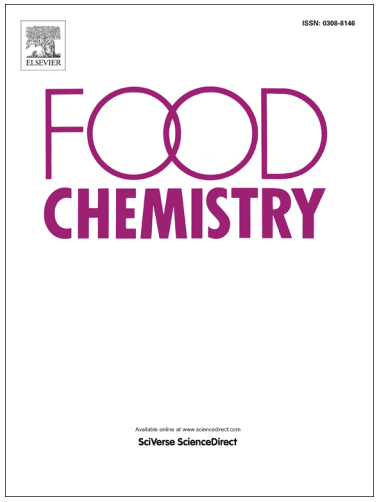

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Antioxidant activity of protocatechuates evaluated by DPPH, ORAC, and CAT methods

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ABSTRACT

Hibiscus sabdariffa L. is a worldwide consumed plant, principally after infusion of its dried sepals and calyces, which are usually discarded. Nevertheless, they represent a potential source of natural bioactive compounds, e.g. polyphenols, which could add value to this under-exploited plant. Protocatechuic acid (PA) was chosen as a model of the phenolic acids that can be extracted from *H. sabdariffa*. In order to modify PA hydrophilic character, which limits its use in lipid-rich food products, PA was esterified to C_1-C_{18} alcohols, and the impact of lipophilization on its antioxidant activity was evaluated in both, an homogeneous (DPPH and ORAC methods) and an heterogeneous (CAT method) system. Results herein obtained showed that, depending on the grafted alkyl chain length, lipophilization could positively affect the antioxidant activity of PA in heterogeneous media; therefore, support its use as an innovative way to synthesize molecules with an improved antioxidant capacity and potential to be used as multifunctional preservatives in food.

HIGHLIGHTS

- Protocatechuic acid (PA) was successfully lipophilized using alcohols from C_1-C_{18}.
- Lipophilization could improve PA antioxidant capacity in heterogeneous media.
- Antioxidant activity is related to compound polarity and method of evaluation.
- Protocatechuates could be considered as potential preservatives in food.
- An innovative way to add-value to phenolic-rich vegetal extracts is proposed.

KEYWORDS: antioxidant, *Hibiscus sabdariffa* L, protocatechuic acid, protocatechuates, ORAC, DPPH, CAT, lipophilization, bioactive compounds, food additive.

1. Introduction

The incorporation of antioxidants, which are molecules capable of preventing and/or delaying the oxidative lipid damage when used in proper conditions, represents a key alternative to overcome the quality deterioration of lipid-based foods products, provoked mainly by the attack of the reactive oxygen species (ROS) (Laguerre et al., 2015). Depending on different factors, such as the physico-chemical characteristics of the media where they are located and their interaction with other compounds, the antioxidants can act as retarders, when they counteract lipid oxidation by protecting target lipids from oxidation initiators; or by hindering the propagation phase, the so-called “chain-breaking” antioxidants. From a kinetic standpoint, chain-breaking antioxidants induce a lag phase where no considerable oxidation occurs, contrary to the retarders, where no distinct lag phase is observed (Laguerre, Lecomte, & Villeneuve, 2007). Between the number of molecules considered as antioxidants, phenolic compounds are particularly important because of their high redox potentials, and also, because they are the most abundant antioxidants found in the diet (Scalbert, Johnson, & Saltmarsh, 2005; Tsao & Deng, 2004).

Protocatechuic acid (3, 4-dihydroxybenzoic acid; PA) has been mainly recognized as a potent antioxidant. Moreover, it posses antibacterial, anticancer, anti-inflammatory, and several others activities (Chao & Yin, 2009; Kakkar & Bais, 2014; Soares et al., 2014; Stojkovic et al., 2013; Yan et al., 2004). PA has been isolated from the dried flowers of *Hibiscus sabdariffa* L., a plant used since ancient times in herbal medicine for its biological properties (Olvera-García et al., 2008; Patel, 2014; Tanaka, Tanaka, & Tanaka, 2011; Tseng et al., 2000), and also, to produce non-alcoholic beverages, jellies, confectionaries, and other food products. Though

hibiscus flower is a powerhouse of phytochemicals, it still remains as an under-exploited plant (Patel, 2014).

Kakkar and Bais (2014) determined that, due to low absorption by oral route, PA is a nontoxic and a relatively safe compound for oral administration. Nevertheless, the incorporation of polyphenols, such as PA, in lipid-rich matrices is complex due to its general low solubility in lipidic media. To counteract this problem, lipophilization of phenolic compounds, by esterification with an acyl or an alkyl donor, has been recently used as a strategy to ameliorate their performance in heterogeneous media, such as food products (Lecomte, Giraldo, Laguerre, Baréa, & Villeneuve, 2010; Sørensen et al., 2014; Trujillo et al., 2006). According to these authors, when grafting a certain carbon chain length, a threshold is reached, called the cut-off effect, after which, a drastic decrease in antioxidant capacity was observed (Laguerre et al., 2009; Laguerre et al., 2010). Esterification of PA with some acyl and alkyl donors has been done and the biological properties of the obtained esters were evaluated. The findings showed that, regardless of carbon chain length, they exhibited fungicidal, antioxidant and antiradical activity (Ha, Shimizu, & Kubo, 2014; Nihei, Nihei, & Kubo, 2003; Saito, Okamoto, & Kawabata, 2004).

In the present work, chemical synthesis of a complete series of PA esters (from C_1 to C_{18}) was successfully made. Newly synthesized protocatechuates were assessed for antioxidant activity in three test systems: two without lipid-water interface (DPPH/alcoholic solution, ORAC/aqueous solution) and another one with such an interface (CAT/oil-in-water emulsion). The ORAC and CAT assays differ from each other on their oxidizable substrate: fluorescein in the ORAC assay and triacylglycerols from stripped tung oil (plus Brij 35 as surfactant) in the CAT assay. Not only did these assays allow us to evaluate the effect of the esterification on the

antioxidant activity of the native molecule, but also demonstrated how the test media was a determinant factor in the antioxidant capacity showed by this kind of molecules. The existence of a cut-off effect within the homologous series of the synthesized protocatechuates is also discussed.

