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Biophenols in Table Olives

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Unprocessed olives are well-known sources of phenolic antioxidants with important biological properties. Processing methods to prepare table olives may cause a reduction of valuable phenols and may deprive the food of precious biological functions. The present work was undertaken to evaluate table olives produced in Greece as sources of biophenols. Commercially available olives were analyzed for their total phenol content by using the Folin−Ciocalteu reagent and for individual phenols by RP-HPLC. Samples were Spanish-style green olives in brine, Greek-style naturally black olives in brine, and Kalamata olives in brine. Most of the types of olives analyzed were found to be good sources of phenols. Hydroxytyrosol, tyrosol, and luteolin were the prevailing phenols in almost all of the samples examined. High levels of hydroxytyrosol were determined mainly in Kalamata olives and Spanish-style green olives, cultivar Chalkidiki (250−760 mg/kg).

KEYWORDS: Table olives; biophenols; hydroxytyrosol; luteolin

INTRODUCTION

The Mediterranean diet, rich in fruits, vegetables, and grains as well as olive oil and olives, has been associated with a lower risk of coronary heart disease and cancer (*1*, *2*). The positive role of olive oil has been related to its fatty acid composition and the presence of phenolic compounds. There is extended literature concerning phenolic compounds in raw olives and virgin olive oil. However, the number of studies focused on the changes of these important constituents during processing and their levels in processed olives is limited (*3*−*6*).

The main biophenols found in unprocessed olives are hydroxytyrosol and tyrosol in free and bound forms (*7*−*11*). The most abundant of these biophenols is the secoiridoid oleuropein, which is a combination of oleoside-11-methyl ester and hydroxytyrosol (*12*). This compound is responsible for the bitter taste of unprocessed olives. Other hydroxytyrosol derivatives present are oleuropein aglycons (*12*), demethyloleuropein (*13*), and hydroxytyrosol glucosides (*14*). The levels of hydroxytyrosol and derivatives in fresh ripe olive fruits from Greek, Italian, Portuguese, and Spanish cultivars, suitable for table olive production, have been found to vary from 100 to 430 mg/kg and from 3670 to 5610 mg/kg, respectively (*15*). Fresh olives from Italian cultivars harvested at optimum ripening stage have been also found to contain verbascoside at levels ranging from 160 to 3200 mg/kg (*16*). Verbascoside, the main hydroxycinnamic acid derivative found in olive fruits, is a caffeoylrhamnosylglucoside of hydroxytyrosol (*17*). Tyrosol derivatives present are tyrosol 1-*O*-glucoside (*18*) and ligstroside, a phenolic compound closely related to oleuropein (*19*). Tyrosol in its free form and tyrosol 1-*O*-glucoside have been found to vary from 30 to 160 mg/kg and from 170 to 260 mg/kg, respectively (*15*). However, ligstroside has been not found in fresh ripe olives (*15*, *16*).

Other phenolic compounds present in unprocessed olives are 3,4-dihydroxyphenylglycol (*20*), anthocyanins, flavonoids, and phenolic acids (*7*). The most abundant anthocyanins found are cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside (*21*). Their levels have been found to vary from 50 to 880 mg/kg and from 250 to 3200 mg/kg, respectively (*16*). Quercetin 3-*O*-glucoside (rutin) and luteolin 7-*O*-glucoside are the two main flavonoids found in raw olive fruits (*17*, *22*). They are present at levels ranging from 110 to 660 mg/kg and from 5 to 600 mg/kg, respectively (*12*, *23*). Hydroxybenzoic, hydroxycinnamic, and hydroxyphenylacetic acids, and also hydrocaffeic acid, have been found to occur in olive flesh (*24*). From the large number of these acids, those found at considerable levels are caffeic acid and *p*-coumaric acid (*15*). The procedure used for the isolation of phenolic compounds from olives plays an important role in the estimated levels of individual phenolic acids (*24*).

