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Production of Triacetylhydroxytyrosol from Olive Mill Waste Waters for Use as Stabilized Bioantioxidant

RENATO CAPASSO,*,† FILOMENA SANNINO,† ANTONIO DE MARTINO,† AND CATERINA MANNA‡

Dipartimento di Scienze del Suolo, della Pianta e dell'Ambiente, Facoltà di Agraria, Università degli Studi di Napoli Federico II, Via Università 100, 80055 Portici (NA), Italy, and Dipartimento di Biochimica e Biofisica "F. Cedrangolo", Facoltà di Medicina e Chirurgia, Seconda Università degli Studi di Napoli, Via de Crecchio 7, 80138 Napoli, Italy

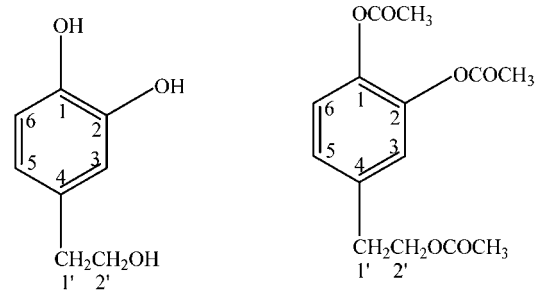
A hydroxytyrosol triacetyl derivative was very efficiently produced as a highly pure stabilized antioxidant compound by a short treatment of olive mill waste water (OMWW) organic extracts, rich in hydroxytyrosol, with an acetylating mixture composed of $HClO_4$-SiO_2 and Ac_2O (Chakborti and Gulhane reaction), in mild and safe conditions. A successive single step of middle pressure liquid chromatography (MPLC) purification of the reaction product was performed, with an overall yield of 35.6%. (This process, including both the Chakborti and Gulhane reaction and the MPLC purification, is protected by an international patent under PCT/IT2005/000781.) The *o*-diphenol triacetyl derivative was also produced by direct reaction of hydroxytyrosol, previously purified by MPLC, with $HClO_4$-SiO_2 and Ac_2O, with an overall yield of 29.5%. A further procedure for the production of the hydroxytyrosol triacetyl derivative was consistent with the direct treatment of raw OMWW with the acetylating agent and a single step of MPLC purification, with an overall yield of 27.6%. The purified natural triacetylhydroxytyrosol confirmed the same strong protective effects against the oxidative stress in human cells as the corresponding synthetic compound, likely because of the biochemical activation of the acetyl derivative into the active parent hydroxytyrosol by esterases. We therefore propose the utilization of OMWW for recovering hydroxytyrosol as a natural antioxidant in a chemically stabilized form, with a good yield, which can be potentially used as a nontoxic functional component in nutritional, pharmaceutical, and cosmetic preparations.

KEYWORDS: Olive mill waste waters; continuous liquid−liquid extraction; chromatographic purification; hydroxytyrosol; triacetylhydroxytyrosol; bioantioxidants

INTRODUCTION

Hydroxytyrosol [4-(2-hydroxyethyl)-1,2-benzenediol, **1a**] (**Figure 1**) is the main polyphenol naturally occurring in olive oil (*1*). This *o*-diphenol is also the most abundant polyphenol present in either olive mill waste waters (OMWW) or in solid−liquid waste (OMW), which are byproducts of three-phase (*2*, *3*) and two-phase olive oil processing (*4*) plants, respectively. Compound **1a** also occurs naturally in virgin olive oil in the form of a monoacetyl derivative (*5*) and has an antioxidant effect very close to the parent compound (*6*).

Compound **1a** is endowed with a free radical scavenging activity (*7*) and has proven to be more active than the usual industrial synthetic antioxidants such as 2,6-di-*tert*-butyl-*p*-hydroxytoluene (BHT) and 3-*tert*-butyl-6-hydroxyanisole (BHA) (*8*). This *o*-diphenol confers cell protection against oxidative damages both in cellular (*9*, *10*) and in vivo experimental models (*11*−*13*); moreover, recent studies have pointed out that **1a** is endowed with significant antithrombotic, antiatherogenic, and anti-inflammatory activities (*14*−*16*).



Hydroxytyrosol (**1a**) Triacetylhydroxytyrosol (**1b**)

Figure 1. Structure of hydroxytyrosol (**1a**) and triacetylhydroxytyrosol (**1b**).

Compound **1a** permeates the cell membrane via a passive diffusion mechanism (*17*). Moreover, it is characterized by high bioavailability in humans when orally administered (*18*), and it

* Corresponding author. Tel: +39 081 2539173. Fax: +39 081 2539186. E-mail: renato.capasso@unina.it.
† Università degli Studi di Napoli Federico II.
‡ Seconda Università degli Studi di Napoli.

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distributes in all organs and tissues when intravenously injected in rats (*19*). Compound **1a** is rapidly metabolized in vivo, yielding metabolites modified either in the aromatic moiety or in the lateral chain, some of them sharing the same antioxidant activity of the parent compound (*19*). Finally, the treatment of rats with up 2 g kg^{-1} induced no significant organ damage, indicating that **1a** can be regarded as a nontoxic compound (*19*). These properties suggest that **1a** could be utilized as a potential antioxidant for nutritional, pharmaceutical, and cosmetic preparations (*20*).

