A Highly Convenient Synthesis of Hydroxytyrosol and Its Recovery from Agricultural Waste Waters

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Hydroxytyrosol, a polyphenol with very interesting antioxidant properties, which naturally occurs in virgin olive oil and mainly in olive oil mill waste waters, was synthesized by reducing 3,4-dihydroxyphenylacetic acid with $LiAlH_4$ in tetrahydrofuran under refluxing for 2 h. The yield of reaction was 82.8%. The spectroscopic and HPLC data of the synthesized compound proved to coincide fully with those of a pure sample obtained by the chromatographic recovery from olive oil mill waste waters (yield = 91 mg/L). This synthetic method appears to be the most convenient compared with those reported in the literature and is more convenient than the chromatographic recovery. The tri- and diacetyl derivatives of the synthetic compound were also prepared for structure−bioactivity relationship studies. A brief discussion is given on the economical and ecological aspects regarding the production of hydroxytyrosol.

Keywords: *Olea europea; olive oil mill waste waters; hydroxytyrosol; acetyl derivatives; antioxidant polyphenol; synthesis; HPLC analysis; spectroscopy; chromatography*

INTRODUCTION

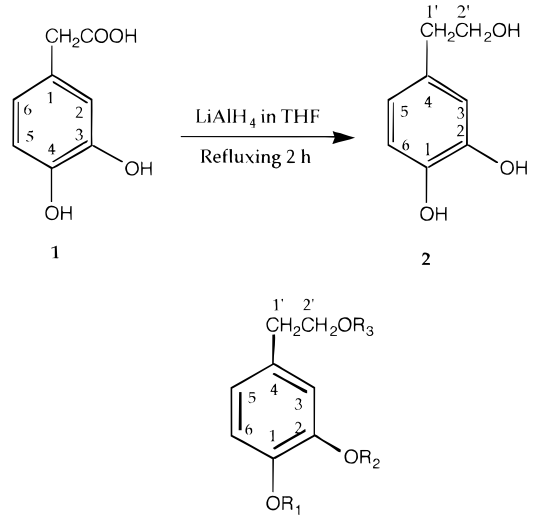
Hydroxytyrosol [4-(2-hydroxyethyl)-1,2-benzenediol, **2**] (Figure 1) is the main natural polyphenolic compound occurring in olive oil mill waste waters (omww) (Ragazzi and Veronesi, 1967; Vazquez Roncero et al., 1974; Capasso et al., 1992), and it is also present in virgin olive oil, allbeit in very small amounts and mainly in the form of natural derivatives (Ragazzi and Veronese, 1973; Vazquez Roncero et al., 1976; Janer del Valle and Vazquez Roncero, 1980; Cortesi and Fedeli, 1983; Forcadell et al., 1987; Montedoro et al., 1992; Mannino et al., 1993; Angerosa et al., 1995). It originates in all likelihood from the hydrolysis of oleuropein by means of an esterase during the mill process (Amiot et al., 1989).

Compound **2** is characterized by a highly antioxidant activity, which is comparable with that of the usual synthetic antioxidants such as 2,6-di-*tert*-butyl-*p*-hydroxytoluene (BHT) and 3-*tert*-butyl-6-hydroxyanisole (BHA) (Chimi et al., 1988).

More recently, Aeschebach et al. (1994) reported a study on the antioxidant activity of **2** compared with that of thymol, carvacrol, 6-gingerol, and zingerone.

Polyphenol **2** contributes to the stability of virgin olive oil (Camurati and Fedeli, 1982; Chimi et al., 1988; Papadoupulos and Boskou, 1991; Angerosa et al., 1995; Visioli et al., 1995a). In vitro it also inhibits the oxidation of low-density lipoproteins (Visioli et al., 1995b; Salami et al., 1995) and confers both cell protection (Galli et al., 1994) and dietetic properties to virgin olive oil (Visioli and Galli, 1995).

Chikamatsu et al. (1996) found that hydroxytyrosol inhibits the formation of melanin and lipidic peroxides,



	R_1	R_2	R_3
3	$COCH_3$	$COCH_3$	H
4	$COCH_3$	$COCH_3$	$COCH_3$

Figure 1. Reduction of 3,4-dihydroxyphenylacetic acid (**1**) into hydroxytyrosol (**2**) and structures of hydroxytyrosol (**2**), diacetylhydroxytyrosol (**3**), and triacetylhydroxytyrosol (**4**).

and they patented its use in topic and bath preparations.

