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Synthesis of new phenolic fatty acid esters and their evaluation as lipophilic antioxidants in an oil matrix

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Abstract

The synthesis of new phenolic-based lipophilic antioxidants is described and the structure–activity relationships of an entire family of phenolic fatty acid esters as potential antioxidants have been investigated. Radical-scavenging activity was evaluated by their activity towards a stable free radical, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{\cdot+}$) and their efficacy as food antioxidants was evaluated in refined olive oil using the Rancimat method. All the phenolic derivatives acylated with palmitic acid, compounds (**1**–**4** and **17**–**20**) showed better radical-scavenging capacities than did the two control food antioxidants, the commonly-used α-tocopherol (**21**) and ascorbyl palmitate (**22**). Moreover, all the di-orthophenolic lipophilic antioxidants prepared (**1**–**12**) showed higher induction times in the Rancimat test than did the control antioxidants. It is important to note that fatty acid esters of dihydrocaffeoyl alcohol (**3**, **6** and **9**) exhibited greater antioxidant ability to stabilize olive oil, than did their corresponding hydroxytyrosol fatty acid esters (**2**, **5** and **8**), clearly showing an effect of the length of the alkyl chain attached to the phenyl ring.

Keywords: Lipophilic antioxidants; Phenols; Hydroxytyrosol; Free radical-scavenging activity; Antioxidizing potency; Structure–activity relationship

1. Introduction

Oxidative stress in living organisms causes the formation of radical species that have been increasingly linked with cardiovascular, neurodegenerative and inflammatory diseases (Parthasarathy, Steinberg, & Witztum, 1992; Regnström, Nilsson, Tornvall, Landou, & Hamsten, 1992; Rice-Evans & Diplock, 1993; Zafrilla et al., 2006), and even with a role in cancer and aging (Beckman & Ames, 1998; Valko, Rhodes, Moncol, Izakovic, & Mazur, 2006). Consequently, the medical use of antioxidants, as one of the possible tools to counteract oxidation damage, is gaining acceptance as a base for novel therapeutic approaches and in the field of preventive medicine (Block, 1992; Rice-Evans, Miller, & Paganga, 1996). In the case of food matrices, oxidation is one of the main causes of food deterioration, especially in oils and fats. Moreover, oxidized lipids, when absorbed in mammals, are incorporated into lipoproteins and this association (especially with low-density lipoprotein) is considered to be one of the main inducers of atherosclerosis (Parthasarathy et al., 1992; Regnström et al., 1992). Antioxidants have been added to food for decades to control the oxidation process and they are widely used today for better food preservation. Development of new antioxidants, with better antioxidant capacity and less toxicity, is desirable for the prevention and/or treatment of a number of diseases and for better food preservation.

Phenolic antioxidants have been broadly employed for food conservation. At the same time, some of these phenols, present in natural sources, have revealed interesting biological properties. For example, resveratrol has shown anticancer and heart-protecting effects (Jang et al., 1997) and olive oil phenols, especially hydroxytyrosol, inhibits

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human LDL oxidation (Visioli, Bellomo, Montedoro, & Galli, 1995), inhibits platelet aggregation (Petroni et al., 1995) and exhibits antiinflammatory (De la Puerta, Ruiz-Gutierrez, & Hoult, 1999) and anticancer properties (Owen et al., 2000). We recently reported (Torres de Pinedo, Peñalver, & Morales, 2007) the antioxidant activity of several phenolic derivatives and found that two of the new compounds, dihydrocaffeoyl alcohol (3-(3,4-dihydroxyphenyl)-1-propanol) and galloyl alcohol (3,4,5-trihydroxybenzylic alcohol), are better antioxidants than hydroxytyrosol as radical-scavengers and for protecting an oil matrix against rancidity. These results may point towards possible interesting biological activity for these new antioxidants.

All naturally occurring antioxidants are strongly hydrophilic and this makes their incorporation into fat and oil matrices difficult. This problem is being approached by the preparation of lipophilic antioxidants from these natural sources, e.g., isoflavone fatty acid esters (Lewis et al., 2000), lipophilic clovamide derivatives (Ley & Bertram, 2003), poly(lauroy(+)-catechin)s (Jin & Yoshioka, 2005) or hydroxytyrosol fatty acid esters (Trujillo et al., 2006). We recently reported (Torres de Pinedo, Peñalver, Rondón, & Morales, 2005) the synthesis of a family of di-orthophenolic fatty acid esters (**1**–**12**) that included several hydroxytyrosol and dihydrocaffeoyl alcohol derivatives (Fig. 1). These antioxidants were prepared enzymatically, using the lipase from *Candida antarctica*, with good to excellent yields.

In this work, we report the synthesis of new phenolic fatty acid esters that incorporate the fatty acid moiety linked to one of the phenolic positions (**13**–**16**) of the phenolic alcohols used to prepare **1**–**12** (Fig. 2), or linked to the primary hydroxyl group when one of the phenol groups is methylated (**17**–**20**) (Fig. 3). In an effort to understand the structural feature that makes a compound an effective lipophilic antioxidant, we have measured the radical-scavenging capacity (using the ABTS method) and the antioxidant potency in food matrices (using the Rancimat method) of a group of phenolic fatty acid esters (**1**–**20**) that varies the position of acylation of the phenol, the length and nature of the fatty acid, the length of the alkyl chain directly connected with the phenol ring and the presence of one methoxy group in the phenol ring. α-Tocopherol (**21**) and ascorbyl palmitate (**22**) served as reference compounds.

2. Materials and methods

2.1. General

Synthesized compounds were purified on a silica gel 60 (200–400 mesh) (Sigma–Aldrich Chemical Co.) column and identified by TLC, MS and NMR analysis. Thin-layer chromatography (TLC) was performed on precoated silica gel 60 Alugram SIL/UV_{254} from Macherey Nagel. FAB mass spectra were collected on a Hewlett–Packard 5988 spectrometer, using a Fisons VG platform or a Fisons VG Autospec-Q. NMR experiments were performed on a Bruker AMX-300, operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C. $CDCl_3$ was used as solvent. Chemical shifts are expressed in δ (parts per million) using the solvent as internal reference. A Cary 100 UV–Vis spectrophotometer from Varian Co. was used in the $ABTS^{\bullet+}$ and log *P* assays. A Rancimat 743 apparatus from Metrohm-Herisau A.G. was used to measure the induction time in oils containing antioxidants.