Since **1a** is not commercially available, many scientists of multidisciplinary areas have been prompted to develop several methods for its production: by direct recovery from OMWW (*2*, *3*) and OMW (*4*) or by chemical (*2*, *21*), biochemical (*22*), or biotechnological (*23*) syntheses starting from a synthetic precursor.

The recent recovery methods have been consistent with the extraction of OMW using a water stream under a pressure of 42 kg cm^{-2} at 160−240 °C in a pilot reactor (*4*), or of three-phase OMWW, using ethyl acetate at room temperature in a polyethylene mixer settler apparatus (*3*). However, because **1a** is unstable, particularly in solution, it has to be preserved dried and in darkness in the absence of air.

Therefore, the recovery of this compound from OMWW as a chemically stable and lipophilic derivative proves to be more advantageous in this form, offering three practical points: (i) higher yield with respect to the recovery from OMWW in the native form; (ii) increased efficiency when added to alimentary, pharmaceutical, or cosmetic matrices as a protective agent against reactive oxygen species (ROS) in human cells; and (iii) possible exploitation as a nontoxic additive to lipophilic matrices. Clearly, a stable derivative of **1a** should be able to be biologically converted in vivo into the native form, which is the effective protective form against ROS in human cells.

In our recent paper, we demonstrated **1a** synthetic triacetyl derivative [4-(acetoxyethyl)-1,2-diacetoxybenzene, **1b**] (**Figure 1**) to be the most suitable compound able to satisfy these objectives since it is transformed into **1a** and nontoxic acetate by esterases in human cells. This investigation, indeed (*20*), showed **1b** to be as effective as the parent **1a** in protecting human cells from oxidative stress-induced toxicity, after the metabolization by esterases, although it is devoid of chemical antioxidant activity. Therefore, the aim of this paper is to select the most efficient method for the production of pure **1b** from OMWW.

In this perspective, both crude samples of OMWW and their organic extracts were comparatively treated with three different acetylating agents, consisting of Ac_2O mixed with H_2SO_4, pyridine, or $HClO_4$ fixed on SiO_2 (Chakborti−Ghulane reagent), and the acetylated mixtures were submitted to middle pressure chromatography (MPLC) purification to obtain pure **1b**. Pure **1b** was also produced by direct acetylation of **1a**, obtained by a previous chromatographic purification of an aliquot of the same organic extracts stated previously. Finally, **1a** and **1b** purified from OMWW were comparatively tested to the corresponding synthetic compounds, to confirm their chemical and biological antioxidant behavior.

The exploitation of natural **1b** from OMWW for use as stabilized lipophilic antioxidant additives in pharmaceutical, nutritional, and cosmetic preparations should be worth while because this waste material represents an economic source of **1a**, a potential high added value substance. Its recovery could also contribute toward resolving the disposal problem of this waste material. A brief comparative study on safety, environmental aspects, and cost of production of natural and synthetic **1b** was also performed.

MATERIALS AND METHODS

Materials. Samples of OMWW (pH 4.5) were supplied by traditional mills located in Marrakech (Morocco). They were lyophilized in a Cryodos−Totelstar lyophilisator apparatus equipped with varian vacuum technologies in the laboratory of Prof. Ismail El Hadrami (Faculty of Semlalia, Marrakech, Morocco) and then redissolved in our laboratory in high purity water (hpw) to the initial volume. Solvents were of analytical and HPLC grade and were purchased from Carlo Erba (Milan, Italy). High purity water was obtained through a double filtration system, consisting of a deionized column and a MilliQ (Millipore) apparatus. Authentic synthetic samples of **1a** and **1b**, which were used as standards for chemical and biological antioxidant activity tests, were synthesized according to our previously reported method (*2*). All of the other materials used for the chemical and biological antioxidant activity tests were the same described in the previous work (*20*).

Analytical TLC. TLC analysis was performed on silica gel plates (Merck, Kieselgel 60 F_{254} 0.25 mm) eluted with acetone/petroleum ether 50:50 (v/v) for monitoring **1a** and acetone/petroleum ether 40:60 (v/v) for monitoring **1b** in the OMWW organic extracts and chromatographic fractions. 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 heating at 110 °C for 10 min. The qualitative monitoring of natural **1a** and **1b** was performed by comparison with the corresponding synthetic standards.

HPLC Analysis. The quantitative and qualitative analysis of **1a** and **1b** was performed using an Agilent 1100 series liquid chromatograph equipped with a DAD array. Detection was accomplished at 264 and 280 nm for the compounds **1b** and **1a**, respectively. A Nucleosil 100-5 C-18 column (stainless steel 250 × 4, Macherey-Nagel, 5 μm) was utilized for both the compounds. An isocratic elution at a flow rate of 1.0 mL min^{-1} with acetonitrile/water (45:55, v/v) as mobile phase was used for **1b** (t_R = 7.62 min), and a flow rate of 1.2 mL min^{-1} with a mixture of acetonitrile (15%) and water (85%, containing 0.35% acetic acid) was used as mobile phase for **1a** (t_r = 3.15 min). A sample volume of 20 μL was used for the injection. HPLC analysis was conducted after every extraction and purification step. The quantitative analysis of **1b** and **1a** from OMWW samples was performed elaborating their corresponding calibration curves (100−2000 and 50−1000 μg/mL, respectively) using the synthetic standards.