2. Materials and methods

2.1. Materials

Protocatechuic acid (3,4-dihydroxybenzoic acid, PA), protocatechuic acid ethyl ester (ethyl 3,4-dihydroxybenzoate), sulfuric acid, fluorescein sodium salt (used as fluorescent tracer), Brij 35 (neutral emulsifier), silica gel (high-purity grade, pore size 60 Å, 70-230 mesh), aluminum oxide (alumina, type CG-20), sodium carbonate (pure dry), molecular sieves (3 Å, beads, 4-8 mesh), alcohols (used for the synthesis: methanol, butanol, hexanol, octanol, decanol, dodecanol, tetradecanol, hexadecanol, octadecanol), and solvents of HPLC or analytical grade (water, acetonitrile, hexane, ethyl acetate, tetrahydrofuran (THF), formic acid, acetic acid) were purchased from Sigma-Aldrich (Saint Quentin, France). Phosphate buffer (PB) solution (pH 7.2) was purchased from Fluka (Saint Quentin, France). Tung oil (China wood oil), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Aldrich (Saint Quentin, France). Trolox was from ACROS Organics™ (Illkirch, France).

2.2. Chemical synthesis and purification of protocatechuates

2.2.1. Chemical synthesis

PA esters were synthesized as described by Reis et al. (2010). Modifications to the general procedure were made to allow the use of fatty alcohols with a longer

carbon chain and facilitate the purification step. Briefly, 1 g (6.49 mmol) of protocatechuic acid was solubilized in 3 mL of THF. Then, 3 molar equivalents (19.47 mmol) of the corresponding alcohol (from methanol to octadecanol, C_1 to C_{18}, respectively) were added. Sulfuric acid was used as catalyst (4 %, v/v of the final reaction mixture volume) and the reaction mixtures were incubated in an orbital shaker (250 rpm) at temperatures varying from 45-65 °C, depending on the alcohol used, for approximately 5 to 10 days, protected from light. To remove water generated during the reaction, molecular sieves were added to the medium (40 mg/mL of final volume). At different time intervals, samples were withdrawn from the reaction mixture, and then analyzed by reversed-phase HPLC (Section 2.3). The reaction was stopped by the addition of 2 mL of sodium carbonate (1 M). The mixture was dissolved in approximately 100 mL of acetonitrile and filtrated (150 mm standard pleated filter, Grosseron, Saint-Herblain, France). The resulting solution was stocked at 4 °C until purification. In the case of the esters with an alkyl chain length from 14 to 18 carbons (solids at room temperature), it was necessary to wash the filter using heated acetonitrile.

2.2.2. Flash chromatography

Protocatechuates were purified using a normal phase column (RediSep® Rf, normal-phase silica Flash column 40 g, Teledyne Isco, Nebraska, USA) in a CombiFlash® Companion® system (Teledyne Isco, Nebraska, USA). Compounds, previously solubilized in acetonitrile, were adsorbed in silica gel to form a pre-column. The mobile phase consisted of A: chloroform, and B: ethyl acetate, with a gradient starting at 0 % B from 0 to 15 min, and to 100 % B, from 15 to 45 min, at a flow rate

of 30 mL/min. Compounds were detected and collected at 260 nm. Fractions were analyzed by thin layer chromatography (TLC).

2.2.3. TLC analysis

Collected samples from flash chromatography were manually applied on silica gel 60 F_{254} plates (5 x 10 cm, Merck Millipore, Darmstadt, Germany). Development was carried out with the upper phase of hexane/ethyl acetate/formic acid (70:30:1, v/v/v). The spots were then visualized after spraying plates with a solution of ferric chloride (0.5 mg/mL) in sulfuric acid/acetic acid/water (5:5:90, v/v/v)) followed by heating at 150 °C for 10 min. Samples without detected residual alcohol were pooled, evaporated under reduced pressure at 37 °C, and put in a vacuum drying oven at room temperature to eliminate traces of solvent. R_f values (from C_4 to C_{18} respectively): 0.39, 0.4, 0.42, 0.45, 0.48, 0.5, 0.5, 0.53.

2.3. HPLC analysis

HPLC analysis were carried out on a Shimadzu LC-20AD equipped with a DAD SPO-M20A and a column oven CTO-10AS_{VP} (Shimadzu, Noisiel, France), using a Kinetex 5 µm C18 column (100 Á, 4.6 x 250 mm; Phenomenex, Le Pecq, France). The mobile phase was A: acetic acid (0.1 %, v/v), and B: methanol (0.1 %, v/v acetic acid). The following gradient was applied: 0–3 min, isocratic at 100 % A; 3–10 min, linear gradient to 100 % B; 10–20 min isocratic at 100 % B; 20-22 min, linear gradient to 100 % A; 22–25 min, equilibration at 100 % A. Compounds showed maximal absorbance at 260 nm. The purified products were also analyzed by LC-MS, using the same solvent system that for the HPLC analysis.

153 *2.4. NMR analysis*

154 Structure of the esterified compounds obtained was confirmed by NMR (^{1}H NMR,
155 500 MHz, MeOD).

156

157 *Data for metyl protocatechuate:* Yield: 88 %; purity: 100 %. ^{1}H NMR (500 MHz,
158 MeOD) δ = 7.51-7.30 (m, 2H), 6.81-6.79 (m, 1H), 3.83 (s, 3H). LC-MS *m/z*: 166.9 [M-
159 H]$^-$.

160 *Data for butyl protocatechuate:* Yield: 82 %; purity: 94.3 %. ^{1}H NMR (500 MHz,
161 MeOD) δ = 7.42-7.39 (m, 2H), 6.79 (dd, *J*=8.1, 0.4, 1H), 4.24 (t, *J*=6.5, 2H), 1.79-1.67
162 (m, 2H), 1.55-1.41 (m, 2H), 0.98 (t, *J*=7.4, 3H). LC-MS *m/z*: 208.9 [M-H]$^-$.

163 *Data for hexyl protocatechuate:* Yield: 82 %; purity: 100 %. δ = 7.49-7.32 (m, 2H),
164 6.79 (dd, *J*=8.1, 0.4, 1H), 4.23 (t, *J*=6.6, 2H), 1.76-1.70 (m, 2H), 1.46-1.42 (m, 2H),
165 1.40-1.29 (m, 4H), 1.02-0.78 (m, 3H). LC-MS *m/z*: 236.9 [M-H]$^-$.

166 *Data for octyl protocatechuate:* Yield: 85 %; purity: 99.4 %. ^{1}H NMR (500 MHz,
167 MeOD) δ = 7.49-7.32 (m, 2H), 6.79 (dd, *J*=8.1, 0.4, 1H), 4.23 (t, *J*=6.6, 2H), 1.76-1.70
168 (m, 2H), 1.54-1.19 (m, 10H), 0.89 (t, *J*=7.0, 3H). LC-MS *m/z*: 265 [M-H]$^-$.