Studies on the relationships of structures with the bioactivity of **2** and its diacetyl (**3**) and triacetyl (**4**) derivatives were also reported (Capasso et al., 1992, 1994b, 1995).

Because **2** is commercially unavailable, chromatographic methods of purification from omww (Ragazzi and Veronesi, 1967; Capasso et al., 1992, 1994a), virgin olive oil (Visioli and Galli, 1995; Chikamatsu et al., 1996; Montedoro et al., 1992), olive leaves (Capasso et al., 1996), and synthetic procedures (Schöpf et al., 1949;

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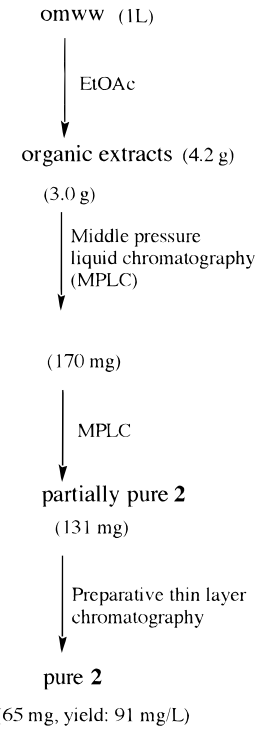

Figure 2. Purification scheme of hydroxytyrosol (**2**) from olive oil mill wastewaters.

Baraldi et al., 1983; Bianco et al., 1988; Verhe et al., 1992) were developed for its production.

In the present paper we report a very simple and highly convenient synthesis of **2** (Figure 1), which could prove useful for laboratory applications as well as for a possible industrial exploitation, and a chromatographic method for recovering it from omww is described, as illustrated in Figure 2; the spectroscopic and HPLC data of synthetic and natural **2** were also compared.

The di- and triacetyl derivatives **3** and **4** (Figure 1) of synthetic **2** were prepared for structure−bioactivity relationship studies.

Last, the economical and ecological aspects regarding the production of synthetic and natural **2** are discussed.

EXPERIMENTAL PROCEDURES

Materials. Fresh omww was supplied by mills from the Isernia province (Italy). Solvents were of HPLC grade and reactives of analytical grade. Pure and dry tetrahydrofuran was obtained by filtration on silica gel and then by distillation over $LiAlH_4$.

Analytical HPLC. The HPLC analyses were performed on a Gyncotec M 480G consisting of a ternary pump and a manual Rheodyne 9125 injector with a 20 μL sample loop and equipped with a Zenith data system Z-433/DX. The data were processed by a Softron Gyncosoft chromatography data system. Sample volume of 20 μL was used for injection. A C-18 Hypersil column (IDS 5 mm, 250 × 4.6 mm) and a Ginkotec UVD 160S as UV detector were used. Separations were performed with an isocratic elution using a mixture of 10% acetonitrile and 90% water from 0 to 2 min, followed by a linear gradient from 10 to 100% of acetonitrile over a period of 30 min and then, finally, by an elution with 100% of acetonitrile for 5 min, at a flow rate of 1 mL/min.

Preparative MPLC. The preparative MPLC was performed with a Büchi 681 pump, using silica gel columns (Merck, Kieselgel 0.040−0.063 mm).

Analytical and Preparative TLC. The analytical and preparative TLCs were performed on silica gel plates (Merck, Kieselgel 60 F_{254} 0.25 and 0.50 mm, respectively); the spots were visualized by exposure to UV radiation and/or by spraying first with 10% sulfuric acid in methanol and then with 5% phosphomolybdic acid in methanol, followed by reacting at 110 °C for 10 min.

NMR Spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz on a Bruker AC 400 spectrometer, using the same solvent as internal standard.

UV Spectroscopy. UV spectra were recorded on a Perkin-Elmer Lambda 7 spectrometer using methanol.

IR Spectroscopy. IR spectra were recorded neat or in $CHCl_3$ on a Perkin-Elmer 1720 X spectrophotometer.

MS Spectrometry. The EIMS spectra were recorded at 70 eV on a Fisons Trio-2000 spectrometer. The FABMS spectra were recorded on a VG ZAB 2SE using cesium as ionizing beam at 8 kV and a mixture of glycerol/thyoglicerol as matrix.