Liquid−Liquid Extraction of OMWW. OMWW samples of 50 mL at native pH (4.5) were extracted with 100 mL of ethyl acetate by refluxing for 8 h in a continuous liquid−liquid Friedrich extractor, obtaining a total organic extract of 1102.0 mg. The amount of **1a** was quantitatively monitored by HPLC analysis during the extraction process until a total recovery of 252.2 mg was obtained.

Middle Pressure Liquid Chromatography (MPLC). The purification procedure was performed using a column of 36 mm (i.d.) × 460 mm (A) packaged with silica gel (Merck, Kieselgel 0.040−0.063 μm) and eluted under middle pressure (20 bar) using a Buchi 681 pump. The homogeneous fractions with the same R_f on the TLC analysis were pooled and evaporated under reduced pressure at 30 °C.

^{1}H NMR, EI-MS, and FAB-MS Analysis. The **1a** and **1b** compounds obtained pure from OMWW were identified by ^{1}H NMR (400 MHz on a Bruker AC 400 spectrometer, using tetradeuterated methanol as solvent for **1a** and deutochloroform for **1b**), EI-MS (70 eV on a Fisons Trio 2000 spectrometer), and FAB-MS (Kratos MS 50, xenon as the bombarding atoms at 8 kV acceleration potential) analyses.

Production of Triacetylhydroxytyrosol [4-(Acetoxyethyl)-1,2-diacetoxybenzene, 1b]. *Preparation of $HClO_4$-SiO_2.* The preparation of the $HClO_4$-SiO_2 catalyst to perform the acetylation reaction with Ac_2O was carried out according to our modified procedure of Chakborti and Gulhane (*24*). $HClO_4$ (1.25 mmol, as a 65% aqueous solution) was added to the suspension of silica gel (2.37 g, 230−400 mesh) in 7.5 mL of diethyl ether. The mixture was kept for 3 h at room temperature under magnetic stirring and successively was heated at 70 °C for 28 h under vacuum to afford $HClO_4$-SiO_2 as a free flowing powder.

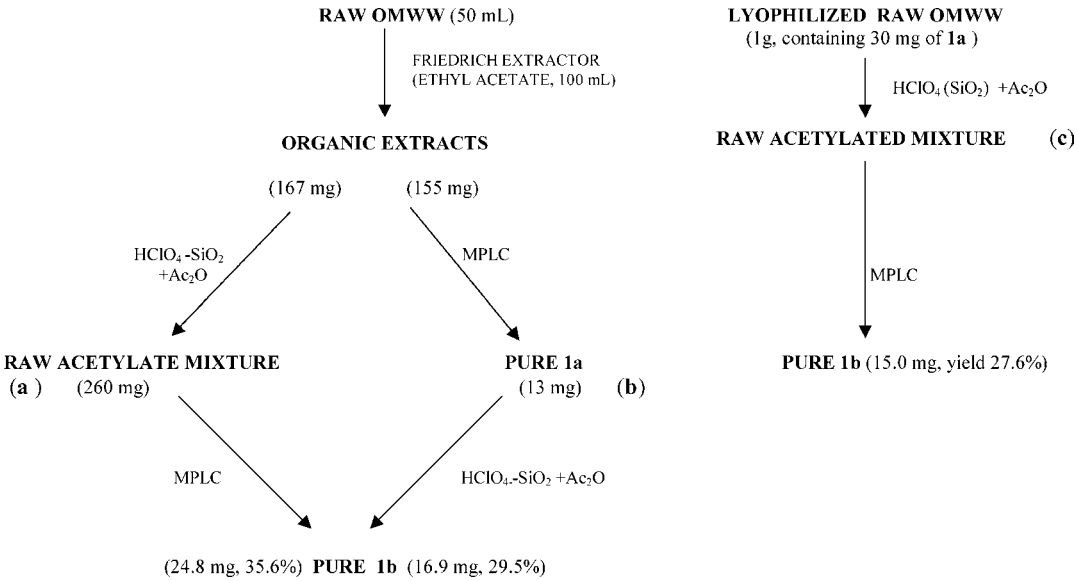

Figure 2. Production scheme of triacetylhydroxytyrosol (**1b**) according to the protocols **a**, **b**, and **c**.

*Production of Pure **1b** by Treatment of OMWW Organic Extracts with a Mixture of $HClO_4$-SiO_2 and Ac_2O and a Successive MPLC Purification Step (**Figure 2**, Protocol **a**).* An aliquot of 167 mg (containing 38.3 mg of **1a**) of extract obtained by liquid−liquid extraction of OMWW was acetylated using as a catalyst $HClO_4$-SiO_2 (22.5 mg) and Ac_2O (1 mL) in the presence of diethyl ether (1 mL) under magnetic stirring and at room temperature. The reaction, monitored by TLC, showed **1b** to be quantitatively obtained after 5 min. The reaction mixture was then washed with ethyl acetate, filtered under vacuum, and evaporated under reduced pressure at 30 °C, leaving a residue of 262 mg. An aliquot of the raw acetylated mixture (260 mg) was purified on column A using as eluent hexane/ethyl acetate 1:1 (v/v) and a flow rate of 2.5 mL/30 s (fraction volume collected was of 2.5 mL). The pooled fractions monitored by TLC and quantitative HPLC showed 24.8 mg of pure **1b**, which was also identified by ^{1}H NMR and EI-MS analyses. These analytical procedures were also carried out on the same compound obtained following the other protocols described next.

