Isolation and Characterization of Virgin Olive Oil Phenolic Compounds by HPLC/UV and GC-MS

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ABSTRACT: This research examined the phenolic fraction of extra virgin olive oil samples from Lianolia variety olives grown in the region of Preveza, Greece. Phenolic compounds were extracted from oil samples, separated by reversed-phase high-performance liquid chromatography (HPLC), and characterized by gas chromatography-mass spectrometry (GC-MS). Both simple and complex phenols were detected with the latter being the most abundant. 3-4-Dihydroxyphenyl ethanol (hydroxytyrosol) and p-hydroxyphenyl ethanol (tyrosol) predominated among the simple phenols. Complex phenolic compounds were further separated by preparative HPLC and analyzed by GC-MS before and after hydrolysis. The presence of hydroxytyrosol and tyrosol derivatives was confirmed. Both derivatives were always present in greater quantities and made up an average exceeding 70% in all samples analyzed.

Key Words: olive oil, phenolic compounds, HPLC, GC-MS

Introduction

VIRGIN OLIVE OIL IS CONSUMED IN ITS NATURAL STATE WITHout refining, thus conserving a number of substances, such as phenolic compounds, which are usually removed from other vegetable oils at various stages of refining. Phenolic compounds have been reported as influencing sensory quality (Esti and others 1998; Ryan and Robards 1998; Brenes-Balbuena and others 1992) and oxidative stability (Chimi and others 1991; Papadopoulos and Boskou 1991; Baldioli and others 1996) of olive oil and as having beneficial biological activity (Shahidi 1997; Aruoma and others 1998; Visioli and Galli 1998; Manna and others 1998).

Phenols make up a part of the "polar fraction" of virgin olive oil. Isolation of these compounds from olive oils is generally achieved by dissolution of the oil with hexane, followed by liquid-liquid extraction of these compounds with various mixtures of water and methanol (Gutfinger 1981; Montedoro and others 1992b). Reversed-phase high-performance liquid chromatography (RP-HPLC) currently is the most popular and reliable technique for the determination of phenolic compounds. Numerous mobile phases have been employed with different modifiers, which include methanol, acetonitrile or tetrahydrofuran, acids (acetic or formic) and/or salts (ammonium phosphate) (Ryan and others 1999). Detection is typically based on the measurement of ultraviolet (UV) absorption, usually at 280 nm, which represents a suitable compromise as most phenols absorb considerably at this wavelength (Tsimidou and others 1992a; Ryan and Robards 1998).

Gas chromatography of phenolic compounds requires the preparation of volatile derivatives and high temperature. Recently this method was used to elucidate the identity of the most complex constituents in olive oil (Angerosa and others 1995).

Cultivar, degree of maturation, climate and type of extraction method selected are among the factors affecting the phenolic content of virgin olive oil (Baldioli and others 1996; Caponio and others 1999; Romani and others 1999). Studies on different olive oil varieties (Amiot and others 1986; Esti and others 1998; Brenes-Balbuena and others 1992) indicate that cultivar has a significant impact on the phenolic composition of virgin olive oil.

Numerous simple and complex phenolic compounds found in virgin olive oil have been identified. The simple phenolic compounds present in olive oil have been identified using commercial standards. However the identification of complex phenols is a more difficult task given that there are different isomers which co-elute in HPLC and there are no commercial standards or spectroscopic data for most of these compounds. Studies on complex phenolic compounds using nuclear magnetic resonance, infrared, and UV spectrometry have allowed the elucidation of the structures of some of these compounds, but their complete chemical nature has not been elucidated (Montedoro and others 1993; Pirisi and others 1997; Ryan and others 1999).

The objective of this work was to isolate and characterize the phenolic fraction of Lianolia variety virgin olive oil. Phenolic compounds were fractionated by reversed-phase HPLC and the main fractions obtained were characterized by gas chromatography-mass spectrometry (GC-MS). The HPLC profiles of phenolic fractions were studied for 3 consecutive crop y (1997/98 1998/99 and 1999/2000) in order to verify the effect, if any, of crop y on the phenolic composition of samples.