Synthesis of Hydroxytyrosol [4-(2-Hydroxyethyl)-1,2-benzenediol, 2]. A 0.2 M solution of 3,4-dihydroxyphenylacetic acid (**1**) in dry tetrahydrofuran (30 mL) was added to a suspension (1.5 g, 39.2 mmol) of $LiAlH_4$ in the same solvent (210 mL) dropwise, under magnetic stirring and at room temperature. Soon after, the mixture was heated until reflux and left under reflux for 2 h, which was the time necessary for the complete transformation of **1** into **2** as monitored by HPLC. Afterward, the reaction mixture was cooled and treated with ethyl acetate (50 mL) and water (10 mL), and the mixture was concentrated under reduced pressure. The residue was suspended in water (50 mL), acidified to pH 2−3 by 6 M HCl, and extracted with ethyl acetate (7 × 100 mL). The combined organic phases were washed with saturated sodium bicarbonate until reaching a permanent pH of 8.5. The combined aqueous phases were then extracted with ethyl acetate (3 × 100 mL). The combined organic phases were dried with sodium sulfate and subsequently dried under reduced pressure, leaving an oil residue (0.76 g, yield = 82.8%). The compound was purified on a silica gel column (1:30, v/v, petroleum ether/ethyl acetate 1:1, v/v) leaving a residue of 0.73 g (yield = 79.0%): HPLC t_R = 5.38 min (single and sharp peak); TLC R_f = 0.42 (petroleum ether/ethyl acetate, 1:1); ^{1}H NMR (CD_3OD) δ 2.67 (*t*, 2H, $J_{1',2'}$ = 7.2 Hz, H-1′), 3.68 (*t*, 2H, $J_{1',2'}$ = 7.2 Hz, H-2′), 6.53 (*dd*, 1H, $J_{3,5}$ = 2.0 Hz, $J_{5,6}$ = 8.0 Hz, H-5), 6.66 (*d*, 1H, J = 2.0 Hz, H-3), 6.68 (*d*, 1H, J = 8.0 Hz, H-6); ^{13}C NMR (CD_3OD) δ 39.0 (C-1′), 63.9 (C-2′), 115.7 (C-3), 116.4 (C-6), 120.6 (C-5), 131.3 (C-4), 143.9 (C-1), 145.2 (C-2); UV (λ_{max} = 218, ϵ = 4300; λ_{max} = 281, ϵ = 1970); EIMS, *m*/*z* (rel int) 154 $[M]^+$ (61), 153 $[M - H]^+$ (20), 136 $[M - H_2O]^+$ (48), 123 $[M - CH_2OH]^+$ (100), 105 $[M - CH_2OH - H_2O]^+$ (87), 87 $[M - CH_2OH - 2 \times H_2O]^+$ (64), 77 $[C_6H_5]^+$ (66), 65 $[C_5H_5]^+$ (49).

Preparation of Diacetylhydroxytyrosol (4-Hydroxyethyl-1,2-diacetoxybenzene, 3) and Triacetylhydroxytyrosol (4-Acetoxyethyl-1,2-diacetoxybenzene, 4). Pyridine (50 μL) was added to a solution of **2** (1.0 g, 6.6 mmol) in acetic anhydride (2.5 mL), kept at −23 °C. The reaction was left at a temperature between −20 and −10 °C. After 30 min, the reaction mixture was treated with ethyl acetate and washed with water to neutrality. The dried organic extracts left a raw oil mixture (1.680 g) after workup. This residue was chromatographed on a silica gel column (ethyl acetate/petroleum ether 1:1) giving two products, **3** (0.65 g, 42%) and **4** (0.69, 38%): HPLC t_R = 13.7 min (**3**), t_R = 18.2 min (**4**). The reaction performed at room temperature led to the complete formation of **4** after 30 min: HPLC t_R = 18.2 min.