*Production of Pure **1b** by Treatment of Purified **1a** with a Mixture of $HClO_4$-SiO_2 and Ac_2O (**Figure 2**, Protocol **b**).* An aliquot of 155 mg (containing 35.5 mg of **1a**) of the organic extracts obtained by liquid−liquid extraction of OMWW was purified by column A using as eluent hexane/ethyl acetate 15:85 (v/v) and a flow rate of 2.5 mL/30 s. The homogeneous fractions with the same R_f of the synthetic standard **1a** were pooled, leaving a residue of 14.6 mg of pure natural **1a**. This compound was analyzed as reported previously. An aliquot of 13 mg of purified **1a** was completely acetylated into **1b** after 5 min (the reaction was monitored by TLC) using the mixture of $HClO_4$-SiO_2 (17.5 mg) and Ac_2O (0.70 mL) in the presence of diethyl ether, under magnetic stirring. The reaction mixture was worked up as described previously, leaving a residue of 16.9 mg of pure **1b**.

*Production of Pure **1b** by Direct Treatment of Lyophilized OMWW with a Mixture of $HClO_4$-SiO_2 and Ac_2O and a Successive MPLC Purification Step (**Figure 2**, Protocol **c**).* A sample of 1 g of lyophilized OMWW (containing 30.24 mg of **1a**) was acetylated under constant stirring using as catalyst $HClO_4$-SiO_2 (106 mg) and Ac_2O (4 mL) in the presence of diethyl ether (6 mL). After 24 h, the reaction mixture was washed with ethyl acetate, filtered under vacuum, and evaporated under reduced pressure at 30 °C. The residue was purified on column A using as eluent hexane/acetone 70:30 (v/v) and a flow rate of 10 mL/50 s (the fraction volume collected was 10 mL). The homogeneous fractions with the same R_f of the synthetic standard **1b** were pooled, leaving a residue of 15.0 mg.

Ferric Reducing Antioxidant Power (FRAP) Assay. The ferric reducing ability of the tested compounds was measured using the FRAP assay, a colorimetric method based on the reduction of a ferric−tripyridyltriazine (ferric−TPTZ) complex to its ferrous form (*25*). Appropriate amounts of antioxidant were added to 1 mL of working solution, prepared by mixing 25 mL of 300 mM acetate buffer [pH 3.6], 2.5 mL of TPTZ solution [10 mM TPTZ in 40 mM HCl], and 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$; after incubation for 6 min at room temperature (23−25 °C), the absorbance was read at 593 nm.

Biological Tests for the Antioxidant Activity of 1a and 1b in Caco-2 Cells and Human Erythrocytes. The antioxidant protective effects on human Caco-2 cells and erythrocytes against oxidative injury exerted by natural **1a** and **1b** isolated from OMWW or by the synthetic ones were measured using the tests of cellular viability and hemolysis, respectively.

Human colon carcinoma (Caco-2) cells were grown, and oxidative stress was induced by *t*-BHP-treatment as previously reported (*20*). At the end of 6 h incubation, *t*-BHP-induced cytotoxicity was measured evaluating cell viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (*26*) , as previously described (*20*).

Human erythrocytes were selected as a second model system to test the antioxidant activity of the tested compounds. Cell suspensions (2% hematocrit) were treated with *t*-BHP (500 μM final concentration). At the end of 2 h of incubation, the extent of hemolysis was measured spectrophotometrically, as previously described (*20*).

Evaluation of Lipoperoxidation. After the removal of the medium, Caco-2 cells were solubilized, and lysates were centrifuged at 12000*g* for 5 min; the obtained supernatants were assayed for TBARS (thiobarbituric acid reactive substances) formation (*9*). Aliquots of supernatants were added to thiobarbituric acid (TBA) in 0.05 N NaOH (0.2% w/v final concentration) and heated in a boiling water bath for 10 min. The absorbance of the developed pink chromophore was determined at 532 nm. For the measure of lipoperoxidation extent in human erythrocytes, samples were mixed with trichloroacetic acid (10% w/v final concentration) and centrifuged at 5000*g* for 15 min; supernatants were finally assayed for lipoperoxidation products as reported previously (*20*).

Statistical Analysis. The results of the biological tests are reported as means ± SD, $n = 4$; Student's paired *t*-test ($p < 0.05$) (Stat-Works software running on Apple Macintosh LC) was routinely utilized.