Materials and Methods

EXTRA VIRGIN OLIVE OIL FROM LIANOLIA VARIETY OLIVES grown in the region of Preveza was used. The olives were collected at different stages of ripeness during these periods: December 1997/January 1998, December 1998/January 1999, and December 1999/January 2000. Oil samples were processed and provided by various local industrial olive oil mills.

Reagents and standards

Acetonitrile, methanol, hexane, acetic acid, and water were all of HPLC grade and were purchased from Merck (Darmstadt, Germany). Methanol and hexane for oil extraction were of pro-analysis and were also purchased from Merck. N,o-bis (trimethyl)trifluoroacetamide (BSTFA)

(99+%) and pyridine (99+%) for silylation were purchased from Aldrich Chemical Co. (Milwaukee, Wis., U.S.A.). The standards tyrosol, syringic acid, ferulic acid, o-coumaric acid, and p-coumaric acid were purchased from Sigma-Aldrich (Steinheim, Germany), and caffeic acid from Merck-Schuchardt (Hohenbrunn, Germany). Hydroxytyrosol was confirmed by mass spectrometry; it had been previously reported (Montedoro and others 1992b).

Extraction method

Phenolic compounds were isolated by extraction of an oil-in-hexane solution with water/methanol (40/60) 3 times. The 3 extracts were combined, evaporated to dryness in a rotary evaporator, and dissolved in methanol for HPLC analysis. Repeatability of extraction was checked by determining total phenol content with Folin-Ciocalteau reagent (Gutfinger 1981).

Preparative HPLC

The HPLC system used was a Waters PrepLC 4000 System equipped with a 7.8 mm id × 300 mm R18 semipreparative column, coupled with a UV detector (Waters 486) and a sample loop of 5 mL capacity. The mobile phase consisted of 2% acetic acid in water (A), methanol (B), and acetonitrile (C) (Montedoro and others 1993) at a flow rate of 3.5 mL/min. The gradient elution program used was: 95% A/5% B in 2 min; 80% A/20% B in 8 min; 60% A/20% B/20% C in 15 min; 40% A/30% B/30% C in 30 min; 30% B/70% C in 40 min and this percentage was maintained for 15 min and finally 95% A/5% B in 3 min. A quantity of phenolic extracts dissolved in methanol and corresponding to 5 mg of the total phenols expressed as caffeic acid equivalent as determined by the Folin-Ciocalteau reaction (Gutfinger 1981) was injected onto the column. The fractions were detected at 280 nm and were collected manually. The collected fractions were checked by analytical HPLC for their separation, then freeze-dried with lyophilization techniques and converted to silyl derivatives for GC-MS analysis.

HPLC methods

A Shimadzu model HPLC system (Shimadzu Corp., Kyoto, Japan) was used, consisting of a solvent delivery module (LC-10AD) with a double-plunger reciprocating pump, UV-VIS detector (SPA-10A), and column oven (CTO-10A). The column used was Apex octadecyl 104 C_{18} (25cm × 0.4cm ID) with 5 μm packing (Jones Chromatography, Lakewood, Colo., U.S.A.).

The elution solvents used were A (2% acetic acid in water), B (methanol), and C (acetonitrile) (Montedoro and others 1992a). The samples were eluted according to the following gradient: 95% A/5% B as initial condition; 60% A/20% B/20% C in 8 min; 40% B/60% C in 17 min, and this percentage was maintained for 10 min; 60% A/20% B/20% C in 10 min; and finally 95% A/5% B in 5 min. Flow rate was 1 mL/min, and the run time 50 min. Column temperature was 32 °C. The sample injection volume was 20 μL. Identification of compounds was achieved by comparing their retention time (RT) values with those of standards. Data were collected and processed using Class-VP Chromatography Laboratory Automated software (Shimadzu Corp).

Hydrolysis

Different hydrolysis procedures were used with 2 N or 4 N HCl at 70 °C or at ambient temperature; 50 mL HCl (2 N or 4 N) was added to 2 to 10 mg of phenolic extracts expressed as caffeic acid equivalent. The mixture was heated at 70 °C for 1 h or was kept at room temperature for 24 h. The reaction products were extracted 3 times with ethyl acetate and brought to dryness in a vacuum rotary evaporator. The residue was subjected to derivatization (silylation) prior to GC-MS analysis.