169 *Data for decyl protocatechuate:* Yield: 89 %; purity: 99.9 %. ^{1}H NMR (500 MHz,
170 MeOD). δ = 7.43-7.40 (m, 2H), 6.80 (d, *J*=8.3, 1H), 4.23 (t, *J*=6.6, 2H), 1.82-1.65 (m,
171 2H), 1.54-1.20 (m, 14H), 0.89 (t, *J*=7.0, 3H). LC-MS *m/z*: 293.1 [M-H]$^-$.

172 *Data for dodecyl protocatechuate:* Yield: 94 %; purity: 98.5%. ^{1}H NMR (500 MHz,
173 MeOD) δ = 7.43-7.40 (m, 2H), 6.80 (d, *J*=8.1, 1H), 4.23 (t, *J*=6.6, 2H), 1.82-1.65 (m,
174 2H), 1.53-1.17 (m, 18H), 0.89 (t, *J*=7.0, 3H). LC-MS *m/z*: 321.1 [M-H]$^-$.

175 *Data for tetradecyl protocatechuate:* Yield: 95 %; purity: 96.6 %. ^{1}H NMR (500 MHz,
176 MeOD) δ = 7.43-7.40 (m, 2H), 6.80 (d, *J*=8.2, 1H), 4.23 (t, *J*=6.6, 2H), 1.75-1.71 (m,
177 2H), 1.49-1.17 (m, 22H), 0.91-0.88 (m, 3H). LC-MS *m/z*: 349.1 [M-H]$^-$.

178 *Data for hexadecyl protocatechuate:* Yield: 59 %; purity: 84.6 %. ^{1}H NMR (500 MHz,
179 MeOD) δ = 7.58-7.38 (m, 2H), 6.89 (d, *J*=8.3, 1H), 4.22 (t, *J*=6.6, 2H), 1.82-1.67 (m,
180 2H), 1.59-1.13 (m, 26H), 0.87 (t, *J*=6.8, 4H). LC-MS *m/z*: 377.2 $[M-H]^-$.
181 *Data for octadecyl protocatechuate:* Yield: 65 %; purity: 96.1 %. ^{1}H NMR (500 MHz,
182 Acetone) δ = 7.61-7.32 (m, 2H), 6.89 (dd, *J*=8.3, 0.9, 1H), 4.22 (t, *J*=6.6, 2H), 1.82-
183 1.58 (m, 2H), 1.55-1.12 (m, 30H), 0.87 (t, *J*=6.5, 3H). LC-MS *m/z*: 405.3 $[M-H]^-$.
184
185 *2.5. Gas chromatography analysis (GC)*
186 Methanolic solutions (10 mM) of the synthesized compounds were analyzed by
187 GC using FOCUS GC apparatus (Thermo Scientific, France) equipped with a flame
188 ionisation detector (FID) and a SPBtm-1 capillary GC column (L x I.D. 30m x 0.32
189 mm; d_f 0.25 µm) (Supelco-Sigma-Aldrich, France). Carrier gas was helium with a flow
190 rate of 1.5 mL/min, and a split ratio of 1/15. The temperature of the injector was 280
191 °C, and that for flame ionization detection was 310 °C. Oven temperature settings
192 were as follows: 60 to 310 °C at 10 °C/min, and hold at 310 °C for 10 min. Chrom-
193 Card software was used for data handling.
194
195 *2.6. Anti-radical and antioxidant activity measurements*
196 *2.6.1. DPPH assay*
197 The anti-radical activity of the protocatechuic acid and its esters was determined
198 by the DPPH method described by Brand-Williams, Cuvelier, and Berset (1995),
199 adapted to microplate assay. Twenty microliters of each sample dilution in methanol
200 (0-30 µM final concentration) and 180 µL of a DPPH methanolic solution (150 µM
201 final concentration) were added to a 96-well microplate (UV-star, flat-bottom, chimney
202 well, µclear, Greiner Bio-One, Frickenhausen, Germany). Absorbance was

immediately read at 515 nm, and every minute during the first 15 min, then at 30, 45, 90, and 120 min, when no lecture variation was detected. Trolox was used as internal control, and for the blank, sample was substituted by methanol. For each antioxidant concentration tested, the loss on the absorbance was measured and the net absorbance was obtained from:

$$Abs_{net} = Abs_{Blank} - Abs_{spl} \quad (1)$$

Where Abs_{Blank} corresponds to the absorbance of the blank and Abs_{spl}, to the absorbance of the sample, both obtained at 120 min of reaction. For each compound, four concentrations were evaluated and the values of the Abs_{net} were plotted to obtain a linear equation from which the anti-radical activity was calculated, as follows:

$$ARA_{value} = (Sample_{slope} / Trolox_{slope}) \times (\text{moles of Trolox/ moles of sample}) \quad (2)$$

Being ARA_{value} the anti-radical activity value of each synthesized antioxidant. A good linear relationship ($R^2 > 0.99$) is needed between Abs_{net} and the antioxidant concentration for all the compounds tested, to make an adequate calculation of the anti-radical activity. The results were expressed as Trolox equivalents (TE) (moles of Trolox/ moles of sample).

2.6.2. Oxygen radical absorbance capacity assay (ORAC assay)

The peroxyl radical scavenging activity of phenolic antioxidants was determined using the method adapted from Ou, Hampsch-Woodill, and Prior (2001). Dilutions (from 0 to 2 µM) of each compound previously solubilized in methanol were prepared

in PB (pH 7.2). It is important to mention that when methanol replaced PB as solvent for dilutions, fluorescence bleaching of fluorescein did not occur through the reaction time, resulting in a no-decay kinetic curve (data not shown). This behavior suggest an influence from the methanol on the absorption and emission spectra of fluorescein (Biswas, Bhattacharya, Sen, & Moulik, 1999; Martin & Lindqvist, 1975). Briefly, 50 µL of sample and 100 µL of a 0.126 µM fluorescein-PB (FL) solution were transferred to a 96-well black microplate (PS, flat- bottom, chimney well, fluotrac 200, black, Greiner Bio-One, Frickenhausen, Germany). After the incubation time (20 min/ 37 ℃/ 600 rpm, light protected), 50 µL of a freshly prepared AAPH-PB solution (32 mM) were added and fluorescence was immediately read at 515 nm (λ_{ex}: 490 nm). The loss of fluorescence was followed every minute for 2 h at 37 ± 0.1 ℃, with 5 s stirring before each measurement. The final mixture (200 µL) in the microplate consisted of 0.063 µM of FL, 8 mM of AAPH, and 4 concentrations of the sample (0-2 µM). Trolox was used as the internal control. Blank was prepared using methanol instead of the sample. The area under the curve (AUC) was calculated as:

$$AUC = 1 + f_1/f_0 + f_2/f_0 + f_3/f_0 + f_4/f_0 + \ldots + f_{120}/f_0 \quad (3)$$