RESULTS AND DISCUSSION

The production of pure **1b** by treatment with $HClO_4$-SiO_2 and Ac_2O and MPLC purification either directly by the organic extracts of OMWW or by the previously purified **1a** was performed according to the respective protocols **a** and **b**, described in the scheme of **Figure 2**. Both methods have a common first step consistent with the continuous liquid−liquid

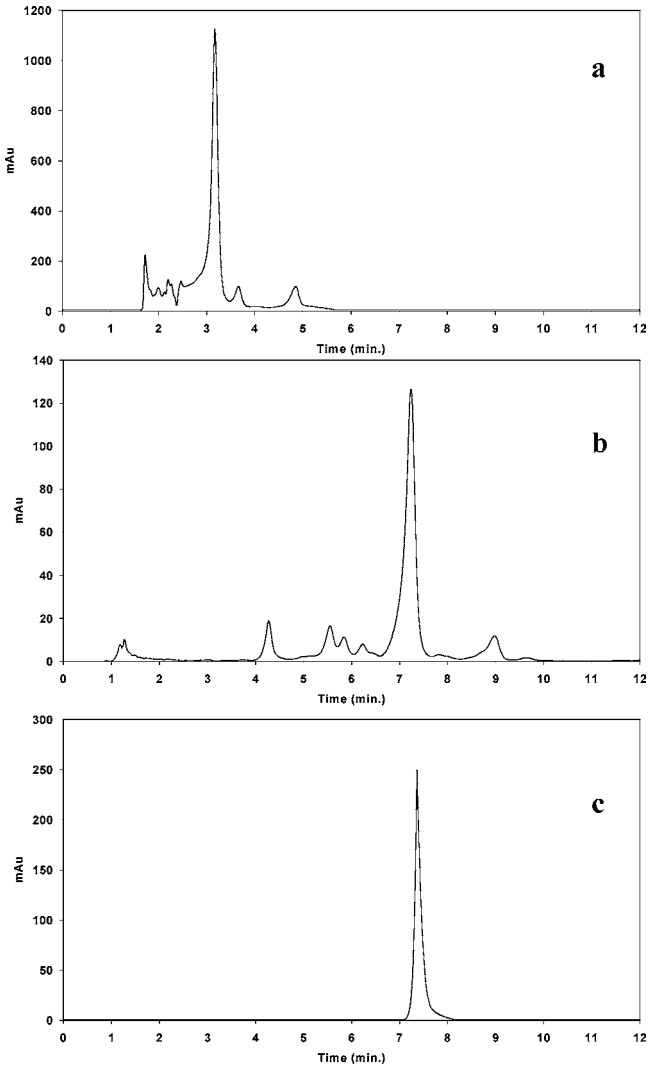

Figure 3. HPLC chromatogram of the OMWW extract (**a**), acetylated OMWW extract (**b**), and triacetylhydroxytyrosol (**1b**) (**c**).

extraction of raw OMWW with ethyl acetate. The total organic extracts showed the maximal amount of 1102.0 mg containing 252.2 mg of **1a**, quantitatively detected by HPLC analysis (**Figure 3a**).

This revealed itself to be the most efficient extraction procedure as compared with those described previously for the extraction of phenols from OMWW (*27*). As shown in the previous work, this method provided higher yield, and the simpler work-up required smaller volumes of ethyl acetate, therefore reducing its overall cost as compared to the continuous liquid−liquid counter-current extraction method or the discontinuous one, in batch mode.

Protocol **a** (**Figure 2**) produced a raw acetylated mixture containing the acetyl derivative **1b** as revealed by TLC (see Materials and Methods) and HPLC controls (**Figure 3b**). Compound **1b** was quantitatively obtained after 5 min and at room temperature. The reacted mixture was chromatographed by a single MPLC step giving pure **1b** (24.8 mg), with a yield of 35.6%, related to the amount of **1a** (38.3 mg) naturally occurring in the organic extracts (167 mg). The high purity grade of **1b** was indicated by the corresponding single and sharp peak at $t_R = 7.62$ min, as shown in the relative HPLC −chromatogram (**Figure 3c**). In addition, the ^{1}H NMR, EI-MS, and FAB-MS data reported in **Table 1** proved to be fully consistent with the structure of this compound (**Figure 1**, **1b**) and were confirmed to be coincident with those of the corresponding synthetic and natural compound **1b** previously described (*2*, *28*, *29*).

The acetylating agent composed of $HClO_4$-SiO_2 and Ac_2O appeared to be the most effective for the production of **1b** (yield of 35.6%), as compared with the mixtures of Ac_2O and H_2SO_4 or pyridine, which gave a yield of 26.1 and 29.8% (experimental data not shown), respectively.

The reaction of Chakborti and Gulhane provides many advantages with respect to those shown by the reaction performed with the other two reagents. In particular, the first reaction is performed in 5 min, at room temperature; in addition, its work-up is simpler, safer, and of lower cost because the reagents of Chakborti and Gulhane are fixed on SiO_2.

Both the mixtures composed of Ac_2O and H_2SO_4 or pyridine represent the usual acetylating agents, described in our previous papers for the acetylation of purified polyphenols, either those naturally occurring in OMWW (*28*) or synthetic ones (*2*). However, the acetyl derivative **1b** was prepared in our previous studies as a main goal, to chemically characterize **1a** and to investigate the mass spectrometry behavior and the structure−bioactivity relationship of the phenol compounds from agricultural waste (*2*, *28*, *29*). The efficiency of the acetylation method and its possible industrial applications were not investigated. The increased efficiency of the catalyst $HClO_4$-SiO_2 with respect to the traditional H_2SO_4 or pyridine can be attributed to the fact that the former probably limits the secondary reactions that occur in the latter reagents, consequently producing a higher yield of the acetyl compounds. The higher yield of **1b** obtained using the first mixture is further confirmation of the efficiency of this reagent for the preparation of acetyl phenols with high yield, according to the results shown by Chakborti and Gulhane (*24*). In addition, these authors demonstrated that the catalyst could also be recycled, with obvious economical advantages in potential industrial applications.