Silylation of phenols

Pyridine (100 μL) and 200 μL BSTFA were added to a dry sample (2 to 10 mg), under inert N_2, and the mixture was heated at 60 °C for 60 min.

GC-MS identification

GC-MS was performed with a GC/MSD Hewlett Packard 5890 Series II instrument equipped with an on-line injection system and mass-selective detector Model HP 5971A. A capillary column BPX5 (30 m × 0.33 mm × 0.25 μm) was used. The carrier gas was helium. Injector and detector temperatures were set at 315 °C. The following oven temperature program was used (Angerosa and others 1995): 70 to 135 °C at 2 °C/min and 10 min at 135 °C; 135 to 220 °C at 4 °C/min and 10 min at 220 °C; 220 to 270 °C at 3.5 °C/min and 20 min at 270 °C.

Results and Discussion

HPLC separation

Figure 1 depicts a representative HPLC chromatogram of phenolic extracts from virgin olive oil of the Lianolia variety. All chromatograms for 20 samples analyzed were similar, with differences relating only to peak areas, and were obtained using a modification of eluents widely used (Montodoro and others 1992a). The typical solvent system for the separation of olive oil phenols is water/methanol. In this study a 3-solvent system was adopted because peak 14 with RT 43.00 min was observed only when acetonitrile was used in the eluent system. The phenolic constituents were grouped into 5 fractions. Fraction I comprised of peaks 1 to 6 corresponding to simple phenolic compounds. Among these compounds, hydroxytyrosol (OHTy) and tyrosol (Ty) predominated. Fractions II, III, and IV were made up of complex phenolic compounds corresponding to peaks 7, 8,

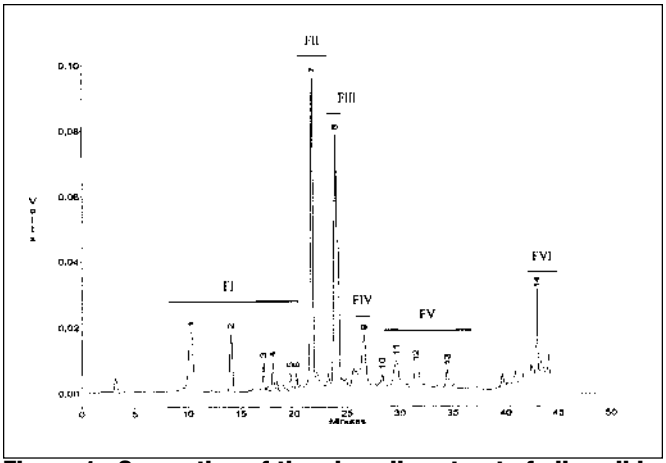

Figure 1—Separation of the phenolic extract of olive oil by reversed-phase HPLC at 280 nm. Peak numbers: (1) hydroxytyrosol; (2) tyrosol; (3) vanillic acid; (4) syringic acid; (5) pcoumaric acid; (6) o-coumaric acid; (7) hydroxytyrosol derivative; (8) tyrosol derivative; (9) hydroxytyrosol derivative; (10) RT 28.37; (11) RT 29.71; (12) RT 31.56; (13) 34.48; (14) RT 43.00. RT=retention time in min.

and 9 with retention times of 21.67, 23.88, and 26.63 min, respectively. Fraction V was comprised of peaks 10 to 13 with RTs of 28.37, 29.71, 31.56, and 34.48 min, respectively, while fraction VI contained peak 14 with a RT of 43.00 min. Fractions V and VI were unidentified.

The simple phenolic compounds found in fraction I have been reported to be present in Italian (Esti and others 1998; Romani and others 1999), Spanish (Brenes-Balbuena 1992), and Greek (Tsimidou and others 1992a) olive oils.

GC-MS analysis was carried out to identify complex phenols in fractions II, III, IV, V, and VI.