Spectroscopic data of diacetylhydroxytyrosol (3): ^{1}H NMR ($CDCl_3$) δ 2.26 (*s*, 6H, Ac), 2.86 (*t*, 2H, $J_{1',2'}$ = 7.0 Hz, H-1′), 3.86 (*t*, 2H, $J_{1',2'}$ = 7.0 Hz, H-2′), 7.06 (*br s*, 1H, H-3), 7.10 (*br s*, 2H, H-5 and H-6); IR, ν_{max} (cm^{-1}) ($CHCl_3$) 3560 and 3450 (alcoholic OH), 1765 (C=O, phenolic ester), 1502 and 1370 (Me of acetyl groups), 1220 (C−O, ester); EIMS, *m*/*z* (rel int) 238 $[M]^+$ (4), 196 $[M - CH_2CO]^+$ (20), 154 $[M - 2 \times CH_2CO]^+$ (95), 136 $[M - CH_2CO - CH_3COOH]^+$ (14), 123 $[M - 2 \times CH_2CO - CH_2OH]^+$ (100), 94 $[M - 2 \times CH_2CO - CH_2OH - HCO]^+$ (5), 77 $[C_6H_5]^+$ (17), 65 $[C_5H_5]^+$ (3), 43 $[C_3CO]^+$ (90).

Spectroscopic data of triacetylhydroxytirosol (4): ^{1}H NMR ($CDCl_3$) δ 2.04 (*s*, 3H, Ac), 2.28 (*s*, 6H, 2MeCO), 2.93 (*t*, 2H, $J_{1',2'}$ = 6.9 Hz, H-1′), 4.28 (*t*, 2H, $J_{1',2'}$ = 6.9 Hz, H-2′), 7.06 (*br s*, 1H, H-5), 7.08−7.19 (*m*, 2H, H-3, H-6); ^{13}C NMR ($CDCl_3$)

δ 170.9 (s, C=O), 168.2 (s, C=O), 168.1 (s, C=O), 142.0 (s, C-1 or C-2), 140.8 (s, C-2 or C-1), 136.7 (s, C-4), 126.7 (d, C-5), 123.8 (d, C-3), 123.3 (d, C-6), 64.3 (t, C-2′), 34.4 (t, C-1′), 20.8, 20.6, and 20.6 (s, 3 MeCO); IR, ν_{max}, cm^{-1} ($CHCl_3$) 1770 (C=O, phenolic ester), 1735 (C=O, alcoholic ester), 1508 and 1371 (Me of acetyl groups), 1259 (C−O, ester); FABMS, *m/z* (rel int) 281 $[MH]^+$ (100), 239 $[MH - CH_2CO]^+$ (25), 221 $[MH - CH_3COOH]^+$ (15), 179 $[MH - CH_3COOH - CH_2CO]^+$ (22), 137 $[MH - CH_3COOH - 2 \times CH_2CO]^+$ (19), 93 $[MH - CH_3COOH - 2 \times CH_2CO - CH_4 - CO]^+$ (7); EIMS, *m/z* (rel int) 279 $[M - H]^+$ (12), 238 $[M - CH_2CO]^+$ (14), 220 $[M - CH_3COOH]^+$ (10), 196 $[M - 2 \times CH_2CO]^+$ (8), 178 $[M - 2 \times CH_2CO - H_2O]^+$ (13), 136 $[M - 3 \times CH_2CO - H_2O]^+$ (100), 107 $[M - 3 \times CH_2CO - H_2O - HCO]^+$ (5), 77 $[C_6H_5]^+$ (5), 65 $[C_5H_5]^+$(5).

Extraction and Chromatographic Purification of Hydroxytyrosol (2) from omww. Fresh omww (1000 mL, pH 5.5) was centrifuged at 7000 rpm for 20 min, and the supernantant was concentrated to 100 mL at 40 °C. This sample was extracted with ethyl acetate (8 × 100 mL). The organic extracts were combined, dried over sodium sulfate, and evaporated under reduced pressure, leaving a residue of 4.2 g. An aliquot (3 g) was chromatographed on a silica gel column (46 × 4.9 cm) eluted with the solvent system acetone/petroleum ether 1:1 under middle pressure (20 bar), with a flow rate of 22 mL/min (fraction volume =11 mL). The homogeneous fractions with the same R_f as a pure sample of synthetic **2** were pooled and evaporated under reduced pressure. The residue, 170 mg, was further chromatographed on a silica gel column (45 × 3 cm) eluted with the solvent system acetone/petroleum ether 1:1 under middle pressure (20 bar), with a flow rate of 8 mL/min (fraction volume = 4 mL). The homogeneous fractions with the same R_f as pure sample of synthetic **2** were pooled and evaporated under reduced pressure, leaving a residue of 131 mg. This residue, which corresponded to a partially pure **2**, was finally purified using silica gel preparative plates, eluting with acetone/pretroleum ether 1:1. The residue was 65 mg. Thus, the total yield of the purified **2** was 91 mg/L.