Where f_0 is the initial fluorescence read at 0 min and f_i is the fluorescence read at time *i*. The net AUC was obtained by subtracting the AUC of the blank from that of each sample. ORAC values were obtained from the net AUC and expressed as TE:

$$\text{ORAC value} = (\text{net AUC}_{spl} / \text{net AUC}_{Trolox}) / (\text{moles of Trolox / moles of sample}) \quad (4)$$

2.6.3. Conjugated autoxidizable triene (CAT) assay

Antioxidant capacity of the synthesized compounds in an microemulsified media was measured by the CAT assay developed by Laguerre et al. (2008), with the improvements to assess both hydrophilic and hydrophobic compounds (Laguerre et al., 2010). This method is based on the oxidation of the triacylglycerols (TAG) from the tung oil (previously stripped of tocopherols), which, due to their high content in octadecatrienoic acid with a conjugated triene, are very sensible to oxidation and exhibit strong absorption in the UV domain at 273 nm; making possible the spectrophotometric measurement. An Ultra Turrax homogenizer (Janke &Kunkel, Staufen, Germany) and an Infinite M1000 PRO microplate reader (Tecan, Gröedig, Austria) equipped with Magellan software, were used. According to the authors, it is possible to make spectrophotometric measurements in this emulsion if we consider it to be likely a microemulsion, whose droplet diameter is small enough to avoid light scattering (Laguerre et al., 2010). Results were expressed in TE, in the same way as in Eq (4), corresponding to the CAT value of each compound.

2.7. Statistical analysis

Results are expressed as means ± standard deviation (SD) of three measurements for the DPPH, ORAC, and CAT assays. The data were subjected to a one-way analysis of variance (ANOVA) using the program JMP software v.8 (SAS Institute, USA). The level of significance was set at $p < 0.05$.

3. Results and discussions

3.1. Chemical synthesis and characterization of protocatechuic acid esters

277 PA was chemically esterified with a series of alcohols (from C_1 to C_{18}), with a
278 moderate to good molar yield (58.6- 94.5 %). The structure (Fig. 1) and the purity of
279 the products were confirmed by NMR, LC-MS and GC. The commercially available
280 ethyl protocatechuate was also used in this study. Molar yields obtained were similar
281 to those previously reported for the PA chemically esterified with shorter alkyl chains
282 (Reis et al., 2010); and also, with those reported for other phenolics enzymatically
283 esterified, such as hydroxytyrosyl esters (Trujillo et al., 2006), and rosmarinic acid
284 esters (Lecomte et al., 2010). It is worthy to mention that the enzymatic esterification
285 of the PA with short chain alcohols using lipase from *Candida antarctica*
286 (Novozymes® CALB EC 3.1.1.3) was also assayed (data not shown), detecting only,
287 or no traces, of the corresponding esters after more than twelve days of reaction.
288 Accordingly, some authors have reported a partial, or even total, enzyme inhibition
289 when the acid function (directly bound to the aromatic ring or via double bound) is
290 conjugated with a phenolic hydroxyl in *para* position, with regard to the side chain
291 bearing the acid, such as in caffeic or in PA (Figueroa-Espinoza, Laguerre,
292 Villeneuve, & Lecomte, 2013; Guyot, Bosquette, Pina, & Graille, 1997; Lecomte et
293 al., 2010; Stamatis, Sereti, & Kolisis, 1999).
294
295 *3.2. Antioxidant evaluation of protocatechuates*
296 In Fig. 2 a comparison between the three different methods is attempted to be
297 made. Such a comparison serves just to give a global idea of the behavior of the new
298 synthesized compounds with respect to their antioxidant activity. From this figure, we
299 can clearly observe that the polarity of the system, but also the length of the esterified
300 chain, have a strong influence on the antioxidant capacity of the synthesized
301 compounds. In DPPH test, protocatechuates were more active than their parent

molecule. In contrast, for the ORAC assay, a markedly diminution of the protocatechuates antioxidant activity was observed as the esterified alkyl chain length increased. A non-linear behavior was shown in the CAT method. Lipophilization of PA with fatty alcohols from short to medium chain lengths (C_1 to C_6) had a positive impact in the behavior of this molecule in an oil-in-water emulsion, which is of great importance having in mind that, from food products to the systems found in cells, we found mostly heterogeneous media. Other parameters of these phenolipids, such as the partition coefficient, are being evaluated.

3.2.1. DPPH assay

The main mechanism of action of phenolic antioxidants is free radical scavenging (Reis et al., 2010). To evaluate this activity, DPPH assay constitutes a widespread and easy-to-use protocol, even if it does not involve an oxidizable substrate (Lopez-Giraldo et al., 2009). Results from the measurement of the scavenging activity of PA and its esters are presented in Fig. 2. Protocatechuates exhibited better antiradical activity than their parent compound. Among them, a slightly but significant difference was observed when increasing from short/medium (C_2-C_8) to longer carbon chains (C_{10}-C_{18}), suggesting that lipophilization positively affected antiradical activity of these compounds, probably due to the increment of their solubility in organic media, as the chain length increased. Accordingly, it was previously reported that PA had a lower DPPH radical scavenging activity than its derivatives, being the protocatechuic propyl ester the most effective of the synthesized compounds (Reis et al., 2010). Nevertheless, chlorogenates and rosmarinates did not show this tendency, being the C_4 and C_8-chlorogenates, and the C_{12}-rosmarinate, the only compounds with better DPPH scavenging activity compared to the unesterified one (Lecomte et al., 2010;

Lopez-Giraldo et al., 2009). Therefore, it has been suggested that the slow radical-scavenging reaction of PA compared to its esters is due to the dissociation of the carboxylic acid function to an electron-donating carboxylate ion, which decreases the electron-withdrawing property of the substituent, and thus decreases the susceptibility of the first formed *O*-quinone towards nucleophilic attack by an alcoholic solvent. Consequently, the ring would be richer in electrons for the acid than for its esters, decreasing the electrophilicity of the carbon (Saito & Kawabata, 2006). Subsequent dimerization was also proposed to explain the increased radical scavenging of protocatechuates in alcohol solvent. Furthermore, a reactivity contribution from the products formed throughout the oxidation reaction was suggested, remarking the importance of adequate assay duration (Lopez-Giraldo et al., 2009; Saito et al., 2004).