GC-MS analysis

Both the fractionated phenols and the unfractionated mixture of phenols were subjected to silylation and further analyzed by GC-MS. Figure 2 shows a typical GC-MS chromatogram of the unfractionated mixture. Tyrosol (RT = 50.46) and hydroxytyrosol (RT = 59.30) were identified by their mass spectra (not shown). Glycerol (RT = 26.80), palmitic acid (RT = 68.49), oleic acid (RT = 75.25), and linoleic acid (RT = 75.54) were also identified by their mass spectra. The 2nd part of the chromatogram (RT = 88.86 – 94.57) is complicated due to the presence of many peaks. Mass spectra (not shown) of these peaks were characterized by the presence of a main peak at m/z 192 or 280 corresponding to compounds containing tyrosol and hydroxytyrosol, respectively, and were identical to those reported by Angerosa and others (1995). Further identification of this fraction is in progress.

GC-MS analysis of fractions FII, FIII, and FIV, which were separated by preparative HPLC, is illustrated in Figure 3. Fraction FII gave a large peak at RT = 59.50 min corresponding to hydroxytyrosol as confirmed by its mass spectra (Figure 3a). Several other peaks, with RTs ranging from 93.56 to 98.09 min and corresponding to complex phenols, were also observed. The mass spectra of these complex phenols have a peak at m/z 280, which corresponds to compounds containing hydroxytyrosol. The GC-MS analysis of fraction FIII, on the other hand, gave a large peak at RT 50.70 min, which corresponds to tyrosol (Figure 3b). Some other complex phenols observed in this fraction have RTs ranging from 88.86 to 92.26 min and m/z of 192, which correspond to compounds containing tyrosol. Fraction-FIV gave a large peak at RT 59.29 min, which corresponds to hydroxytyrosol, and 2 peaks at RT 97.65 and 108.0 min, corresponding to a complex phenol containing hydroxytyrosol with m/z 280. It must be noted that, although the preparative HPLC-separated fractions (FII, FIII, FIV) consisted of complex phenols, their respective GC-MS chromatograms gave simple phenols in addition to complex phenols. This is due to experimental derivatization and analysis conditions leading to hydrolysis of complex phenols. Fraction FV (peaks 10 to 13, Figure 1), after separation by preparative HPLC into 4 individual peaks, did not give a peak during GC analysis, probably because its concentration was too low to be detected. Fraction FVI also did not give any peak. Perhaps it is not of a phenolic nature.

The GC-MS of hydrolyzed fractions FII, FIII, and FIV gave peaks corresponding only to OHTy, Ty, and OHTy, respectively, confirming the assumption that 3 fractions separated by HPLC corresponded to derivatives of OHTy (FII and FIV) and Ty (FIII).

The above results are in accordance with the findings of Montedoro and others (1993), who identified 3 successively eluting complex phenolic compounds corresponding to: (a) dialdehydic form of elenolic acid linked to OHTy, (b) dialde-

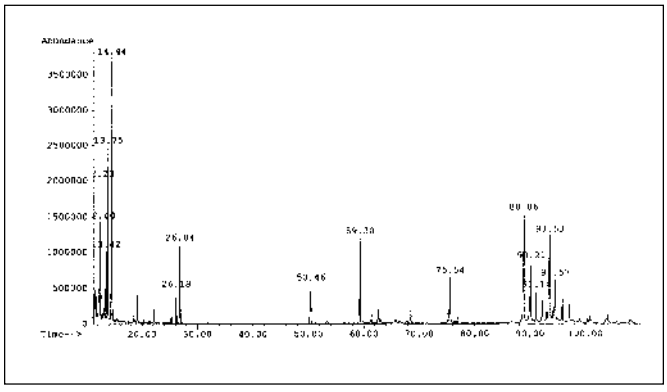

Figure 2—Separation of phenolic extract of olive oil by GC-MS. Retention times: (26.84) glycerol; (50.46) tyrosol; (59.30) hydroxytyrosol; (68.49) palmitic acid; (75.25) oleic acid; (75.54) linoleic acid; (88.86-94.57) compounds linked to tyrosol and hydroxytyrosol.