2.2. Chemicals

Homoprotocatechuic acid (3,4-dihydroxyphenylacetic acid), vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol), homovanillyl alcohol (4-hydroxy-3-methoxyphenethyl alcohol), ferulic acid (4-hydroxy-3-methoxycinnamic acid), L-ascorbic acid 6-palmitate, α-tocopherol and 2,2-

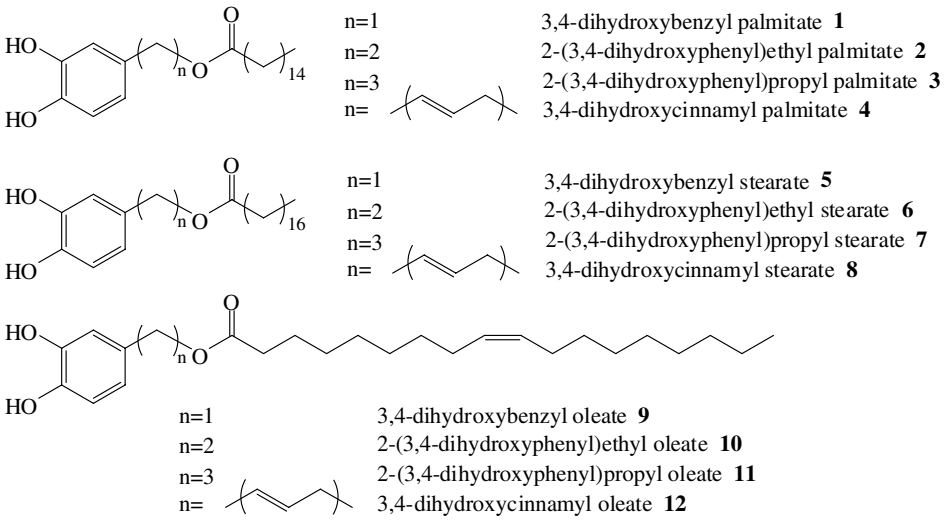

Fig. 1. Chemical structures of di-orthophenolic lipophilic antioxidants under study.

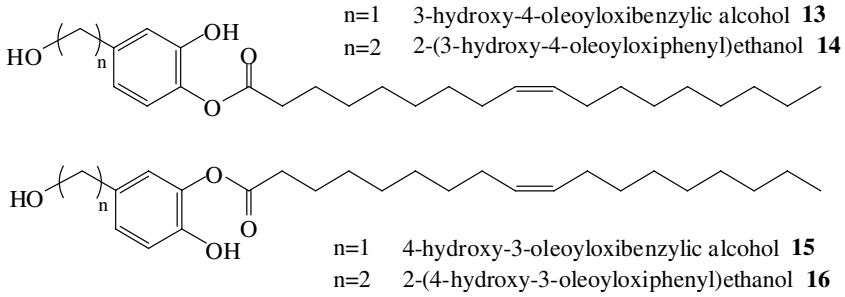

Fig. 2. Chemical structures of phenolic-acylated lipophilic antioxidants under study.

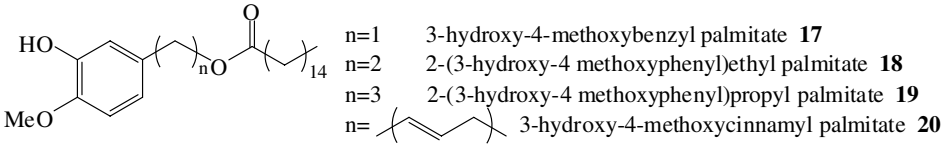

Fig. 3. Chemical structures of methoxylated-phenolic lipophilic antioxidants under study.

azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) were purchased from Sigma–Aldrich Chemical Co. Protocatechuic acid (3,4-dihydroxybenzoic acid) and hydroferulic acid (3-(4-hydroxy-3-methoxyphenyl)propionic acid) were purchased from Fluka. Immobilized lipase from *C. antarctica* B (Novozym 435) was kindly donated by Novozymes A/S. All other reagents and solvents were of analytical, spectrometric or HPLC grade.

2.3. Synthesis

2.3.1. General

Protocatechuyl alcohol (3,4-dihydroxybenzylic alcohol) and hydroxytyrosol (2-(3,4-dihydroxyphenyl)ethanol) are starting materials in the synthesis of lipophilic antioxidants **13**–**16**. These phenolic alcohols were prepared from their corresponding carboxylic acids by reduction with lithium aluminium hydride (Atkinson, Brown, & Gilby, 1973; Capasso, Evidente, Avolio, & Solla, 1999). Preparation of lipophilic antioxidants **13**–**16** was carried out by chemical acylation of the corresponding alcohols following the procedure described below.

2.3.2. General procedure for the preparation of mono-acyl phenol-substituted lipophilic antioxidants

To a suspension of oleic acid (0.2 ml, 0.66 mmol) and 1-ethyl-3-(3-dimethylaminopropyl carbodiimide)hydrochloride (EDCI) (145 mg, 0.7 mmol) in anhydrous THF (10 ml) at 0 °C under argon atmosphere, one equivalent of the corresponding alcohol and 4-dimethylaminopiridine (25 mg, 0.19 mmol) were added. The reaction mixture was stirred and allowed to reach room temperature. After 3 h, the reaction was stopped by filtration of the precipitated urea. The filtrate was evaporated to dryness, residue dissolved in dichloromethane (25 ml), washed with a saturated solution of $NaHCO_3$ (50 ml) and a saturated solution of sodium chloride (50 ml). The organic phase was dried (Na_2SO_4), concentrated to dryness and purified by flash column chromatography (hexane–ether (1:1)).