3.2.2. ORAC assay

ORAC values obtained for the PA and its derivatives are shown in Fig. 2. A higher protective capacity was observed for PA than for its derivatives, also predicted from the behavior in the fluorescence decay curves, where the esters lost fluorescence approximately from the beginning of the reaction (Fig. 3 and Fig. 4). Contrary to the behavior observed in the DPPH assay, the grafting of an alkyl chain to the protocatechuic acid, negatively affected the antioxidant capacity of the molecules. Therefore, in this case, the decrease in the antioxidant activity can be explained by the decrease of the solubility of the lipophilized compounds in water. Nevertheless, protocatechuates from C_1 to C_8 were more effective than Trolox (ORAC value from 1.6 to 1 TE, respectively). These results are in agreement with others previously reported, e. g. the *p*-hydroxyphenylacetic acid (HPA) and its conjugates, where HPA

was better than its dodecyl and butyl ester (Yuji et al., 2007); in the case of nitrohydroxytyrosyl esters, side chains with 6 or more carbon atoms induced a negative effect on the antioxidant activity (Trujillo et al., 2014). The possible formation of micelles due to the high hydrophobicity of the synthesized compounds, that decreases the accessibility of the hydroxyl group to the free radicals, is also proposed (Yuji et al., 2007).

3.2.3. CAT assay

The antioxidant capacity of a homologous series of PA and its alkyl esters was evaluated by the CAT improved protocol (Laguerre et al., 2010). In contrast to the assays previously discussed, the CAT assay is carried out in a heterogeneous media (emulsified lipid system), as this is closer to natural compartmentalized conditions. Results obtained from the oxidation curves, expressed as TE, are shown in Fig. 2. All the synthesized compounds were capable to delay AAPH-induced oxidation of stripped tung oil, in a concentration depending manner (Fig. 5). In general, protocatechuates with short to medium chain's length (from C_1 to C_6) acted as better antioxidants than those with the longest chains.

In Fig. 5 it can be observed that esters possessing higher CAT values, e.g. C_1 and C_6-protocatechuates, showed an important difference in the pseudolag phase, which also depends on the ester concentration, compared to the curves from esters with the poorest antioxidant capacity, e.g. C_{14} and C_{18}-protocatechuates, where the pseudolag phase was practically absent. The existence of a pseudolag phase is characteristic of chain-breaker antioxidants, which could interrupt the free radical chain reaction. In other words, they rather scavenge the propagator lipoperoxyl radicals derived from stripped tung oil ($LOO^{\bullet}$), than stabilize the initiator peroxyl

radicals derived from AAPH ($ROO^{\bullet}$) (Laguerre et al., 2008). This would mean that chain-breaker protocatechuates should be near or at the interface oil-water to reduce $LOO^{\bullet}$, see their mobility and solubility, they could easily approach to it. Nevertheless, as they are well solubilized in the aqueous phase, it can be considered that short alkyl chain protocatechuates also reduce radicals derived from AAPH, being C_2 and C_4-protocatechuates the ones that are better distributed into all the emulsion parts (Fig. 6). In contrast, even when PA showed better antioxidant activity than its derivatives with medium to long alkyl chains (C_8 to C_{18}), a pseudolag phase in its decay kinetic curve was not observed, meaning that it is acting mostly as a retarder, scavenging AAPH-derived peroxyl radicals, rather than a pure chain-breaking antioxidant. This behavior is associated to the relative hydrophilic nature of PA, therefore, its predominant tendency to move away from the oil-water interface, where lipid oxidation reactions would be greater (Decker, Warner, Richards, & Shahidi, 2005; Laguerre et al., 2007). This suggests that lipophilization, since it affects the polarity of the PA, could change its localization within the system and the way it interacts or reacts with its chemical environment. Nevertheless, this polarity modulation from lipophilization, can be only visible in heterogeneous media, such as CAT assay, and not in the homogeneous one (ORAC, DPPH).

Therefore, the stronger or weaker antioxidant capacity of the esters may be seen as a result of their localization toward the oxidizable substrate. As represented in Fig. 6, we can suppose that the short-chain alkyl esters (from 1 to 6 carbons) are distributed in the aqueous phase near to the oil-water interface, or even inserted in the interfacial layer of the oil droplet, where the oxidation process occurs, explaining its higher CAT value. Thus, a cut-off effect could be suggested at the hexyl protocatechuate, from which an important loss of activity is observed (Fig. 2).

When comparing our results, where the most efficient alkyl esters were the C_2 and C_4-protocatechuates (benzoic acid derivatives) (Fig. 2), to those from similar alkyl chain length series of esters, such as caffeates, ferulates and coumarates (Sørensen et al., 2014), or chlorogenates and rosmarinates (Laguerre et al., 2009; Laguerre et al., 2010) (cinnamic acid derivatives), which presented a bell shaped curve chain length, being the medium/long chain esters (from C_8 to C_{12} depending on the phenolic compound) the most efficient, it is likely that the partition in the emulsion also depends on the phenolic structure itself. Based on this, one cannot generalize on the behavior of the phenolic esters even when they are tested in the same heterogeneous media. Thus, to find the optimum chain length to have the higher CAT value, each series of phenolic esters have to be evaluated, since the behavior of a given antioxidant in an emulsion is under multifactorial control.

As for example, Sørensen et al. (2014) reported that in general, the caffeates were better antioxidants than the ferulates and coumarates tested in the CAT assay. PA possesses two hydroxyl groups attached to the aromatic ring as the caffeic acid, but it has not the propenoic chain that contributes to the resonance stabilization and facilitates the homolytic dissociation of the O–H bond in caffeic acid, making it a better antioxidant than PA. Besides, PA possesses an electron withdrawing COOH group directly bonded to the aromatic ring at *para* position, which raises the O–H bond dissociation enthalpy (BDE) (the higher the BDE, the lower the antioxidant activity) (Laguerre et al., 2011). This could also contribute to explain the higher antioxidant CAT value of caffeates than that of protocatechuates.