Oleuropein and hydroxytyrosol are known to possess several biological properties, many of which are attributed to their antioxidant and free radical scavenging ability (*25*). Dietary antioxidants present in virgin olive oil and olives were found to increase the resistance of low-density lipoproteins to oxidation (*26*, *27*). Hydroxytyrosol and oleuropein were shown to increase NO production from macrophages (*28*). Hydroxytyrosol was also reported to inhibit peroxynitrite-dependent DNA damage (*29*), to protect human erythrocytes against hydrogen peroxide-induced oxidative alterations (*30*), and to reduce the urinary excretion of the F_2-isoprostane 8-iso-PGF_{2a}, a biomarker of oxidative stress (*31*). Oleuropein is also believed to have many functional properties. Olive tree leaf extracts, which are a rich

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source of this secoiridoid (*32*), are used as folk medicines against many pathogenic conditions.

Olives are consumed after processing for removal, at least partially, of their natural bitterness. Three types of table olives are of great importance in international trade and are mainly produced on an industrial scale: Spanish-style green olives in brine, Greek-style naturally black olives in brine, and California-style black olives in brine (*33*). The latter do not necessarily require a fermentation process, as in the case of Spanish-style green olives in brine and Greek-style naturally black olives in brine. The treatments during processing and the type of fermentation may cause several alterations in the profile and level of valuable biophenols and may deprive the final product of precious biological functions.

The aim of this work is to examine if processed table olives are a good source of hydroxytyrosol and other biophenols and may contribute to the benefits of the Mediterranean diet. Samples of table olives were collected from the retail market and analyzed for their content in total and individual phenols. Total phenols were measured by a colorimetric method using the Folin−Ciocalteu reagent. TLC was used to screen individual phenols in the purified olive extract. Then RP-HPLC coupled with a diode array detector was employed to identify and quantify those compounds present in abundance.

MATERIALS AND METHODS

Samples. Twenty-five samples of table olives, packed in cans, glass bottles, flexible packages, or barrels, were purchased from the local market.

Reference Compounds. Hydroxytyrosol was synthesized according to the procedure of Baraldi et al. (*34*). Its purity was checked by HPLC analysis. The chemical structure was verified with ^{1}H and ^{13}C NMR and GC-MS analysis of the TMS ether derivative. Tyrosol, vanillic acid, and *p*-coumaric acid were purchased from Aldrich (Steinheim, Germany); apigenin, luteolin, and oleuropein were from Roth (Karlsruhe, Germany); and caffeic acid was from Riedel de Haën (Seelze, Germany).

Apparatus. High-perfomance liquid chromatographic analysis of the individual phenols was performed with a system consisting of a Thermoquest SpectraSystem quaternary pump model P4000 (Austin, TX), a Rheodyne model 9725i injection valve with a 20 μL loop, a Laballiance column oven model 505 (State College, PA), and a Thermoquest SpectraSystem diode array detector model UV6000LP. The data were processed electronically by the Thermoquest model ChromQuest chromatography workstation (plus spectral software). A UV−vis spectrophotometer Hitachi model U-2000 (Tokyo, Japan) was used in the colorimetric determination of total phenols.

Extraction and Purification of Phenolic Compounds. Six medium or large size fruits from each table olive sample were destoned. The obtained plant material was weighed and then immediately subjected to freeze-drying. The dry mass was crushed in a porcelain crucible, and 1 g of the powder was extracted three times with 15 mL of 80% (v/v) ethanol, containing 0.5% (w/v) sodium metabisulfite, for 20 min. The extracts were combined, and ethanol was evaporated under vacuum at 40 °C. The residue was dissolved in water (8 mL) and extracted twice with petroleum ether (15 mL) for the removal of pigments and most of the lipids. Methanol (20%, v/v), ammonium sulfate (20%, w/v), and metaphosphoric acid (2%, w/v) were added to the water solution, for acidification and reinforcement of the ionic strength. The phenolic compounds were then extracted by ethyl acetate (20 mL). The collected extracts of four successive extractions were dried with anhydrous sodium sulfate, the solvent was evaporated at 40 °C, and the dry residue was dissolved in methanol (5 mL). Aliquots of this solution were used for both the colorimetric and chromatographic analysis. The repeatability of the extraction procedure was checked for both colorimetric determination of total phenols (CV% = ±4.2, n = 5) and HPLC quantification of individual phenols: hydroxytyrosol (CV% = ±2.7, n = 5), tyrosol (CV% = ±3.4, n = 5), and luteolin (CV% = ±4.9, n = 5).