HPLC, EI-MS, and ^{1}H NMR data proved to coincide fully with those of a sample of the synthetic **2**.

RESULTS AND DISCUSSION

Hydroxytyrosol [4-(2-hydroxyethyl)-1,2-benzenediol, **2**] was obtained by the reduction of 3,4-dihydroxyphenylacetic acid (**1**, Figure 1) with $LiAlH_4$ in tetrahydrofuran for 2 h, under refluxing, with a yield of 82.8% (see Experimental Procedures). The yield decreased slightly to 79.0% after chromatographic purification, due to the elimination of an impurity already present in the commercial **1**, as was detected at $t_R = 20.76$ min by an HPLC control.

Compound **2** was identified by ^{1}H NMR, EI-MS, and UV (see Experimental Procedures), proving these data to be fully in accordance with those reported previously in the literature for naturally occurring **2** (Capasso et al., 1992).

In addition, this compound was further characterized by original HPLC and ^{13}C NMR data. With regard to the HPLC analysis, a single and sharp peak of **2** was detected at $t_R = 5.38$ min. With regard to ^{13}C NMR data, the spectrum of **2** showed signals at δ 63.9 and 39.6 in the region of aliphatic carbons corresponding to the C-2′ and C-1′ carbons, respectively, whereas it showed signals at δ 145.2, 143.9, 131.3, 120.6, 116.4, and 115.7 in the region of aromatic carbons, corresponding to the carbons C-2, C-1, C-4, C-5, C-6, and C-3 of the benzene ring, respectively.

Other synthetic methods for the production of **2** have been previously reported in the literature (Schöpf et al., 1949; Baraldi et al., 1983; Bianco et al., 1988; Verhe et al., 1992; Chikamatsu et al., 1996). In particular, those reported by Schöpf et al. (1949) and by Verhe et al. (1992) consisted of several steps with a global yield of 40%, and that reported by Bianco et al. (1988) consisted of two steps with a global yield of 80%. The synthetic methods developed by Baraldi et al. (1983) and Chikamatsu et al. (1996) were fully identical and consisted of two steps, giving a yield of 66%.

Synthesis of **2**, which we independently developed, started directly from commercially available **1**, which was reduced into **2** with $LiAlH_4$ in dry tetrahydrofuran under refluxing for 2 h and with a yield of 82.8%. This higher yield can be attributed to a high dilution of the solution used by us that not only improved compound **1** solubility but also stopped the latter from being incorporated into the salts forming during the reaction. Furthermore, the chromatographic purification of **2** we performed caused only a slight decrease of the yield of the reaction from 82.8 to 79.0%, which was mainly to attributable to the fact that commercial **1** was not completely pure (the impurity was detected at $t_R = 20.76$ min by HPLC). Thus, the synthesis of **2** we performed is the most convenient as it is carried out in only one step, in 2 h, and with a yield of 82.8%.

With a view to carrying out further studies on structure−bioactivity relationships, of which many results are already reported in previous articles (Capasso et al., 1992, 1994b, 1995), synthetic **2** was transformed into the respective diacetyl **3** and triacetyl **4** derivatives (Figure 1) by treating it with acetic anhydride and pyridine in the range of −20 to −10 °C for 30 min. The two compounds were chromatographically separated, obtaining yields of 42 and 38%, respectively.

Acetyl derivatives **3** and **4** were characterized by spectroscopic and HPLC analysis (see Experimental Procedures). The respective data proved to coincide fully with those previously reported in the literature for the corresponding acetyl derivatives **3** (Capasso et al., 1994b) and **4** (Capasso et al., 1992) of naturally occurring **2**.

With regard to the production of **2** from natural sources, some reports describe its isolation from virgin olive oil (Montedoro et al., 1992) by means of extraction and an HPLC procedure and from omww (Ragazzi and Veronesi, 1967; Capasso et al., 1992, 1994a), by extraction and chromatography at normal and low pressures.

In the present paper we report a new chromatographic method using the silica gel column middle pressure (20 bar) (two steps) and preparative silica gel TLC (one step) (see Experimental Procedures) to purify **2** from the organic extracts of omww with a final yield of 91 mg/L, as illustrated in Figure 2.