The antioxidant activity of amphiphilic molecules implicates the mobility and partition between different phases in an emulsion. Thus, alkyl protocatechuates would partitionate between the aqueous phase, the interface and the oil phase

427 according to their saturated alkyl chain length and depending on the emulsion
428 composition as well. Their antioxidant polar groups scavenge AAPH radicals and/or
429 lipid hydroperoxyl radicals according to their location in the system and to their
430 concentration. In function of the alkyl length chain, and their hydrophilic/lipophilic
431 balance, some protocatechuates could also contribute to physically stabilize the
432 emulsion in the CAT assay. Moreover, since tung oil-emulsion is considered as a
433 water-in-oil microemulsion (Laguerre et al., 2010), it can be supposed that long chain
434 protocatechuates form micelles in the oil droplet, by directing their polar head to a
435 water droplet; also, they could be distributed into the oil droplet (Fig. 6)

436 According to our results, it is likely that protocatechuates present critical chain
437 length (CCL) at C_2 and C_4. Nevertheless, this result has to be considered cautiously,
438 as it is possible that antioxidant activity is overestimated for the short length
439 protocatechuates. When analyzing the antioxidant kinetics in CAT (Fig. 5), it can be
440 observed that from C_1 to C_6-protocatechuates, there is a pseudo lag phase, which
441 disappears when increasing the alkyl chain length. If all esters presented the same
442 kinetics (chain-breaker or retarder), a direct comparison of CAT value calculated from
443 the AUC would present no problem, as it was made for chlorogenates (Laguerre et
444 al., 2009) or rosmarinates (Laguerre et al., 2010). In the case of protocatechuates,
445 when the pseudolag phase is present, it increases the AUC, then, overestimating the
446 overall antioxidant activity, by comparison with those protocatechuates which have
447 no pseudolag phase. Apparently, it is the first time that such an antioxidant behavior
448 in the CAT assay with an analogous series of alkyl esters is observed. Thus, it would
449 be better to compare molecules with the same antioxidant kinetics profile, what
450 means that the best chain length for the chain-breaking protocatechuates would be
451 either 2 or 4 carbons, for its good solubility and mobility in the media, and the dodecyl

ester for the retarder protocatechuates, due to its better migration into the interface where the phenolipid would be at the highest concentration.

C_1 to C_6-protocatechuates could act as chain-breakers in the CAT assay and were the most efficient in the ORAC assay. They would scavenge the peroxyl radical induced by AAPH or the lipoperoxyl radical derived from the stripped tung oil in the ORAC or CAT assay, respectively. It is interesting to note that in both assays, ORAC and CAT, in the presence of octyl protocatechuates the activity fell down. It is likely that for PA esters, from C_8 alkyl chain length, solubility in the water phase decreases and probably they aggregate into micelles, strongly affecting their antioxidant capacity, as it has been already discussed. C_{10} to C_{18}-protocatechuates presented low antioxidant activity in the ORAC assay and no lag phase in their CAT kinetic curves, meaning that they would act as retarders by stabilizing the initiator peroxyl radicals derived from AAPH in the CAT assay.

Antiradical activity of protocatechuates in homogeneous media was related to the antioxidant solubility, as demonstrated by the DPPH and ORAC values, which were inversely correlated. The partition of protocatechuates in the lipid-water interface on the CAT assay in function of their chain length is also a factor of importance in the antioxidant activity in a non homogeneous system.

4. Conclusion

The present work showed, in one hand, the importance of combining different methods to assess antioxidant capacity to better determine the properties and mechanism of action of compounds that can be used as antioxidants in food products. On the other hand, here we provide novel data supporting that lipophilizing PA with the correct alkyl chain length can be a strategy to improve its antioxidant

activity, by suggesting that the protocatechuates obtained could have potential as natural preservatives in food. Finally, this research shows the potential use of the tropical vegetal by-products, as those from *H. sabdariffa* L., issued i.e. from the beverage industry, for the production of high added value extracts rich in phenolic compounds that could be modified by lipophilization to obtain amphipilic molecules with improved antioxidant capacity.

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FIGURES LIST

Fig. 1. Acid catalyzed esterification of protocatechuic acid (PA) with alcohols of various chain lengths.

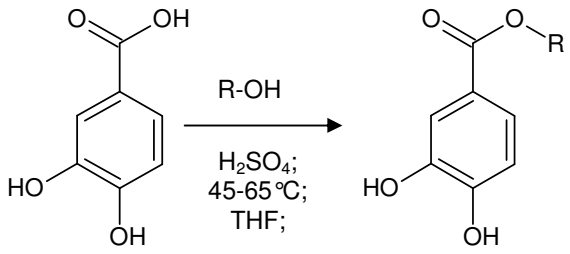
Fig. 2. Antioxidant capacities of PA and protocatechuates expressed as Trolox equivalents, measured by different methods.

Fig. 3. Fluorescence decay curves induced by AAPH in the presence of A) different concentrations of protocatechuic acid (PA): (—) 0 µM, (■) 0.5 µM, (▲) 1.0 µM, (◆) 1.5 µM, (○) 2.0 µM.

Fig. 4. Fluorescence decay curves induced by AAPH in the presence of PA and its alkyl protocatechuates (PA-C1 to PA-C18) at 1 µM.

Fig. 5. Kinetics of stripped tung oil oxidation in the presence of protocatechuic acid and its esters at A) 0.25 µM and B) 1 µM. Proposed behavior of the antioxidants with regard to the presence (chain-breaking) or absence (retarder) of the *pseudolag* phase.

Fig. 6. Proposed scheme of the distribution of protocatechuic acid and alkyl protocatechuates in an oil-in-water microemulsion in the CAT assay.



R= $-CH_3$	Methyl protocatechuate = PA-C_1
R= $-CH_2-CH_3$	Ethyl protocatechuate= PA-C_2
R= $-(CH_2)_3-CH_3$	Butyl protocatechuate= PA-C_4
R= $-(CH_2)_5-CH_3$	Hexyl protocatechuate= PA-C_6
R= $-(CH_2)_7-CH_3$	Octyl protocatechuate= PA-C_8
R= $-(CH_2)_9-CH_3$	Decyl protocatechuate= PA-C_{10}
R= $-(CH_2)_{11}-CH_3$	Dodecyl protocatechuate= PA-C_{12}
R= $-(CH_2)_{13}-CH_3$	Tetradecyl protocatechuate= PA-C_{14}
R= $-(CH_2)_{15}-CH_3$	Hexadecyl protocatechuate= PA-C_{16}
R= $-(CH_2)_{17}-CH_3$	Octadecyl protocatechuate= PA-C_{18}