2.3.3. 4-Hydroxy-3-oleoyloxybenzylic alcohol (*13*) and 3-hydroxy-4-oleoyloxybenzylic alcohol (*15*)

Ninety-three milligrams (0.66 mmol) of protocatechuyl alcohol were added to the reaction mixture. A white solid was obtained after column purification (176 mg, 67%) that corresponded to a 1:1 mixture of lipophilic antioxidants **13** and **15**. ^{1}H NMR (300 mHz, $CDCl_3$) δ 7.03–6.74 (m, 6H, ar), 5.34 (m, 4H, HC=CH), 4.54 (s, 2H, –CH_2OH), 4.48 (s, 2H, –CH_2OH), 2.58 (*t*, $J = 7.4$ Hz, 4H, -ar-OOC–CH_2–), 1.99 (m, 8H, –CH_2–HC=CH–CH_2–), 1.74 (m, 4H, -ar-OOC–CH_2–CH_2–), 1.25 (m, 56H, –CH_2–), 0.87 (*t*, $J = 6.9$ Hz, 6H, –CH_3); ^{13}C NMR (75 mHz, $CDCl_3$) δ 174.3 (–COO–), 174.0 (–COO–), 131.5, 131.1 (–HC=CH–) 149.0, 148.1, 141.0, 139.9, 139.3, 134.9, 127.2 123.8, 122.9, 120.4, 119.1, 117.6 (ar), 65.9 (PhCH_2OH), 35.6, 35.6, 33.3, 31.2, 31.1, 30.9, 30.7, 30.6, 30.5, 30.5, 28.6, 28.6, 26.3, 24.1 (–CH_2–HC=CH–CH_2–, –CH_2–), 15.5 (–CH_3). HRFABMS$^+$ calcd. for $C_{25}H_{40}O_4Na$ $[M+Na]^+$ 427.2821, found 427.2819.

2.3.4. 2-(4-Hydroxy-3-oleoyloxyphenyl) ethanol (*14*) and 2-(3-hydroxy-4-oleoyloxyphenyl) ethanol (*16*)

One hundred milligrams (0.65 mmol) of hydroxytyrosol were added to the reaction mixture. A white solid was obtained after column purification (177 mg, 65%) that corresponded to a 1:1 mixture of lipophilic antioxidants **14** and **16**. ^{1}H NMR (300 mHz, $CDCl_3$) δ 6.95 (*d*, $J = 8.1$ Hz, 1H, ar), 6.90 (dd, $J = 5.3$, 2.1 Hz, 2H, ar), 6.92 (s, 1H, ar), 6.80 (*d*, $J = 2.1$ Hz, 1H, ar), 6.72 (dd, $J = 8.2$, $J = 2.1$ Hz, 1H, ar), 6.15 (m, OH), 5.75 (m, OH), 5.34 (m, 4H, HC=CH), 3.79 (*t*, $J = 6.4$ Hz, 4H –CH_2OH), 2.76 (*t*, $J = 6.4$ Hz, 4H, -ar-CH_2–), 2.56 (*t*, $J = 7.4$ Hz, 4H,

-ar-OOC–CH_2–), 1.99 (m, 8H, –CH_2–HC=CH–CH_2–), 1.75 (m, 4H, -ar-OOC–CH_2–CH_2–), 1.25 (m, 56H, –CH_2–), 0.87 (*t*, $J = 6.9$ Hz, 6H, –CH_3); ^{13}C NMR (75 mHz, $CDCl_3$) δ 172.5 (–COO–), 131.5, 131.1 (–HC=CH–) 149.0, 148.1, 141.0, 139.9, 139.3, 134.9, 127.2 123.8, 122.9, 120.4, 119.1, 117.6 (ar), 63.5 (–CH_2OH), 38.6, 38.3 (PhCH_2–) 35.6, 35.6, 33.3, 31.2, 31.1, 30.9, 30.7, 30.6, 30.5, 30.5, 28.6, 28.6, 26.3, 24.1 (–CH_2–HC=CH–CH_2–, –CH_2–), 14.2 (–CH_3); HRFABMS$^+$ calcd. for $C_{26}H_{42}O_4Na$ $[M+Na]^+$ 441.2978, found 441.2973.

Coniferyl alcohol (4-hydroxy-3-methoxycinnamylic alcohol) and hydroconiferyl alcohol (3-(4-hydroxy-3-methoxyphenyl)propanol) are starting materials in the synthesis of lipophilic antioxidants **19** and **20**, respectively. These phenolic alcohols were synthesized from their corresponding carboxylic acids by reduction with lithium aluminium hydride in anhydrous tetrahydrofuran (see experimental in Torres de Pinedo et al., 2007). Lipophilic antioxidants **17**–**20** were prepared by enzymatic acylation of the corresponding phenolic alcohols, using lipase from *C. antarctica*, following a previously published procedure (see experimental in Torres de Pinedo et al., 2005).

2.3.5. 3-Hydroxy-4-methoxybenzyl palmitate (*17*)

The reaction mixture was purified by flash column chromatography, using a hexanes/diethyl ether (3:1) mixture, to yield a white solid (95%). ^{1}H NMR (300 mHz, $CDCl_3$) δ 6.87 (m, 3H, ar), 5.02 (s, 2H, PhCH_2OOC–), 3.89 (s, 3H, OMe), 2.32 (*t*, $J = 7.5$ Hz, 2H, –OOC–CH_2–), 1.62 (*q*, $J = 7.2$ Hz, 2H, –OOC–CH_2–CH_2), 1.25 (m, 24H, –CH_2–), 0.87 (*t*, $J = 6.4$, 3H, –CH_3); ^{13}C NMR (75 mHz, $CDCl_3$) δ 173.8 (–COO–), 146.5, 145.8, 128.1, 122.0, 114.4, 111.3 (ar), 66.3 (–CH_2OCO–), 56.0 (–OCH_3), 34.5, 32.0, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 25.0, 22.7 (–CH_2–), 14.1 (–CH_3). HRFABMS$^+$ calcd. for $C_{24}H_{40}O_4Na$ $[M+Na]^+$ 415.2824, found 415.2824.