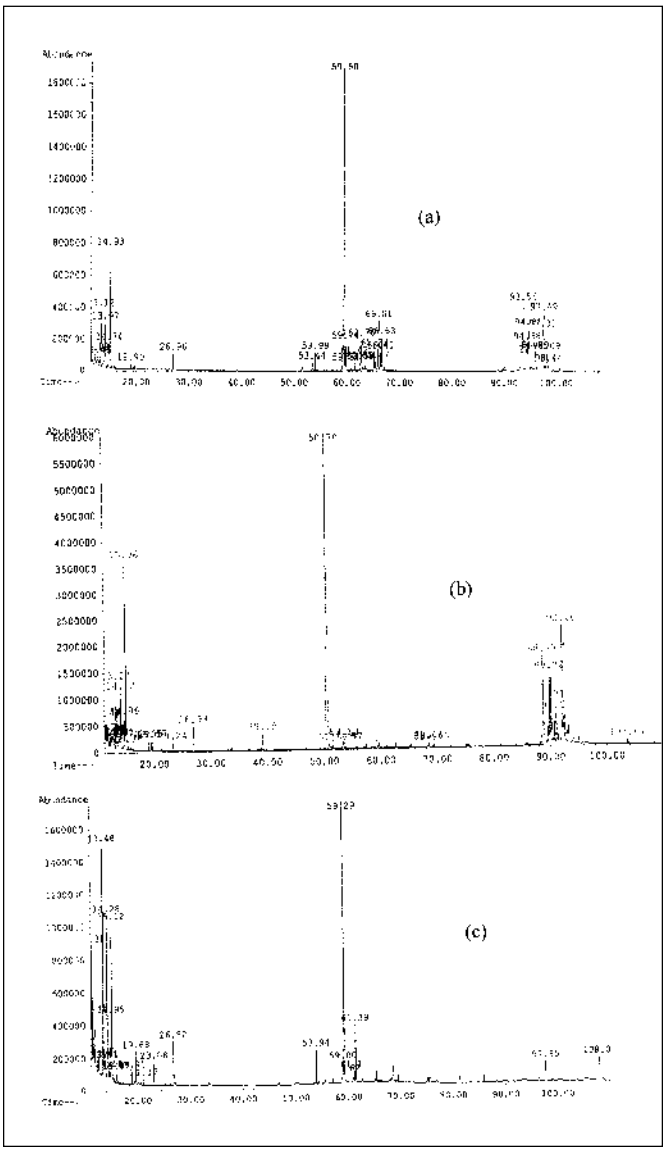

Figure 3—GC-MS of fractionated phenolic compounds. (a) Fraction II, (b) Fraction III and (c) Fraction IV.

Table1: Phenolic composition (%) of Lianolia variety virgin olive oil

Samples	Fraction I (peaks 1-6)	Fraction II (peak 7)	Fraction III (peak 8)	Fraction IV +V (peaks 9-13)
1	4.37	28.06	46.06	21.51
2	6.85	25.05	49.45	18.65
3	5.51	25.24	47.88	21.37
4	3.79	28.37	48.99	18.85
5	6.95	24.30	49.95	18.80
6	6.77	27.18	42.27	23.78
7	5.53	21.94	49.30	23.23
8	6.58	26.67	48.47	18.28
9	5.86	25.81	44.68	23.65
10	3.38	25.90	49.06	21.66
11	4.69	30.21	42.65	22.45
12	4.97	26.92	47.36	20.75
13	4.04	29.36	48.32	18.28
14	3.40	29.52	44.47	22.61
15	4.86	27.65	46.45	21.04
16	6.34	28.56	44.12	20.98
17	6.31	24.73	47.55	21.41
18	5.69	28.97	45.25	20.09
19	6.93	26.74	46.02	20.31
20	4.68	25.05	47.37	22.90
Simple phenols (%)		Complex phenols (%)		
Fraction I (mean ± SD)[a]		Fraction II (mean ± SD)[a]	Fraction III (mean ± SD)[a]	Fraction IV (mean ± SD)[a]
5.37±1.20		26,81±2.09	46,78±2.28	21,03±1.78