With regard to the production of **1b**, obtained with the protocol b (**Figure 2**), the organic extracts first underwent MPLC purification obtaining pure **1a** (yield 41%). The HPLC analysis of this compound showed a corresponding single and sharp peak at $t_R = 3.15$ min (data not shown). Moreover, its respective ^{1}H NMR and EI-MS data (**Table 1**) proved to be fully consistent with the corresponding structure (**Figure 1**). The purified **1a** was then acetylated using the same procedure followed protocol **a** (**Figure 2**), with a yield of 72%. Therefore, the overall yield for the production of **1b** using this latter protocol proved to be 29.5%.

It is worth noting that the preparation of **1a** from the same OMWW source for the production of **1b** was also motivated by the necessity to homogeneously compare the data on the chemical and biological antioxidant effects of the *o*-diphenol with those of its corresponding acetylated form. The findings of this comparative investigation are illustrated later.

In summary, protocol **a** appeared to be more efficient than protocol **b** (**Figure 2**), **1b** being produced by the first method with a yield of 35.6% and by the second with an yield of 29.5%. Very probably, compound **1a** is adsorbed more strongly than **1b** on the silica gel stationary phase and undergoes oxidative and photochemical processes during the preparation by protocol **b**, whereas it is protected by the acetyl groups in **1b** during the preparation by protocol **a**.

The only work that describes the production of ester derivatives of **1a** with the perspectives of industrial applications regards a recent patent (*30*). This work reports a general production of **1a** esters that proved to be more resistant than

Table 1. ^{1}H NMR , EI-MS, and FAB-MS Data of **1a** and **1b**

	1a			**1b**		
	^{1}HN MR (δ) (CD_3 OD)	EI-MS [*m/z*] (rel. int.)		^{1}HNMR (δ) ($CDCl_3$)	EI-MS [*m/z*] (rel. int.)	FAB-MS [*m/z*] (rel. int.)
2H-1'	2.61 (t, 2H, $J_{1',2'} = 7.2$ Hz)	154 $[M]^+$ (58)	2H- 1'	2.91 (t, 2H, $J_{1',2'} = 6.9$ Hz)	279 $[M - H]^+$ (8)	281 $[MH]^+$ (100)
2H-2'	3.67 (t, 2H, $J_{1',2'} = 7.2$ Hz)	136 $[M - H_2O]^+$ (45)	2H-2'	4.25 (t, 2H, $J_{1',2'} = 6.9$ Hz)	238 $[M - CH_2CO]^+$ (12)	239 $[MH - CH_2CO]^+$ (30)
		123 $[M - CH_2OH]^+$ (100)	H-3, H-6	7.05–7.17 (m, 2H)	220 $[M - CH_3COH]^+$ (7)	221 $[MH - CH_3COOH]^+$ (18)
H-3	6.65 (d, 1H, $J = 2$ Hz)	105 $[M - CH_2OH - H_2O]^+$ (82)	H-5	7.02 (brs, 1H)	196 $[M - 2CH_2CO]^+$ (5)	179 $[MH - CH_3CO\ OH - CH_2CO]^+$ (28)
H-5	6.55 (dd, 1H, $J_{3,5} = 2.0$ Hz, $J_{5,6} = 8$ Hz)	43 $[M - CH_2OH - CHO]^+$ (43)	$CH_3CO - 3$	2.26 (s, 3H)	178 $[M - 2CH_2CO - H_2O]^+$ (10)	137 $[MH - CH_3COOH - 2CH_2CO]^+$ (22)
H-6	6.70 (dd, 1H, $J = 8$ Hz)	87 $[M - CH_2OH - 2H_2O]^+$ (60)	$CH_3CO - 4$	2.26 (s, 3H)	136 $[M - 3CH_2CO - H_2O]^+$ (100)	93 $[MH - CH_3COOH - 2CH_2CO - CH_4 - CO]^+$ (10)
		77 $[C_6H_5]^+$ (64)	$CH_3CO_2C_2H_4 - 1$	2.02 (s.3H)	107 $[M - 3CH_2CO - H_2O - HCO]^+$ (4)	
		65 $[C_5H_5]^+$ (46)			77 $[C_6H_5]^+$ (3)	
		51 $[C_4H_3]^+$ (55)			65 $[C_5H_5]^+$ (3)	
					43 $[CH_3CO]^+$ (10)	

1a to air oxidation in an edibile matrix at 120 °C. Moreover, this work reports on formulations in juice, nutritional beverages, and butter preparations and on beneficial effects against oxidative stress and inflammatory diseases. In particular, it describes the production of a tristearyl derivative of **1a** by the reaction of **1a** with stearic acid, with a yield of 32%. This production obviously requires a preliminary preparation of synthetic or natural **1a**, which is usually obtained with a yield ranging between 41%—the amount of the natural compound obtained in the present work—and 79%, which is the amount reported in the literature (*2*). Therefore, the production of a tristearyl derivative of **1a** can be obtained with an overall maximum yield of 25.6%. In our work, the production of the acetyl derivative **1b** according to the protocol **a** (**Figure 2**) is more convenient (yield of 35.6%) because it is obtained by the direct treatment of the OMWW organic extracts with the acetylating mixture, without any previous isolation and purification of **1a**.