2.3.6. 2-(3-Hydroxy-4-methoxyphenyl) ethyl palmitate (*18*)

The reaction mixture was purified by flash column chromatography, using a hexanes/diethyl ether (3:1) mixture, to yield a white solid (97%). ^{1}H NMR (300 mHz, $CDCl_3$) δ 6.83 (dd, $J = 6.5$, 2.1 Hz, 1H, ar), 6.71 (s, 1H, ar), 6.69 (d, 1.8 Hz, 1H, ar), 4.24 (*t*, $J = 7.1$ Hz, 2H, –CH_2OOC–), 3.87 (s, 3H, OMe), 2.85 (*t*, $J = 7.1$ Hz, 2H, ar-CH_2–), 2.27 (*t*, $J = 7.5$ Hz, 2H, –OOC–CH_2–), 1.58 (q, $J = 7.2$ Hz, 2H, –OOC–CH_2–CH_2), 1.24 (m, 24H, –CH_2–), 0.87 (*t*, $J = 6.4$, 3H, –CH_3); ^{13}C NMR (75 mHz, $CDCl_3$) δ 173.9 (–COO–), 146.5, 145.4, 129.7, 121.7, 114.4, 111.4 (ar), 65.0 (–CH_2OCO–), 55.9 (–OCH_3), 34.9, 34.4, 32.0, 29.7, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 25.0, 22.7 (–CH_2–), 14.1 (–CH_3); HRFABMS$^+$ calcd. for $C_{25}H_{42}O_4Na$ $[M+Na]^+$ 429.2981, found 429.2977.

2.3.7. 3-(3-Hydroxy-4-methoxyphenyl) propyl palmitate (*19*)

The reaction mixture was purified by flash column chromatography, using a hexanes/diethyl ether (3:1) mixture, to yield a white solid (95%). ^{1}H NMR (300 mHz, $CDCl_3$) δ 6.82 (m, 1H, ar), 6.67 (m, 2H, ar), 4.08 (*t*, $J = 6.5$ Hz, 2H, –CH_2OOC–), 3.86 (s, 3H, OMe), 2.61 (*t*, $J = 8.1$ Hz, 2H, ar-CH_2–), 2.30 (*t*, $J = 7.6$ Hz, 2H, –OOC–CH_2–), 1.88 (*q*, $J = 8.3$ Hz, 2H, –CH_2–), 1.62 (*q*, $J = 7.2$ Hz, 2H, –OOC–CH_2–CH_2), 1.25 (m, 24H, –CH_2–), 0.87 (*t*, $J = 6.9$, 3H, –CH_3); ^{13}C NMR (75 mHz, $CDCl_3$) δ 174.0 (–COO–), 146.5, 143.9, 133.2, 121.0, 114.4, 111.0 (ar), 63.6 (–CH_2OCO–), 55.9 (–OCH_3), 34.4, 32.0, 31.9, 30.6, 29.7, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 25.0, 22.7 (–CH_2–), 14.1 (–CH_3); HRFABMS$^+$ calcd. for $C_{26}H_{44}O_4Na$ $[M+Na]^+$ 443.3137, found 443.3132.

2.3.8. 3-Hydroxy-4-methoxycinnamoyl palmitate (*20*)

The reaction mixture was purified by flash column chromatography, using a hexanes/diethyl ether (3:1) mixture, to yield a white solid (82%). ^{1}H NMR (300 mHz, $CDCl_3$) δ 6.92 (s, 1H, aromático), 6.81 (s, 2H, aromático), 6.51 (*d*, $J = 15.8$ Hz, 1H, Ph–C*H*=CH–CH_2–), 6.09 (dt, $J = 15.8$, 6.6 Hz, 1H, Ph–CH=C*H*–CH_2–), 4.69 (d, $J = 6.5$ Hz, Ph–CH=CH–CH_2–), 3.87 (s, 3H, OMe), 2.33 (*t*, $J = 7.4$ Hz, 2H, –OOC–CH_2–), 1.63 (*q*, $J = 7.0$ Hz, 2H, –OOC–CH_2–CH_2), 1.25 (m, 24H, –CH_2–), 0.87 (*t*, $J = 6.3$, 3H, –CH_3); ^{13}C NMR (75 mHz, $CDCl_3$) δ 173.8 (–COO–), 134.4 (Ph–*C*H=CH–), 121.0 (Ph–CH=*C*H–), 146.7, 146.0, 128.9, 120.7, 114.5, 108.5 (Ar), 65.1 (–CH_2OCO–), 55.9 (–OCH_3), 34.4, 32.0, 29.7, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 25.0, 22.7 (–CH_2–), 14.1 (–CH_3); HRFABMS$^+$ calcd. for $C_{26}H_{42}O_4Na$ $[M+Na]^+$ 441.2981, found 441.2976.

2.4. ABTS radical-scavenging assay

A stock solution of ABTS was prepared by mixing 5 ml of a 7 mM 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) aqueous solution with 88 μλ of a 140 mM potassium persulphate aqueous solution (Re et al., 1999; Rice-Evans et al., 1996). The stock solution was kept in the dark for 16 h, allowing it to form the ABTS radical ($ABTS^{\cdot+}$). Finally, the stock solution was diluted with ethanol (approx. 1/88) to obtain an absorbance of 0.7 ± 0.02 at 734 nm. For the spectrophotometric assay, 2 ml of the $ABTS^{\cdot+}$ diluted solution were mixed with 2 ml of the test compounds at different concentrations in ethanol, so that the final concentrations of the antioxidants in the mixture were 0.5, 1, 2, 5 and 10 μM. Samples were mixed vigorously for 30 s and allowed to stand for 10 min in the dark at room temperature. The absorbance for each sample was measured at 734 nm and corrected for the absorbance of an ABTS blank (2 ml ethanol). All tests were performed in triplicate. The radical-scavenging activity of the samples was expressed as % reduction of $ABTS^{\cdot+}$ absorbance (% reduction $= [(A_{control} - A_{test})/A_{control})] \times 100$, where $A_{control}$ is the absorbance of the control ($ABTS^{\cdot+}$ solution without test sample) and A_{test} is the absorbance of the test sample ($ABTS^{\cdot+}$ solution plus compound). α-Tocopherol (**21**) and ascorbyl palmitate (**22**) were used as reference compounds.

2.5. Rancimat test

Measurement of the antioxidant potency of the new compounds in a food matrix (oil) was performed using the well-established Rancimat method. Moreover, the Rancimat method correlates well with the active oxygen method (Läubly & Bruttel, 1986). A Rancimat apparatus from Metrohm-Herisau A.G. was operated at 120 °C. A dry air flow of 20 l/h was passed through the oil sample (5 ± 0.001 g) containing the antioxidant. The volatile oxidation products, arising from the oxidation of the oil, are dissolved in cold milliQ water (60 ml) causing an increase of the electrical conductivity parameter value. All tests were performed in triplicate. The time (in hours) taken to reach a specific conductivity value, corresponding to the flex point of the peroxidation curve, was considered as the induction time (IT). The longer the induction times, the greater were the antioxidant potencies of the compounds.