Colorimetric Determination of Total Phenols. Total phenols were determined by using the Folin−Ciocalteu reagent according to the method of Gutfinger (*35*). A calibration curve with equation: $y = 0.0111x + 0.0081$ ($R^2 = 0.9996$) was constructed using caffeic acid solutions within the range of 1−10 mg/L. The determination was performed twice for each olive extract (CV% = ±2.5, n = 5).

Characterization of Phenolic Compounds by TLC. The phenolic extract was subjected to chromatographic analysis on TLC plates precoated with silica gel 60. A solvent system of trichloromethane/ethyl acetate/formic acid (50:40:5, v/v/v) was used (*36*). The phenolic compounds were detected by spraying the TLC plates with diluted Folin−Ciocalteu reagent. Their characterization was based on the comparison of the R_f values with those of reference compounds and on the color developed after the TLC plates had been sprayed with ferric chloride (1%, w/v) and aluminum chloride (1%, w/v) in ethanol (*37*). They were also characterized by HPLC and UV spectroscopy after extraction from the silica layers.

Separation, Identification, and Quantification of Phenolic Compounds by RP-HPLC Diode Array Detection. Elution was performed on a Spherisorb ODS-2 (5 μm, 25 cm × 4.6 mm i.d.) analytical column from Anachem (Luton, U.K.) after a suitable adaptation of the system used. It was applied according to the procedure of Pirisi et al. (*38*). The gradient elution system, consisting of solution A and mixture B, was 4% B for 1 min, 4−30% B in 25 min, 30−60% B in 10 min, 60−98% B in 30 min, 98−4% B in 4 min, and 4% B for 10 min, where A was a 1% (v/v) formic acid solution and B was methanol/acetonitrile (50:50, v/v). The flow rate was 0.9 mL/min. The injection volume was 20 μL. The identification of the chromatographic peaks was made by comparing the retention times with those of reference compounds and by recording the UV spectra of the peaks in the range 220−360 nm by means of the diode array detector. The external standard method was used for the quantification of the individual phenolic compounds. The hydroxytyrosol and tyrosol contents (at 280 nm) of the samples were estimated in triplicate from the calibration curves using the equations $y = 84307x + 32765$ ($R^2 = 0.9955$) and $y = 58436x - 41101$ ($R^2 = 0.9953$), respectively. Luteolin content (at 346 nm) was also estimated in triplicate from a calibration curve using the equation $y = 543494x - 38507$ ($R^2 = 0.9999$).

RESULTS AND DISCUSSION

The olive samples, with their characteristics, are presented in **Table 1**. They are representative of the three main Greek cultivars, Conservolea (Amfissa), Nychati Kalamata, and Chalkidiki.

Conservolea is the economically most important cultivar in Greece. This cultivar is processed in two ways. When green mature, the olives are used for the production of Spanish-style green olives in brine. The processing includes lye treatment, washing, and lactic acid fermentation in brine (*33*). Black mature fruits are used for Greek-style naturally black olives in brine. The treatment in this case is milder and involves washing, natural fermentation in brine, and color improvement, mainly during sorting and grading before packing. For the fermentation a mixed microflora of bacteria and yeasts is responsible (*33*).

Nychati Kalamata is the second most important cultivar. It is mainly used for the production of table olives known as "Kalamata olives in brine", which are very popular in Greece. They are now of increasing importance in the European Union, the United States, Canada, and Australia. This is due to the texture and flavor of the final product, which are appreciated by a wide range of consumers. Kalamata olives are a special type of Greek-style naturally black olives in brine produced by brining for 5−8 days (the liquid is changed two or three times each day) and immersion in wine vinegar for 1−2 days (*33*).