The spectroscopic data of natural **2** obtained using this procedure proved to be fully coincident with those of the synthetic one.

The remarkable decrease of production of **2** through all of the various purification steps indicated by the scheme of Figure 2 can be attributed not only to the roughing out of the initial organic extracts but also to both the retention and chemical modification processes compound **2** undergoes on the chromatographic stationary phase.

The chromatographic production of **2** could be of industrial interest (i.e., for a large-scale production) at a second step level, because of the possible use of chromatographic columns for industrial purposes, although at this level the degree of purity is 80% (evaluated by TLC control).

This kind of production of **2** from omww may prove a very interesting method from two points of view: (1) it

could allow a natural and nontoxic antioxidant to be obtained and (2) recovery of **2** could be a useful process for recycling omww, thus resolving its disposal problems, albeit partially. However, despite that at present this kind of production is more convenient than the other methods (Ragazzi and Veronesi, 1967; Capasso et al., 1992, 1994a), it gives scarce yields, because **2** undergoes both retention and chemical modification processes on the stationary phase during the chromatographic purification.

Complete chromatographic purification of **2** is achieved by a third step using preparative TLC (scheme shown in Figure 2), and consequently at this level it could be only used for laboratory uses.

The synthetic method we propose thus appears to be the most convenient for three reasons with respect to the other methods reported in the literature: it consists of only one step, the reaction is completed in 2 h, and it gives a yield of 82.8%, starting from **1**, which is a commercially available product. It is also more convenient than the chromatographic purification methods from omww reported in this paper and previously (Ragazzi and Veronesi, 1967; Capasso et al., 1992, 1994a) because they produce **2** in small yield and are more expensive than the synthesis method.

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LITERATURE CITED

Aeschbach, R.; Loeliger, J.; Scott, B.; Murcia, A.; Butler, J.; Halliwell, B.; Aruoma, O. L. Antioxidant actions of thymol, carvacrol, 6-gingerol, zingerone and hydroxytyrosol. *Food Chem. Toxicol.* **1994**, *32*, 31−36.

Amiot, M. J.; Fleuriet, A.; Macheix, J. J. Accumulation of oleuropein derivatives during olivematuration. *Phytochemistry* **1989**, *28*, 67−69.

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

Baraldi, P. G.; Simoni, D.; Manfredini, S.; Menziani E. Preparation of 3,4-Dihydroxy-1-benzeneethanol: A Reinvestigation. *Liebigs Ann. Chem.* **1983**, *83*, 684−686.

Bianco, A.; Passacantilli, P.; Righi; G. Improved procedure for the reduction of esters to alcohols by sodium borohydride. *Synth. Commun.* **1988**, *18*, 1765−1771.

Camurati, F.; Fedeli, E. Attività antiossidante di estratti fenolici delle acque di vegetazione delle olive. *Riv. Ital. Sostanze Grasse* **1982**, *49*, 623−626.

Capasso, R.; Cristinzio, G.; Evidente, A.; Scognamiglio, F. Isolation, spectroscopy and selective phytotoxic effects of polyphenols from vegetable waste waters. *Phytochemistry* **1992**, *12*, 4125−4128.

Capasso, R.; Evidente, A.; Visca, C. Production of hydroxytyrosol from olive oil vegetation water. *Agrochimica* **1994a**, *38*, 165−172.

Capasso, R.; Evidente, A.; Tremblay, E.; Sala, A.; Santoro, C.; Cristinzio, G.; Scognamiglio, F. Direct and mediated effects on *Bactrocera oleae* (Gmelin) of natural polyphenols and some related synthetic compounds: structure−activity relationships. *J. Chem. Ecol.* **1994b**, *20*, 1189−1199.

Capasso, R.; Evidente, A.; Schivo, L.; Orrù, G.; Marcialis, M. A.; Cristinzio, G. Antibacterial polyphenols from olive oil mill wastewaters. *J. Appl. Bacter.* **1995**, *79*, 393−398.

Capasso, R.; Evidente, A.; Visca, C.; Gianfreda, L.; Maremonti, M.; Greco, G., Jr. Production of glucose and bioactive aglycone by chemical and enzymatic hydrolysis of purified oleuropein from *Olea europea*. *Appl. Biochem. Biotechnol.* **1996**, *60*, 365−377.