2.6. log P Solubility measurement

Dispersions (0.3 mM) of each compound were prepared in 1-octanol and were heated to 60 °C for 1 h to help to solubilize all of the antioxidant. UV spectra for each solution were measured and absorbance at the maximum was determined (A_0). Equal volumes of the organic solution (2 ml) and a phosphate buffer (0.1 M, pH 7.4) were vigorously mixed using a vortex mixer for 1 min. The mixture was allowed to stabilize and separate for 30 min; then the organic phase was obtained and its absorbance determined (A_x). Partition coefficient ($\log P$) was determined from the fraction $P = A_x/(A_0 - A_x)$. All tests were performed in triplicate. α-Tocopherol (**21**) and ascorbyl palmitate (**22**) were used as reference compounds.

3. Results and discussion

3.1. Synthesis

Di-orthophenolic fatty acid esters (Fig. 1) (**1**–**12**) were prepared from the corresponding phenolic alcohols by enzymatic transesterification, as previously reported, (Torres de Pinedo et al., 2005). The acylating agents used in the reaction were vinyl palmitate, stearate or oleate and the lipase was Novozym 435 (immobilized lipase from *C. antarctica*). Lipophilic antioxidants **1**–**12** were obtained in good to excellent yields. In the case of methoxylated-phenolic antioxidants **17**–**20** (Fig. 3), enzymatic synthesis was also used in order to achieve selective esterification at the primary hydroxyl group. Two of the phenolic alcohols needed were commercially available (vanillyl alcohol and homovanillyl alcohol) and the other two were prepared from ferulic acid and dihydroferulic acid, respectively, by reduction with lithium aluminium hydride (Capasso et al., 1999; Freudenberg, Swaleh, Aufbau, & Abbau, 1969). The synthesis of lipophilic antioxidants **17**–**20** was performed using experimental conditions similar to those for antioxidants **1**–**12**.

Phenolic-acylated lipophilic antioxidants **13**–**16** (Fig. 2) were synthesized by chemical acylation of benzylic alcohol and hydroxytyrosol. These phenolic alcohols were prepared from their corresponding carboxylic acids by reduction with lithium aluminium hydride (Atkinson et al., 1973; Capasso et al., 1999). Acylation of benzylic alcohol with oleic acid was carried out in the presence of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDCI) and 4-methylaminopyridine (DMAP) in anhydrous THF. A 1:1 mixture of phenolic-acylated regioisomers (**13** and **15**) was obtained that could not be separated by column chromatography. Similar results were achieved for hydroxytyrosol, giving a mixture of lipophilic antioxidants **14** and **16**. These 1:1 mixtures were used, as such, in the experiments to determine their antioxidant capacity.

3.2. Radical-scavenging activity

The ABTS assay is a widely used method for measuring the ability of antioxidants to trap free radicals (Re et al., 1999). The radical scavenging capacity of the phenolic-based lipophilic antioxidants in the study is shown in Fig. 4. Two controls, traditional food antioxidants α-tocopherol (**21**) and ascorbyl palmitate (**22**), were also included. Standard deviation values for all compounds were below 3.5%. Surprisingly, lipophilic antioxidants **18** and **19**, homovanillyl alcohol and coniferyl alcohol derivatives, respectively, acylated at the primary alcohol and including a phenolic alcohol and a methoxy group at the ring, were the most effective radical-scavengers. They are closely followed by antioxidant **17**, also a methoxylated-phenolic derivative, and by the series of acylated di-ortho-phenolic antioxidants **1**–**4**. These results contrast with those obtained for the corresponding non-acylated phenolic alcohols, where di-orthophenolic alcohols (benzylic alcohol, hydroxytyrosol, dihydrocaffeoyl alcohol and caffeoyl alcohol) showed better radical-scavenging activity in a DPPH test than did the corresponding mono-methoxylated-phenolic alcohols (vanillyl alcohol, homovanillyl alcohol, dihydroconiferyl alcohol and coniferyl alcohol) (Torres de Pinedo et al., 2007). Moreover, benzylic alcohol derivatives, **5**, **9** and the **13**–**15** mixture, showed less radical-scavenging activity than did the previously mentioned compounds (**1**–**4** and **17**–**19**) but still more than the control compounds **21** and **22**. Only compounds **12** and the **14**–**16** mixture had less radical inhibition capacity than the controls.

Unexpectedly, the major factor affecting radical-scavenging activity seems to be the type of the fatty acid chain since lipophilic antioxidants substituted with palmitic acid (**1**–**4** and **17**–**19**) were the most effective scavengers of the studied compounds with the exception of compound **20**. Stearate- or oleate-substituted compounds had radical-scavenging potencies only slightly higher than or similar to the control compounds **21** and **22**. The position of the

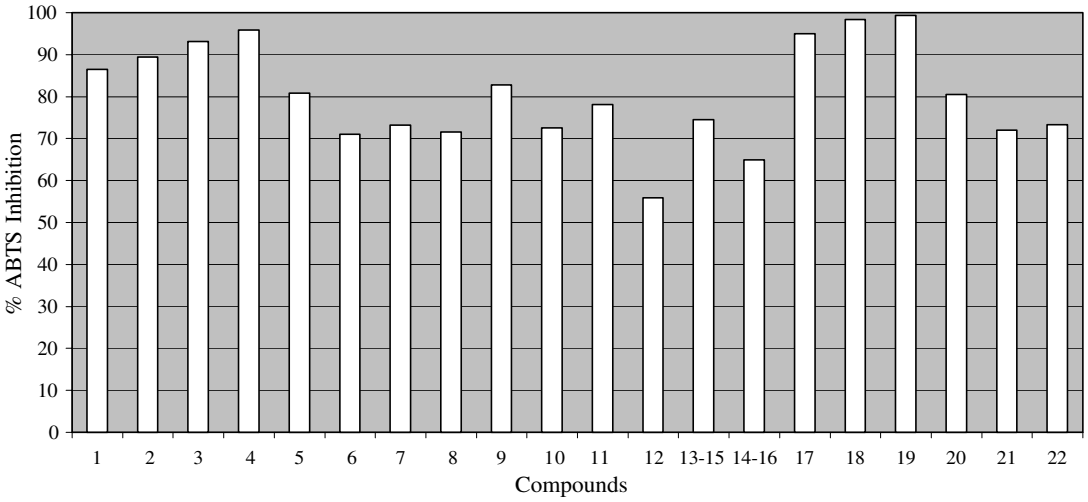

Fig. 4. $ABTS^{\cdot+}$ radical-scavenging activity of phenolic acid esters. Final concentration of compounds was 10 μM. All standard deviations values were below 3.5%.