Table 1. Table Olive Samples

sample	cultivar	type of table olives	flavored with	additives[a]
1	Chalkidiki	Spanish-style green olives in brine		E270
2	Chalkidiki	Spanish-style green olives in brine		E270
3	Chalkidiki	Spanish-style green olives in brine		E270
4	Chalkidiki	Spanish-style green olives in brine		E270
5	Chalkidiki	Spanish-style green olives in brine		E270
6	Conservolea	Spanish-style green olives in brine		E270, E300, E330
7	Conservolea (biological cultivation)	Spanish-style green olives in brine		E270
8	Conservolea	Spanish-style pitted green olives stuffed with red pepper in brine		E270, E330
9	Conservolea	Greek-style naturally black olives in brine		E270
10	Conservolea	Greek-style naturally black olives in brine		E270
11	Conservolea	Greek-style naturally black olives in brine		E270
12	Conservolea	Greek-style naturally black olives in brine	vinegar, oregano	
13	Conservolea	Greek-style naturally black olives in brine	olive oil, vinegar, oregano	
14	Conservolea	Greek-style naturally black olives in brine		E270
15	Nychati Kalamata	Kalamata olives in brine	virgin olive oil, vinegar	E270
16	Nychati Kalamata (biological cultivation)	Kalamata olives in brine	vinegar	
17	Nychati Kalamata	Kalamata olives in brine	virgin olive oil, vinegar, oregano	
18	Nychati Kalamata	Kalamata olives in brine	virgin olive oil, vinegar	
19	Nychati Kalamata	Kalamata olives in brine	virgin olive oil, vinegar	E270
20	Nychati Kalamata	Kalamata olives in brine	virgin olive oil, vinegar	
21	Nychati Kalamata	Kalamata olives in brine	virgin olive oil, vinegar	
22	Nychati Kalamata	Kalamata olives in brine	virgin olive oil, vinegar	
23	Nychati Kalamata	Kalamata olives in brine	extra virgin olive oil, wine vinegar	E270
24	Thassos	naturally black olives in dry salt		
25	Thassos	naturally black olives in dry salt	olive oil, oregano	

[a] E270, lactic acid; E300, ascorbic acid; E330, citric acid.

The third cultivar is Chalkidiki. These olives have a potential as the raw material suitable for the production of Spanish-style green olives in brine.

Samples 24 and 25 in the table represent naturally black olives in dry salt, cultivar Thassos. This treatment (olives layered with table salt) is applied only domestically (*33*).

Table 2 presents the results obtained for total phenol and HPLC data for the main phenols.

Total phenol content, expressed as caffeic acid, was found to range from 178 to 1718 mg/kg of flesh. Kalamata olives showed the highest mean content in total phenols (1046 mg/kg), probably because of the mild treatment. Lower mean values were found in Greek-style naturally black olives (708 mg/kg) and Spanish-style green olives (632 mg/kg). The two samples of untreated naturally black olives in dry salt contained similar amounts of total phenols.

A preliminary screening of the extracts with TLC indicated the presence of a limited number of individual phenolic compounds. HPLC analysis confirmed the presence of hydroxytyrosol, tyrosol, and luteolin. Oleuropein, other bound forms of phenols, and free phenolic acids were not found. These findings can be explained by the composition of unprocessed olives and the treatment.

Lye treatment hydrolyzes ester bonds. Oleuropein gives hydroxytyrosol and elenolic acid glucoside (*5*). Verbascoside is hydrolyzed to caffeic acid and hydroxytyrosol 1-*O*-rhamnosylglucoside. Glycosides, including luteolin 7-*O*-glucoside, are either partially hydrolyzed during lactic acid fermentation or pass completely into the brine (*3*) and therefore are not found in the fermented fruits. This explains the presence of only hydroxytyrosol, tyrosol, and luteolin in the flesh of the examined Spanish-style green olive samples.