[a] n=20

hydic form of elenolic acid linked to Ty, and (c) an isomer of oleuropein aglycon. The above compounds (a, b, and c) correspond to fractions II, III, and IV in the present study. On the other hand, Pirisi and others (1997) reported the elution of complex phenolic compounds in the following order: (a) deacetoxy oleuropeine alglycone followed by oleuropeine aglycone, (b) dialdehydic form of elenolic acid linked to Ty, and (c) dialdehydic oleuropeine derivative. They proposed an isomerization mechanism between (a) and (c) derivatives in acidic solvents. In the above 2 studies differences exist with regards to structure and substitutes (dialdehydic form, deacetoxy) of elenolic acid as well as differences in the elution time of oleuropein aglycon, which appears before or after Ty derivatives. Our results show that there are at least 6 different compounds in each of the fractions II and III, and 2 compounds in fraction IV (Figure 3), indicating the presence of significantly larger number of components in these fractions as compared to those already identified.

The phenolic compounds of virgin olive oil have been reported as having significant antioxidant activity (Chimi and others 1991; Tsimidou and others 1992b; Baldioli and others 1996). Studies on antioxidant activity of phenolic compounds showed that biphenols such as oleuropein hydroxytyrosol, had higher antioxidant activity than tyrosol which is a monophenol (Chimi and others 1991; Papadopoulos and Boskou 1991). In addition, OHTy and Ty as well as their derivatives (fractions II, III, and IV) have been reported as being responsible for the bitter and pepper-like sensation that is occasionally dominant in the taste of olive oil (Kiritsakis 1998; Morales and Tsimidou 2000).

Knowledge of virgin olive oil phenolic composition is useful to obtain a better understanding of the influence of individual phenols on the overall oxidation stability of the oil. Given the fact that the separation, identification, and quantification of all the phenolic compounds occurring in olive oil is a difficult task, the quantification of the above compounds as groups with similar characteristics were suggested. Table 1 shows the percentage composition of simple and complex phenols determined in the extra virgin olive oil samples analyzed. Fraction I contained all the simple phenols and did not exceed 6% of the total fractions. Fraction II contained OHTy derivatives (27%), and fraction III Ty derivatives (47%). Both fractions II and III were always present in greater quantities. Fraction IV also contained OHTy derivatives but occurred in much lower amount than fraction II. Fraction V was comprised of the unidentified peaks. Both fractions IV and V made up an average of 21% of the total fractions. It must be noted that all these compound have different response factors (Tsimidou and others 1992a; Pirisi and others 1997), therefore there is no direct relationship between peak area percentage and absolute fraction weight.

Conclusion

USING ANALYTICAL HPLC AND A COMBINATION OF SEMI-preparative HPLC and GC-MS, the composition of phenolic compounds present in Lianolia virgin olive oil has been determined. The main phenolic compounds identified were those of fractions FI, FII, FIII, and FIV. Fraction FI was comprised of the simple phenols with OHTy and Ty predominating. FII and FIV contained OHTy derivatives, while FIII contained Ty derivative. The HPLC profiles of all samples analyzed were similar for the 3 consecutive years studied, with differences relating only to peak areas. Among the complex phenols, 2 peaks (FII and FIII) corresponding to OHTy and Ty derivatives predominated (> 70%). Simple phenols make up a relatively minor (< 6%) fraction.

References

Amiot MJ, Fleuriet A, Macheix JJ. 1986. Importance and evolution of phenolic compounds in olive during growth and maturation. J Agric Food Chem 34:823-826.

Angerosa F, d'Alessandro N, Konstantinou P, Di Giacinto L. 1995. GC-MS evaluation of phenolic compounds in virgin olive oil. J Agric Food Chem 43: 1802-1807.

Aruoma IO, Deiana M, Jenner A, Halliwell B, Kaur H, Banni S, Corongiu PF, Dessi MA, Aeschbach R. 1998. Effect of hydroxytyrosol found in extra virgin olive oil on oxidative DNA damage and on low-density lipoprotein oxidation. J Agric Food Chem 46:5181-5187.