alkyl chain also influenced scavenging capacity. Compounds **9** and **10**, with oleic acid linked to its primary alcohol, showed higher radical-scavenging activity than did the corresponding compound mixtures **13**–**15** and **14**–**16**, with oleic acid linked to one of the phenolic groups. These differences are probably based on the fact that **9** and **10** possess a di-orthophenolic structure whereas **13**–**15** and **14**–**16** mixtures posses one phenolic alcohol and one primary alcohol. It is known that the presence of a catechol ring in the structure of an antioxidant lowers the O–H bond dissociation enthalpy and increases the rate of H-atom transfer to peroxyl radicals with respect to a phenol structure (Lucarini & Pedulli, 1994; Shahidi & Wanasundara, 1992). Moreover, the existence of the extra primary alcohol in the structures of the **13**–**15** and **14**–**16** mixtures does not seem to be enough to equal the scavenging potencies of **9** and **10**.

It is important to note that a small effect of the length of the alkyl chain in radical-scavenging activity is also observed. For example, an increase of the length in the series of antioxidants **1**–**3** and **17**–**19** leads to an increase in the radical-scavenging capacity. Actually, a similar effect was observed for the corresponding non-acylated phenolic alcohols (Torres de Pinedo et al., 2007). The role of the double bond of the alkenyl chain is not so clear, since compound **4** is a better radical-scavenger than its alkyl analogue **3**, whereas compound **20** is a less effective scavenger than its analogue **19**. Contradictory results were also obtained for the corresponding non-acylated phenolic alcohols (Torres de Pinedo et al., 2007).

3.3. Antioxidant activity in oils

Antioxidant capacity in oils was evaluated by measuring the oxidative stability of refined olive oil spiked with each antioxidant (final concentration of 100 mg/l) using the Rancimat method. This is a commonly used method for comparing the oxidative stabilities of fats and oils. Several control measurements were also carried out, such as addition of traditional food antioxidants **21** or **22**, and blank oil with no antioxidants added. The results for induction time in refined olive oil are shown in Fig. 5. Standard deviation values were between 0.3 and 1.3 h. All new lipophilic phenolic-based antioxidants, except methoxylated compounds **17**–**20**, showed longer induction times (IT) than did α-tocopherol (control compound **21**). These results contrast with those obtained for radical-scavenging activity. Even more relevant is the fact that the di-orthophenolic lipid antioxidants **2**–**4** and **6**–**12** had longer ITs than had the commonly-used fat-soluble antioxidant ascorbyl palmitate (control compound **22**). It appears that the cathecol structure imparts effective lipid antioxidant activity, whereas the presence of a phenolic group and a methoxy group in the ring structure yields less effective lipid antioxidants.

The type of fatty acid (palmitate, stearate or oleate) acylating the phenolic alcohols appears to yield very few differences in ITs for the lipid antioxidants **1**–**12**. Only a slight increase of IT was observed in the benzylic alcohol series when going from palmitate to stearate and then oleate (**1**, **5** and **9**), and a slight decrease in IT in the dihydrocaffeoyl alcohol series, again going from palmitate to stearate and then oleate (**3**, **7** and **11**). Similar results have been reported recently by Trujillo et al. (2006), where hydroxytyrosol and hydroxytyrosol fatty acid derivatives, with palmitic acid, stearic acid, oleic acid and linoleic acid, yielded almost the same IT values. In fact, a very similar pattern was observed for antioxidant groups **1**–**4**, **5**–**8** and **9**–**12**. The nature of this pattern was actually due to the effect of the varying length and nature of the side chain covalently linked to the phenol ring. When the length of alkyl chain increased (**1**–**3**, **5**–**7** and **9**–**11**), an increase in IT was observed. This increase was more marked in the case of the palmitate **1**–**4** series and resulted in the compound with the highest IT (14 h), compound **3**, 2-(3,4-

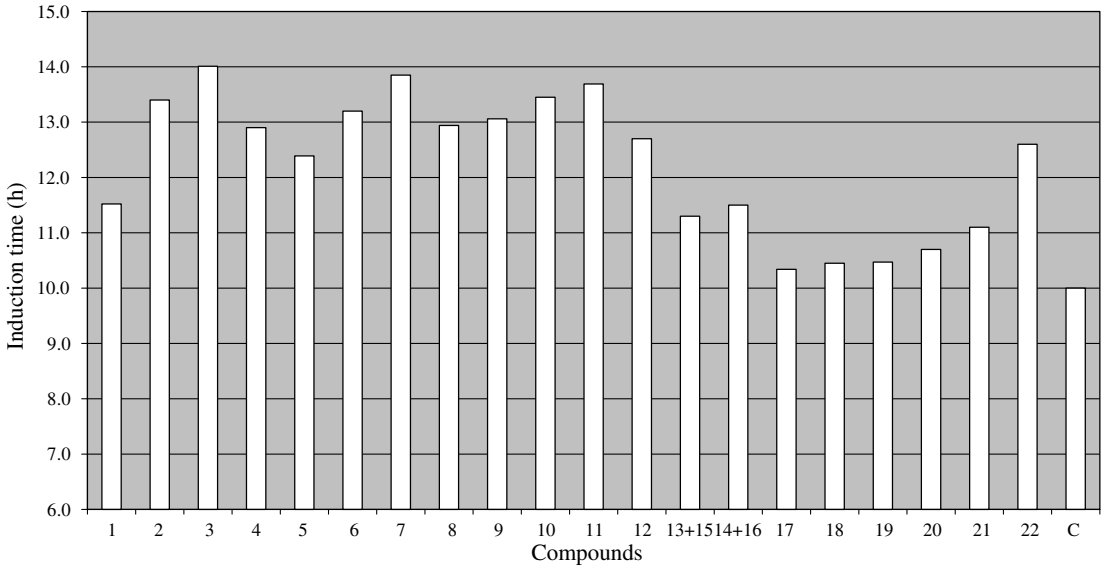

Fig. 5. Average induction time values of phenolic acid esters. C is the control olive oil with no added compounds. Standard deviations values were between 0.3 and 1.3 h.