During the long processing period (>6 months) for preparing Greek-style naturally black olives, a diffusion of polar phenolic compounds into the brine and a complete hydrolysis of oleuropein and its aglycons in olive flesh take place (*33*). This is related to *Lactobacillus plantarum* strains, which hydrolyze the rest of oleuropein and its aglycons by means of β-glycosidase and esterase action (*39*, *40*). Thus, bound forms of hydroxytyrosol are absent in the flesh of the fermented fruits. Oleuropein, oleuropein aglycons, and hydroxytyrosol are known for their

Table 2. Total Phenol, Hydroxytyrosol, Tyrosol, and Luteolin Contents of Table Olive Samples

sample	total phenols (as caffeic acid), mg/kg	hydroxytyrosol,[a] mg/kg	tyrosol,[a] mg/kg	luteolin,[a] mg/kg
1	1222	450 ± 12	99 ± 8	7 ± 0.1
2	985	371 ± 17	23 ± 1.5	12 ± 0.2
3	640	499 ± 10	106 ± 2	3 ± 0.1
4	703	513 ± 3	87 ± 0.6	4 ± 0.1
5	398	287 ± 3	64 ± 0.6	5 ± 0.2
6	354	233 ± 6	45 ± 1	2 ± 0.2
7	579	169 ± 4	30 ± 1	6 ± 0.1
8	178	43 ± 1	9 ± 1	6 ± 0.1
9	490	219 ± 9	41 ± 2	27 ± 1.5
10	615	101 ± 5	24 ± 1	26 ± 1.0
11	843	204 ± 14	38 ± 4	31 ± 1.4
12	1014	339 ± 9	34 ± 1	36 ± 0.6
13	1074	209 ± 3	19 ± 0.4	55 ± 0.9
14	210	0	13 ± 0.6	1 ± 0.1
15	994	475 ± 13	101 ± 3	43 ± 2.1
16	1527	431 ± 13	166 ± 7	74 ± 1.5
17	1718	761 ± 49	86 ± 3	25 ± 0.2
18	1303	591 ± 8	129 ± 2	45 ± 0.8
19	623	254 ± 5	53 ± 2	33 ± 1.0
20	888	462 ± 5	93 ± 1	32 ± 0.3
21	1027	395 ± 7	91 ± 1	52 ± 1.1
22	538	343 ± 1	69 ± 0.2	49 ± 0.2
23	795	388 ± 1	86 ± 0.5	66 ± 0.8
24	623	63 ± 6	14 ± 1	33 ± 1.4
25	829	78 ± 5	19 ± 1	22 ± 0.9

[a] Results based on triplicate analysis.

inhibitory effect against lactic acid bacteria growth (*39*). However, *L. plantarum* strains are grown when the total phenol content of the olives is relatively low, as in the case of Conservolea cultivar fruits, and the fermentation brine contains no more than 8% salt (the current processing in Greece).

High levels of hydroxytyrosol were determined in Kalamata olives (250−760 mg/kg), Spanish-style green olives (170−510 mg/kg), and Greek-style naturally black olives (100−340 mg/kg). Natually black olives in dry salt had lower levels of hydroxytyrosol. Tyrosol and luteolin levels did not exceed 170 and 74 mg/kg, respectively.

With regard to the levels of hydroxytyrosol, it can be concluded that table olives are generally good sources of this biophenol. This is nutricially important because the only other edible source of hydroxytyrosol is virgin olive oil. It should be noted also that in the oil the bound forms prevail.

So far olives have been used in culinary purposes mainly because of their pleasant flavor and color. Their possible nutritive value has been rather overlooked. This explains why processing methods have been applied to speed up artificially color development with the use of aeration and chemicals such as ferrous salts. These techniques, although popular, deprive the product of the valuable *o*-diphenols (*4*, *6*). As shown in **Table 2**, sample 14 contains no hydroxytyrosol. Taking into account the appearance characteristics, this sample is probably subjected to artificial blackening. The latter technique is not permitted in Greece for the processing of olives. Therefore, the sample should not be categorized as Greek-style naturally black olives. These olives are obviously illegally produced in Greece or are imported and misbranded. In view of the importance of and the enthusiasm about the Mediterranean diet, the industry should be encouraged to produce table olives that, apart from appearance and taste, could contribute also to the antioxidant daily intake.

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