	5
idem (^{1}H-NMR)	50	20	5	10	10	5
Taggiasca (HPLC)	57	21	11	6		5
idem (^{1}H-NMR)	55	20	10	5		10
Cassanese (HPLC)	73	6	9	6		6
idem (^{1}H-NMR)	70	5	10	5		10
Carolea (HPLC)	71	11	6	6		6
idem (^{1}H-NMR)	75	10	5	5		5

[a] A, HTy; B, Ty; C, caffeic acid; D, *p*-coumaric acid; E, hydroxycaffeic acid; F, other not identified biophenols.

Finally, the data for "Hojiblanca" and "Douro" cultivars, which were examined in different stages of ripening (green, cherry and black), confirm previously obtained results (Amiot, Fleuriet & Macheix, 1989; Donaire, Sanchez, Lopez-Georgè & Recalde, 1975), with a decrease of oleuropein **2** during olive maturation and a contemporary increase of oleuropein derivatives.

The two other protocols yield the amounts of free and alkali-hydrolysable biophenols (protocol B), and of the total biophenolic contents (protocol C). In both cases, the crude biophenolic fraction was analyzed by HPLC and by ^{1}H-NMR and the results, summarized in the Tables 2 and 3, are generally in good agreement.

Protocol B is characterized by an alkaline work-up which quantitatively extracts the whole FAHBP fraction. Information on the ratio between FBP and AHBP in olives is lost, while glucosides of tyrosol and hydroxytyrosol are not detected by this method, remaining soluble in the aqueous phase.

Table 2 shows no significant differences between HPLC and NMR data. Table 2 indicates that HTy is always the major BP component in the FAHBP fraction of examined olives. Italian cultivars are characterized by higher HTy contents (70% in "Cassanese" and 50% in "Taggiasca"), than other cultivars, (40–45%). Greek cultivars are typically characterized by the presence of hydroxycaffeic acid (5–10%) and by significant quantities of Ty (20–25%) and *p*-coumaric acid (10%). In the "Hojiblanca" cultivar, caffeic acid is present in large amounts in green and cherry olives (26%), becoming identical to that of HTy in black olives (40%). The characteristic feature of the "Douro" cultivar is the large amount of Ty in green and cherry olives (40%, identical to HTy), which decreased to 9% in black olives with a contemporary increase of caffeic acid.

Alkaline treatment is used for the industrial debittering of table olives; this treatment, as demonstrated by our results, causes a high decrease of biophenols content and deprives the food of precious compounds from a nutritional point of view.

Protocol C, characterized by an acid work-up, gives information regarding the TBP fraction, formed by both ester and glycosidic functional groups sensitive to acid hydrolysis. The experimental conditions thus adopted, do not cause phenol oxidation and the estimated loss of BP can be negligible.

The reported data (Table 3) show no significant differences between HPLC and NMR data. The general trend of the BP distribution, obtained by method C in relation to the cultivars, is summarized in Table 3, and is analogous to that described in Table 2. As previously described (Bianco & Uccella, 2000), the complete hydrolysis of bound BP does not significantly alter the relative quantities of the main BP components.

The NMR technique, thus investigated, could be applied to indicate molecular composition of BP found in olive samples.

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