dihydroxyphenyl)propyl palmitate. It is important to remark that fatty acid esters of dihydrocaffeoyl alcohol (**3**, **7** and **11**) exhibited higher antioxidant capacities than did their corresponding hydroxytyrosol fatty acid esters (**2**, **6** and **10**) to stabilize olive oil. The presence of an alkenyl chain instead of an alkyl chain resulted in a reduction in IT, which can be observed by comparing **3** with **4**, **7** with **8** and **11** with **12**. The effect of the nature and length of the alkyl chain is similar to that observed in the corresponding non-acylated phenolic alcohols (Torres de Pinedo et al., 2007).

Finally, the position of acylation of the phenolic alcohol seems to be an important factor in determining the effectiveness of lipid antioxidant activity. When oleic acid is attached to the primary alcohol of the phenolic alcohols, such as in compounds **9** and **10**, much higher ITs were observed than when oleic acid is attached to one of the phenolic groups, such as in the corresponding compound mixtures **13**–**15** and **14**–**16**.

3.4. Solubility of the antioxidants

The partition coefficient ($\log P$) was measured for all the compounds in the study, to address the real lipophilicity of the new phenolic esters and its possible correlation with the Rancimat values (see Fig. 6). Standard deviation values for all compounds were below 5%. All phenolic fatty acid esters are clearly hydrophobic, but only compounds **17**–**20** show $\log P$ values similar to those of the control compounds, α-tocopherol (**21**) and ascorbyl palmitate (**22**).

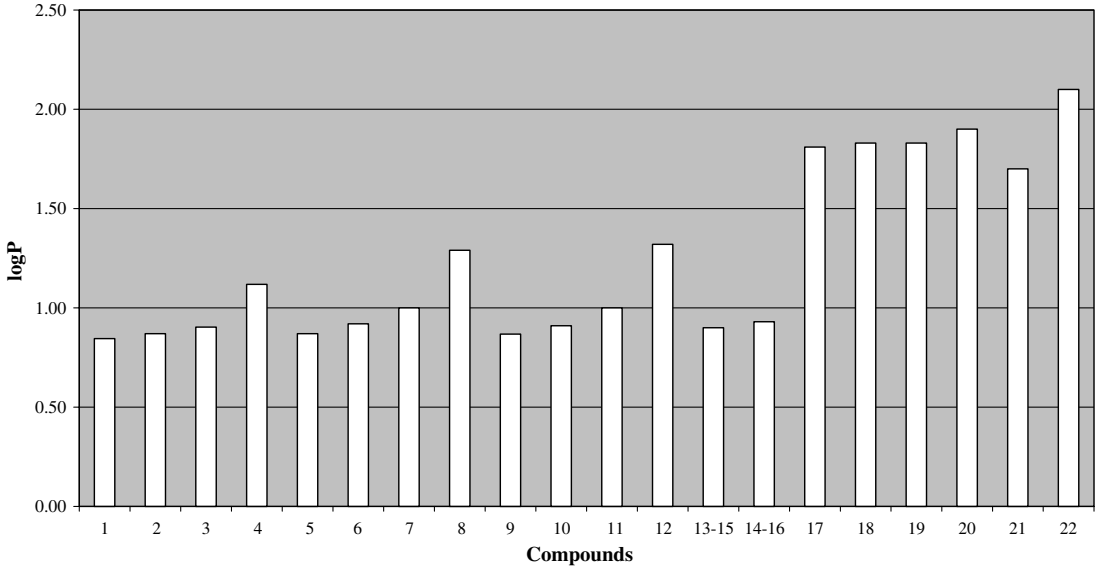

Fig. 6. $\log P$ values of phenolic acid esters. Standard deviations values were below 5%.

All other phenolic esters in the study **1**–**16** possess log *P* values around 1.0, and are clearly less hydrophobic than **17**–**20** and controls **21** and **22**.

When the lipophilicity data of the phenolic fatty acid esters (Fig. 6) were compared with their Rancimat values (Fig. 5), in general terms, the more polar compounds **1**–**16** were better antioxidants in an oil matrix, with the exception of compound **1** and compound mixtures **13**–**15** and **14**–**16**. This is in accordance with the proposed polar paradox where more polar antioxidants are more effective in less polar media (Frankel, Huang, Kanner, & German, 1994; Porter, 1993). When a detailed comparison is carried out, this correlation is not always true. For example, compounds with an alkenyl chain attached to the phenolic ring (**4**, **8** and **12**), which have polarities similar to those of their analogues with an alkyl chain (**3**, **7** and **11**), actually show lower ITs, in accordance with the polar paradox. But, in contrast, compound mixtures **13**–**15** and **14**–**16**, with similar polarities to their analogues **9** and **10**, have higher ITs.

4. Conclusion

We have prepared new phenolic-based lipophilic antioxidants (**13**–**20**) and studied the structure–activity relationships of an entire family of phenolic fatty acid esters (**1**–**20**) as potential antioxidants. Radical-scavenging activity was evaluated using the ABTS method and oxidative stability of oils was measured by the Rancimat method. No clear correlation can be drawn between radical inhibition capacities of the phenolic fatty acid esters and their induction time values. The type of fatty acid seems to play an important role in radical-scavenging capacity, since all the palmitate phenolic derivatives, compounds **1**–**4** and **17**–**20** are better scavengers than are stearate (**5**–**8**) or oleate derivatives (**9**–**16**). In contrast, induction times show small differences for different fatty acid derivatives.