Baldioli M, Servili M, Perretti G, Montedoro GF. 1996. Antioxidant activity of tocopherols and phenolic compounds of virgin olive oil. JAOCS 73:1589-1593.

Brenes-Balbuena M, Garcia-Garcia P, Garrido-Fernandez A. 1992. Phenolic compounds related to the black color formed during the processing of ripe olives. J Agric Food Chem 40:1192-1196.

Caponio F, Alloggio V, Gomes T. 1999. Phenolic compounds of virgin olive oil: influence of paste preparation techniques. Food Chemistry 64:203-209.

Chimi H, Cillard J, Rahmani M. 1991. Peroxyl and Hydroxyl radical scavenging activity of some natural phenolic antioxidants. JAOCS 68:307-311.

Esti M, Cinquanta L, La Notte E. 1998. Phenolic compounds in different olive varieties. J Agric Food Chem 46:32-35.

Gutfinger T. 1981. Phenols in olive oils. JAOCS 58:966-998.

Kiritsakis AK. 1998. Flavor components of olive oil-a review. JAOCS 75:673-681.

Manna C, Galletti P, Cucciolla V, Montedoro G, Zappia V. 1998. Olive oil hydroxytyrosol protects human erythrocytes against oxidative damages. J Nutr Biochem 10:159-165.

Montedoro G, Servili M, Baldioli M, Miniati E. 1992a. Simple and hydrolyzable phenolic compounds in virgin olive oil. 1. Their extraction, separation, and quantitative and semiquantitative evaluation by HPLC. J Agric Food Chem 40:1571-1576.

Montedoro G, Servili M, Baldioli M, Miniati E. 1992b. Simple and hydrolyzable phenolic compounds in virgin olive oil. 2. Initial characterization of the hydrolyzable fraction. J Agric Food Chem 40:1571-1580.

Montedoro G, Servili M, Baldioli M, Selvaggini R, Miniati E, Macchioni A. 1993. Simple and hydrolyzable phenolic compounds in virgin olive oil. 3. Spectroscopic characterization of the secoiridoid derivatives. J Agric Food Chem 41:2228-2234.

Morales MT, Tsimidou M. 2000. The role of volatile compounds and polyphenols in olive oil sensory quality. In: Harwood J, Aparicio R, editors. Handbook of olive oil, analysis and properties. Maryland: Aspen Publishing. p 393-458.

Papadopoulos G, Boskou D. 1991. Antioxidant effect of natural phenols on olive oil. JAOCS 68:669-671.

Pirisi FM, Angioni A, Cabras P, Garau VL, di Teulada Sanjust MT, dos Santos MK, Bandino G. 1997. Phenolic compounds in virgin olive oils. 1. Low-wavelength quantitative determination of complex phenols by high-performance liquid

chromatography under isocratic elution. J Chromatography A 768: 207-213.
Romani A, Mulinacci N, Pinelli P, Vincieri FF, Cimato A. 1999. Polyphenolic content in 5 Tuscany cultivars of Olea Europaea L. J Agric Food Chem 47:964-967.
Ryan D, Robards K. 1998. Phenolic compounds in olives. Critical review. Analyst 123:31R-44R.
Ryan D, Robards K, Lavee S. 1999. Determination of phenolic compounds in olives by reversed-phase chromatography and mass spectrometry. J Chromatography A 832: 87-96.
Shahidi F, editor. 1997. Natural Antioxidants: Chemistry, Health Effects, and Applications. Champaign, Ill: AOCS Press.
Tsimidou M, Papadopoulos G, Boskou D. 1992a. Determination of phenolic compounds in virgin olive oil by reversed-phase HPLC with emphasis on UV detection. Food Chemistry 44:53-60.
Tsimidou M, Papadopoulos G, Boskou D. 1992b. Phenolic compounds and stability of virgin olive oil to Part I. Food Chemistry 45:141-144.
Visioli F, Galli C. 1998. Olive oil phenols and their potential effects on human health. J Agric Food Chem 46:4292-4296.
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