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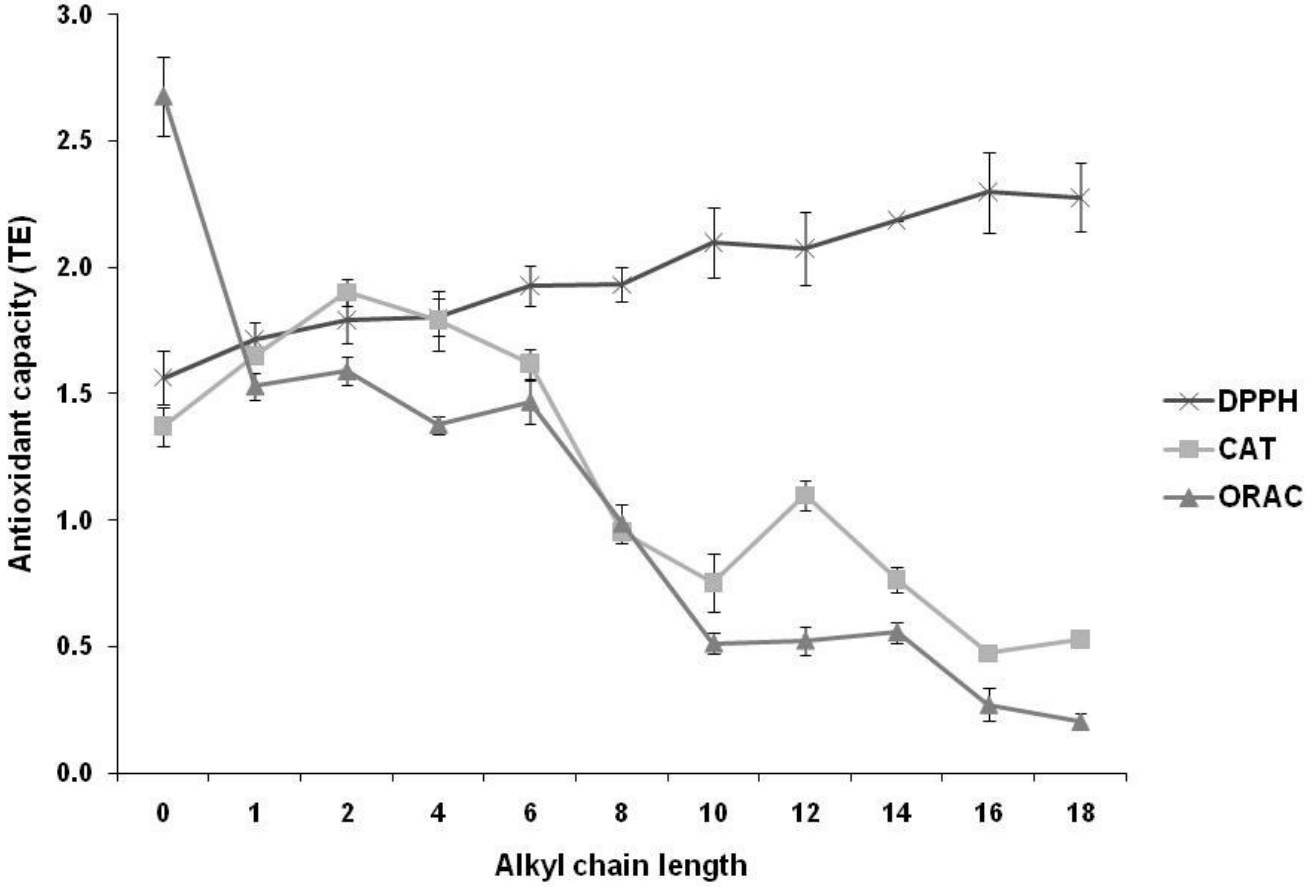

Fig. 2. Antioxidant capacities of PA and protocatechuates expressed as Trolox equivalents (TE), measured by different methods.

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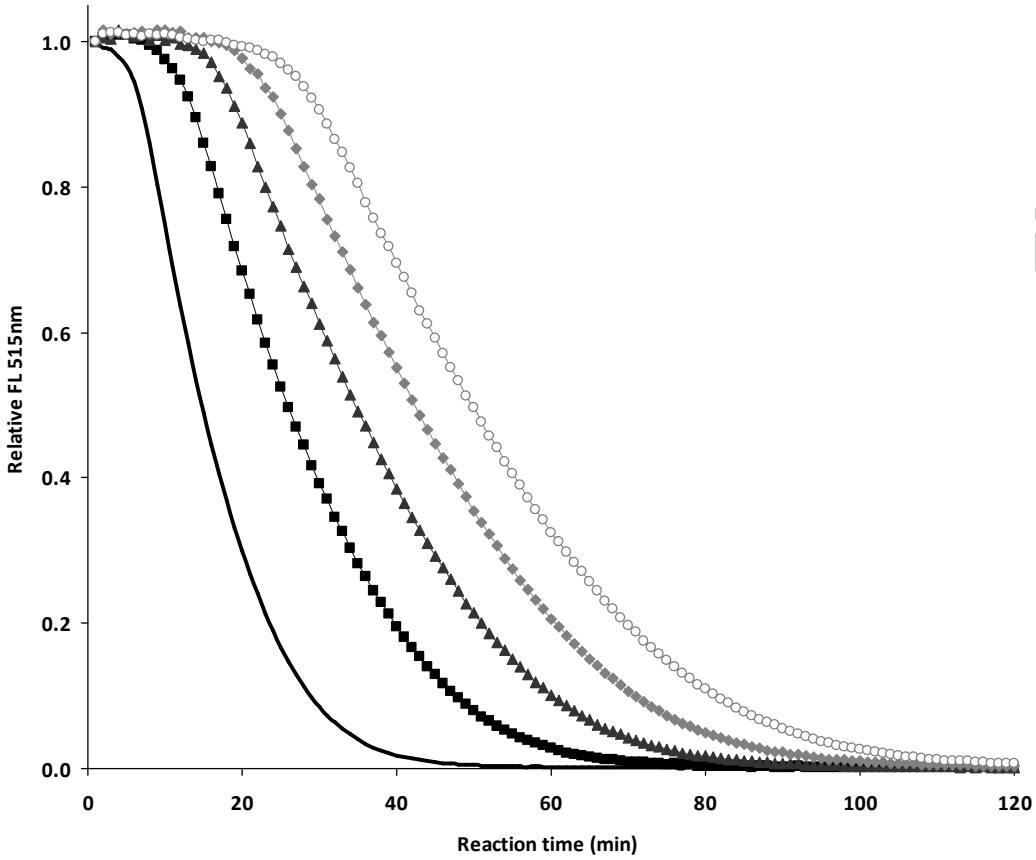