The di-orthophenolic lipophilic antioxidants **1**–**12** showed, in general, higher induction times in the Rancimat test than did the control antioxidants. There is a clear effect of the length of the alkyl chain attached to the phenyl ring in antioxidant potency, resulting in fatty acid esters of dihydrocaffeoyl alcohol (**3**, **6** and **9**) with greater antioxidant ability to stabilize olive oil than their corresponding hydroxytyrosol fatty acid esters (**2**, **5** and **8**). The polar paradox hypothesis can be used to explain the antioxidant capacity of these new phenolic antioxidants from a very general perspective.

References

Atkinson, P. W., Brown, W. V., & Gilby, A. R. (1973). Phenolic compounds from insect cuticle, identification of some lipid antioxidant. *Insect Biochemistry, 3*, 309–315.

Beckman, K. B., & Ames, B. N. (1998). The free radical theory of aging matures. *Physiological Reviews, 78*, 547–581.

Block, G. (1992). A role for antioxidants in reducing cancer risk. *Nutrition Reviews, 50*, 207–213.

Capasso, R., Evidente, A., Avolio, S., & Solla, F. (1999). A highly convenient synthesis of hydroxytyrosol and its recovery from agricultural waste waters. *Journal of Agricultural and Food Chemistry, 47*, 1745–1748.

De la Puerta, R., Ruiz-Gutierrez, V., & Hoult, J. R. (1999). Inhibition of leukocyte 5-lipooxygenase by phenolics from virgin olive oil. *Biochemical Pharmacology, 57*, 445–449.

Frankel, E. N., Huang, S.-W., Kanner, J., & German, J. B. (1994). Interfacial phenomena in the evaluation of antioxidants: Bulk oils vs emulsions. *Journal of Agricultural and Food Chemistry, 42*, 1054–1059.

Freudenberg, K., Swaleh, M., Aufbau & Abbau (1969). Desku¨nstlichen Lignins aus Coniferylalkohol-[γ-^{14}C] (preparation and degradation of synthetic lignins from coniferyl alcohol-[γ -^{14}C]). *Chemische Berichte, 102*, 1316–1319.

Jang, M., Cai, L., Udeani, G. O., Slowing, K. V., Thomas, C. F., Beecher, C. W. W., et al. (1997). Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science, 275*, 218–220.

Jin, G., & Yoshioka, H. (2005). Synthesis of lipophilic poly-lauroyl-(+)-catechins and radical scavenging activity. *Bioscience Biotechnology and Biochemistry, 69*, 440–447.

Läubly, M. W., & Bruttel, P. A. (1986). Determination of oxidative stability of fats and oil: Comparison between the active oxygen method (AOCS CD 12-57) and the "Rancimat" method. *Journal of the American Oil Chemists' Society, 63*, 792–795.

Lewis, P. T., Wähälä, K., Hoikkala, A., Mutikainen, I., Meng, Q.-H., Adlercreutz, H., et al. (2000). Synthesis of antioxidant isoflavone fatty acid ester. *Tetrahedron, 56*, 7805–7810.

Ley, J. P., & Bertram, H. J. (2003). Synthesis of lipophilic clovamide derivatives and their antioxidant potential against lipid peroxidation. *Journal of Agricultural and Food Chemistry, 51*, 4596–4602.

Lucarini, M., & Pedulli, G. F. (1994). Bond dissociation enthalpy of α-tocopherol and other phenolic antioxidants. *Journal of Organic Chemistry, 59*, 5063–5070.

Owen, R. W., Giacosa, A., Hull, W. E., Haubner, R., Spiegel-halder, B., & Bartsch, H. (2000). The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. *European Journal of Cancer, 36*, 1235–1247.

Parthasarathy, S., Steinberg, D., & Witztum, J. L. (1992). The role of oxidized low-density lipoproteins in the pathogenesis of atherosclerosis. *Annual Review of Medicine, 43*, 219–225.

Petroni, A., Blasevich, M., Salami, M., Papini, M., Montedoro, G. F., & Galli, C. (1995). Inhibition of platelet aggregation and eicosanoid production by phenolic component of olive oil. *Thrombosis Research, 78*, 151–160.

Porter, W. L. (1993). Paradoxical behavior of antioxidants in food and biological systems. *Toxicology & Industrial Health, 9*, 93–122.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. A. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine, 26*(9–10), 1231–1237.

Regnström, J., Nilsson, J., Tornvall, P., Landou, C., & Hamsten, A. (1992). Susceptibility to low-density lipoprotein oxidation and coronary atherosclerosis in man. *Lancet, 339*, 1183–1186.

Rice-Evans, C. A., & Diplock, A. T. (1993). Current status of antioxidant therapy. *Free Radical Biology and Medicine, 15*, 77–96.

Rice-Evans, A. C., Miller, N. J., & Paganga, G. (1996). Structure–antioxidant activity relationship of flavonoids and phenolic acids. *Free Radical Biology and Medicine, 20*, 933–956.

Shahidi, F., & Wanasundara, P. K. J. P. D. (1992). Phenolic antioxidants. *Critical Reviews in Food Science and Nutrition, 32*, 67–103.

Torres de Pinedo, A., Peñalver, P., Rondón, D., & Morales, J. C. (2005). Efficient lipase-catalyzed synthesis of new lipid antioxidants based on a catechol structure. *Tetrahedron, 61*, 7654–7660.

Torres de Pinedo, A., Peñalver, P., & Morales, J. C. (2007). Synthesis and evaluation of new phenolic-based antioxidants: Structure–activity relationship. *Food Chemistry, 103*, 55–61.

Trujillo, M., Mateos, R., Collantes de Teran, L., Espartero, J. L., Cert, R., Jover, M., et al. (2006). Lipophilic hydroxytyrosyl esters. Antioxidant activity in lipid matrices and biological systems. *Journal of Agricultural and Food Chemistry, 54*, 3779–3785.

Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M., & Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biological Interactions, 160*, 1–40.

Visioli, F., Bellomo, G., Montedoro, G., & Galli, C. (1995). Low-density lipoprotein oxidation is inhibited in vitro by olive oil constituents. *Atherosclerosis, 117*, 25–32.

Zafrilla, P., Mulero, J., Xandri, J. M., Santo, E., Caravaca, G., & Morillas, J. M. (2006). Oxidative stress in Alzheimer patients in different stages of the disease. *Current Medical Chemistry, 13*, 1075–1083.