Another method for the production of **1b**, described in **Figure 2**, protocol **c**, was consistent with the direct treatment of lyophilized OMWW (see Materials and Methods) with $HClO_4$-SiO_2 and Ac_2O and a successive MPLC step. But, the yield of this procedure was lower (27.6%) than that obtained by treating the organic extracts because of the chemical complex composition of the raw lyophilized OMWW mixture. In addition, this procedure requires proportionally larger amounts of a chromatographic stationary phase and solvents for the purification of the acetylated product considered, and consequently, it is more laborious and expensive than the other methods considered previously. The comparative yield for the production of **1b**, obtained by the different methods, is reported in **Table 2**.

In conclusion, the production of **1b** appears to be much more efficient and advantageous using the method that employs the treatment of the phenol extracts with $HClO_4$-SiO_2 and Ac_2O (protocol **a**, **Figure 2**), as compared with the other methods summarized in **Table 2**.

A brief evaluation of the production of **1b** from OMWW as compared to that of the synthetic form, using as a common step the Chakborti and Gulhane reagent, with regard to safety, environmental aspects, and cost, led us to assess the production of natural **1b** to be more effective than the corresponding synthetic compound. In particular, assuming the production of natural **1b** to be obtained according to protocol **a** of **Figure 2**, it is performed in three steps, with an overall yield of 35.6%. Instead, the production of synthetic **1b** would require four steps: (i) the synthesis of **1a** from 3,4-hydroxyphenylacetic acid; (ii) the work-up of this reaction; (iii) the chromatographic purification of the impure **1a** [these first three steps and the corresponding yield % are reported in detail by Capasso et al. (*2*)]; and (iv) the Chakborti and Gulhane reaction. The yield of this production is calculated to be 56%. The production of synthetic **1b** involves additional costs with respect to that of the natural compound: (i) the reactor for the synthesis of **1a**; (ii) 3,4-hydroxyphenylacectic acid and reagents and solvents related to the transformation of the substrate into **1a**; (iii) the disposal or recycle of the considered reagents and solvents; and (iv) the expenses for one additional step (cost efficiency). Even if the yield % of the production of synthetic **1b** is higher than that of the corresponding natural compound by almost 20%, this increased production is not likely to justify the additional costs considered previously. Moreover, the first step relative to the synthesis of **1a** also implies additional environmental and safety problems with respect to the production of **1b** from OMWW. Finally, the utilization of this agricultural waste material for the recovery of the natural stabilized antioxidant

Table 2. Comparative Yield of Overall Production of **1b** and Tristearyl-**1a** from OMWW

	overall yield%
production of **1b** by treatment of OMWW organic extracts with $HClO_4$-SiO_2 and Ac_2O	35.6
production of **1b** by treatment of OMWW organic extracts with H_2SO_4 and Ac_2O	26.1
production of **1b** by treatment of OMWW organic extracts with pyridine and Ac_2O	29.8
production of **1b** by treatment of preliminarily purified **1a** from OMWW organic extracts with $HClO_4$-SiO_2 and Ac_2O	29.5
production of tristearyl-**1a** by reaction of purified **1a** with stearic acid[a]	25.6
production of **1b** by treatment of lyophilized OMWW with $HClO_4$-SiO_2 and Ac_2O	27.6

[a] This method has been published in a recent patent (*30*). It is reported with a yield of 32% because it is related to the reaction with a preliminary purified synthetic or natural **1a**. Since the production of pure **1a** is reported in literature with a maximum yield of 80% (*2*, *3*), we calculated the maximal yield of 25.6% considering the overall process.