Chikamatsu, Y.; Ando, H.; Yamamoto, A.; Kyo, S.; Yamashita, K.; Dojo, K. Hydroxytyrosol as melanin formation inhibitor and lipid peroxide formation inhibitor and its application to topical preparations and bath preparations. Jpn. Patent 8119825, 1996; pp 1−10.

Chimi, H.; Sadik, B.; Le tutour, M.; Rahmani, M. Contribution à l'étude comparative des pouvoirs antioxidants dans l'huile d'olive du tyrosol, de l'hydroxytyrosol, de l'acide caféique, de l'oleuropeine et du BHT. *Rev. Fr. Corps Gras* **1988**, *8/9*, 339−344.

Cortesi, N.; Fedeli, E. I composti polari di oli di oliva vergine (Nota I). *Riv. Ital. Sostanze Grasse* **1983**, *60*, 341−351.

Forcadell, M. L.; Comas, M.; Miquel, X.; De la Torre, M. C. Tyrosol and hydroxytyrosol determination in virgin olive oils. *Rev. Fr. Corps Gras* **1987**, *34*, 547−549.

Galli, C.; Petroni, A.; Visioli, F. Natural antioxidants, with special reference to those in olive oil, and cell protection. *Eur. J. Pharm. Sci.* **1994**, *2*, 67−68.

Janer del Valle, C.; Vazquez Roncero, A. A study of the poplar compounds in olive oil by gas chromatography. *Grasas Aceites* **1980**, *31*, 309−316.

Mannino, S.; Cosio, M.; Bertuccioli, M. High performance liquid chromatography of phenolic compounds in virgin olive oils using amperometric detection. *Ital. J. Food Sci.* **1993**, *4*, 363−370.

Montedoro, G.; Servili, M.; Badioli, M.; Miniati, E. Simple and hydrolyzable phenolic compounds in virgin olive oil. Their extraction, separation and quantitative and semiquantitative evaluation by HPLC. *J. Agric. Food Chem.* **1992**, *40*, 1571−1576.

Papadoupulos, G.; Boskou, D. Antioxidant effect of natural phenols on olive oil. *J. Am. Oil Chem. Soc.* **1991**, *68*, 669−671.

Ragazzi, E.; Veronesi, G. Ricerche sui costituenti idrosolubili delle olive. I. Zuccheri e fenoli. *Ann. Chim.* **1967**, *57*, 1476−1486.

Ragazzi, E.; Veronese, G. Indagini sui componenti fenolici degi oli di oliva. *Riv. Ital. Sostanze Grasse* **1973**, *50*, 443−452.

Salami, M.; Galli, C.; De Angelis, L.; Visioli, F. Formation of F2-isoprostanes in oxidized LDL: protective effect of hydroxytyrosol. *Pharm. Res.* **1995**, *31*, 225−279.

Schöpf, C.; Göttmann, G.; Meisel, E. M.; Neuroth, L. Über *β*-(3,4-Dioxyphenyl)-äthylalkohol. *Liebigs Ann. Chem.* **1949**, *563*, 86−93.

Vazquez Roncero, A.; Maestro Duran, R.; Graciani Costante, E. Componentes fenolicos de la la aceituna II. Polifenoles del alpecin. *Grasas Aceites* **1974**, *25*, 341−345.

Vazquez Roncero, A.; Janer del Valle, C.; Janer del Valle, L. Componentes fenolicos de la la aceituna. III. Polifenoles del aceite. *Grasas Aceites* **1976**, *27*, 185−191.

Verhe, R.; Papadopoulos, G.; Boskou, D. Preparation of hydroxytyrosol. *Bull. Liason Groupe Polyphenols* **1992**, *15*, 237−244.

Visioli, F.; Galli, C. Natural antioxidants and prevention of coronary heart disease: the potential role of olive oil and its minor constituents. *Nutr. Metabol. Cardiovasc. Dis.* **1995**, *5*, 306−314.

Visioli, F.; Vinceri, F.; Galli, C. Wastewaters from olive oil production are rich in natural antioxidants. *Experientia* **1995a**, *51*, 32−34.

Visioli, F.; Bellomo, G.; Montedoro, G.; Galli, C. Low-density lipoprotein oxidation is inhibited, i*n vitro*, by olive oil constituents. *Atherosclerosis* **1995b**, *117*, 25−32.

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