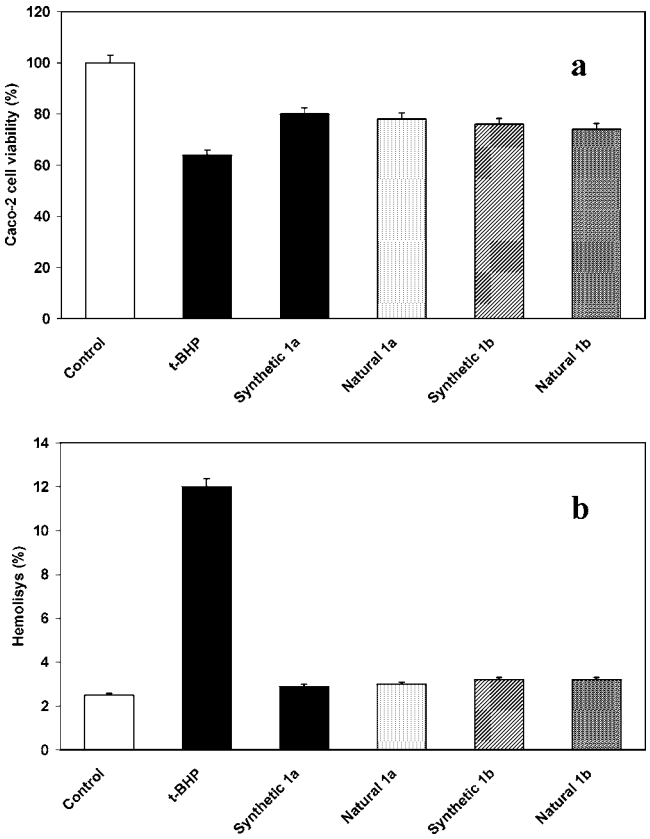

Figure 4. Comparative antioxidant effects of natural and synthetic hydroxytyrosol (**1a**) and triacetylhydroxytyrosol (**1b**) on *t*-BHP-induced oxidative stress in Caco-2 cells (**a**) and erythrocytes (**b**). These effects were measured using the tests of cellular viability (MTT assay) and hemolysis, respectively.

can contribute to an integrative solution for disposal problems since the exhausted material from the phenol organic extraction is recyclable potentially as an amendment because of its high organic content (*31*, *32*).

We then comparatively tested the chemical antioxidant activity of natural **1a** and its acetyl derivative **1b**. As expected, differently from its precursor **1a** endowed with a high antioxidant capacity, the acetylated compound does not shown any ferric reducing activity, as measured using the FRAP assay, confirming that **1b**, lacking the *o*-diphenol moiety, is totally inactive as a hydrogen donor and therefore does not exert any antioxidant effect (data not shown).

The biological antioxidant activity of natural **1b** was evaluated using human cells, including colon carcinoma cells (Caco-2 cells) and erythrocytes (**Figure 4a**,**b**). Preincubation in the presence of 50 μM **1b** significantly prevents the decrease in cell viability induced by t-BHP in Caco-2 cells to the same extent of the native compound **1a** (**Figure 4a**). Similarly, a complete protection against oxidative stress-induced hemolysis was observable in the antioxidant-treated erythrocytes, the antioxidant effect of **1b** being of some order of magnitude of **1a** (**Figure 4b**). Finally, **1a** and its acetyl analogue equally prevented at the same concentration the oxidative alterations of membrane phospholipids, as indicated by TBARS evaluation as a lipoperoxidation marker (data not shown).

It is worth noting that natural **1a** and **1b**, obtained by treatment of OMWW extracts as described in the present work, show protective effects to be very close to those of the corresponding synthetic compounds (**Figure 4**) prepared and assayed as described in our previous work (*20*), indicating the efficiency as biological antioxidants also of the compounds obtained from the OMWW source; the data also indicate that both the synthetic and the natural compounds have been obtained with the same high purity grade.

All together, the findings of both the chemical and the biological tests performed using natural **1a** and **1b** confirm our previous data (*20*), indicating that synthetic **1b** is endowed with a strong biological antioxidant activity similar to that of the parent compound, although devoid of ferric reducing ability, likely after its activation into **1a** at a cellular level by esterases. It should be stressed, in this respect, that stable **1b** that is preserved against possible chemical oxidation and is activated into **1a** only at the cellular level could ensure the highest antioxidant activity and therefore the highest protecting effect in vivo.

On the basis of these considerations, we propose a possible industrial production of natural **1b** for its use as a nontoxic functional component in a wide range of biological matrices for human purposes, not only in cosmetic and pharmaceutical preparations, but mainly as an active ingredient in food preparation.

The food industry, indeed, is deeply involved in the identification of new functional ingredients with health-promoting properties for designing high added value so-called functional foods, innovative foodstuffs claiming to improve health and/or to reduce the risk of diseases, by beneficially affecting one or more target functions in the body (*33*, *35*).

Antioxidants have being amply used by the food industry as additives in food production to prevent any alteration in their taste, appearance, and safety. They play such an important role in delaying the spoiling of foodstuff, including oxidation, a chemical process due to exposure to air or to the effects of heat and light, responsible for a decrease in the shelf life of products. It is important to underline that protection against oxidation exerted by antioxidants results in a loss of their concentration in food, and a further decrease can occur during cooking; therefore, only a small part of the antioxidant originally present in the raw material is ingested. Therefore, specific compounds, such as **1b**, which do not exert antioxidant properties in food

but are converted in vivo in the active antioxidant compound, could be regarded as a tool designed to specifically increase the antioxidant potential in humans for delaying the progression of several ROS-related pathologies, including CHD. However, there are two important aspects that must always be taken into account, including bioavailability and safety of the potential additive. As pointed out in the Introduction, several observations on both humans (*11*, *14*) and animal (*12*, *13*) models indicate the high bioavailability and safety of **1a**.

Referring to the possibility to use **1b** in cosmetic preparations, several authors have proposed that topically applied phenolic antioxidants could represent a tool for protecting the skin against UV-mediated oxidative damage (*36*, *37*). In particular, recent data indicate that caffeic acid and, at a higher degree, ferulic acid (because of its higher lipophilicity) are able to permeate through the stratum corneum and to exert significant protection against UVB-induced skin erythema in healthy human volunteers.

Moreover, based on the important biological and pharmacological activities exerted by **1a**, we consider that its acetylated form could be proposed as a therapeutic agent in pharmacological preparation. In this case, however, further studies should provide unequivocal evidence of the therapeutic efficacy of **1b** and, more important, its safety at pharmacological doses.

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