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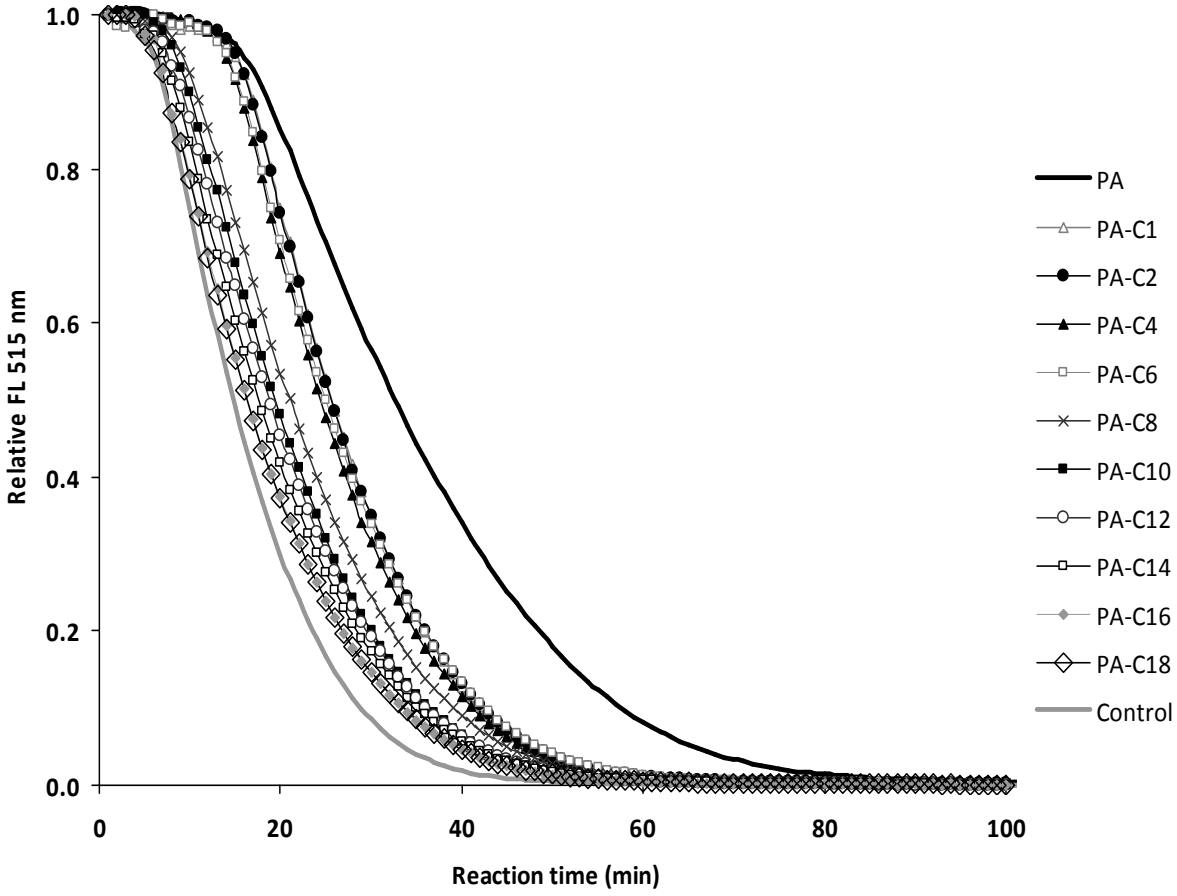

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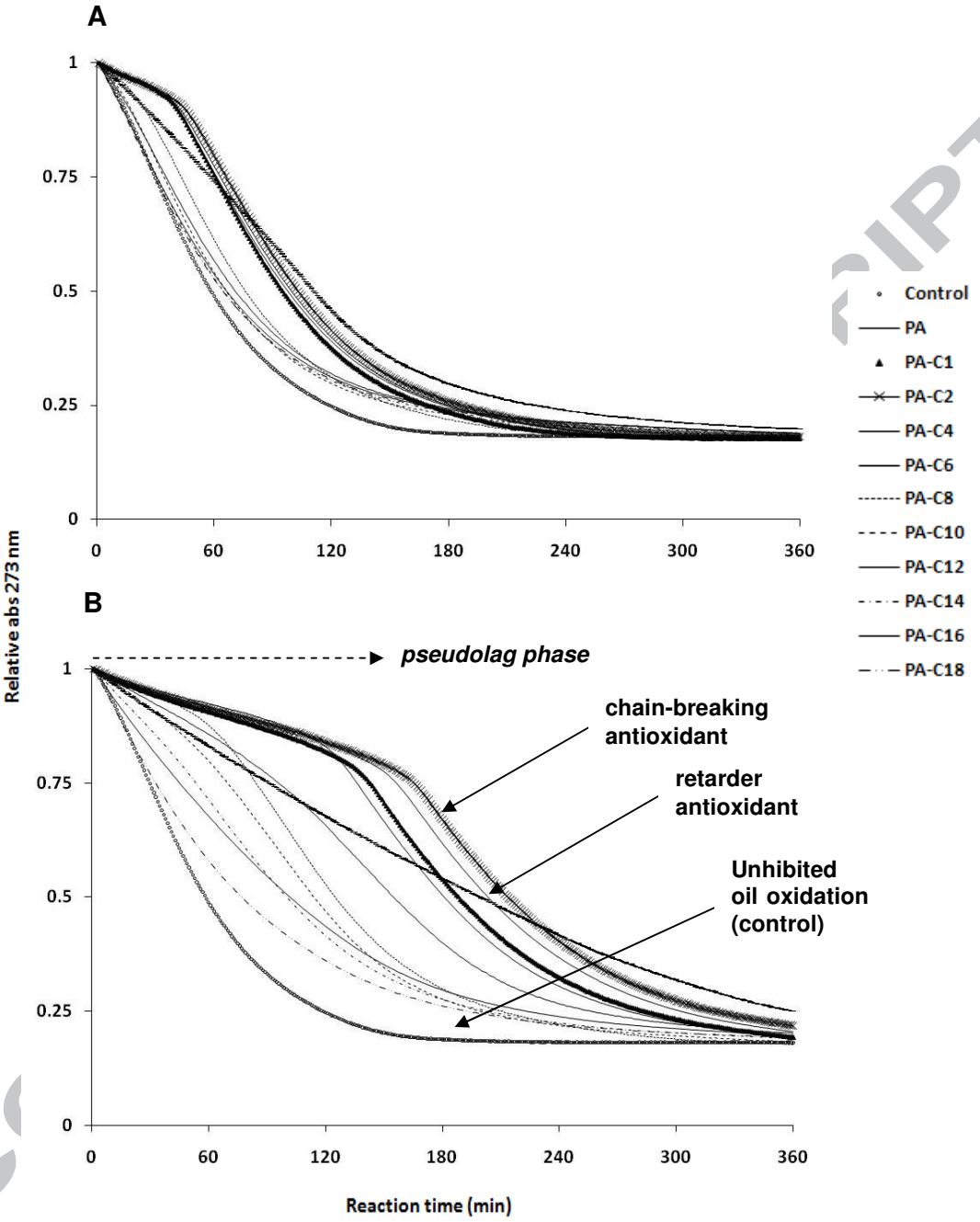

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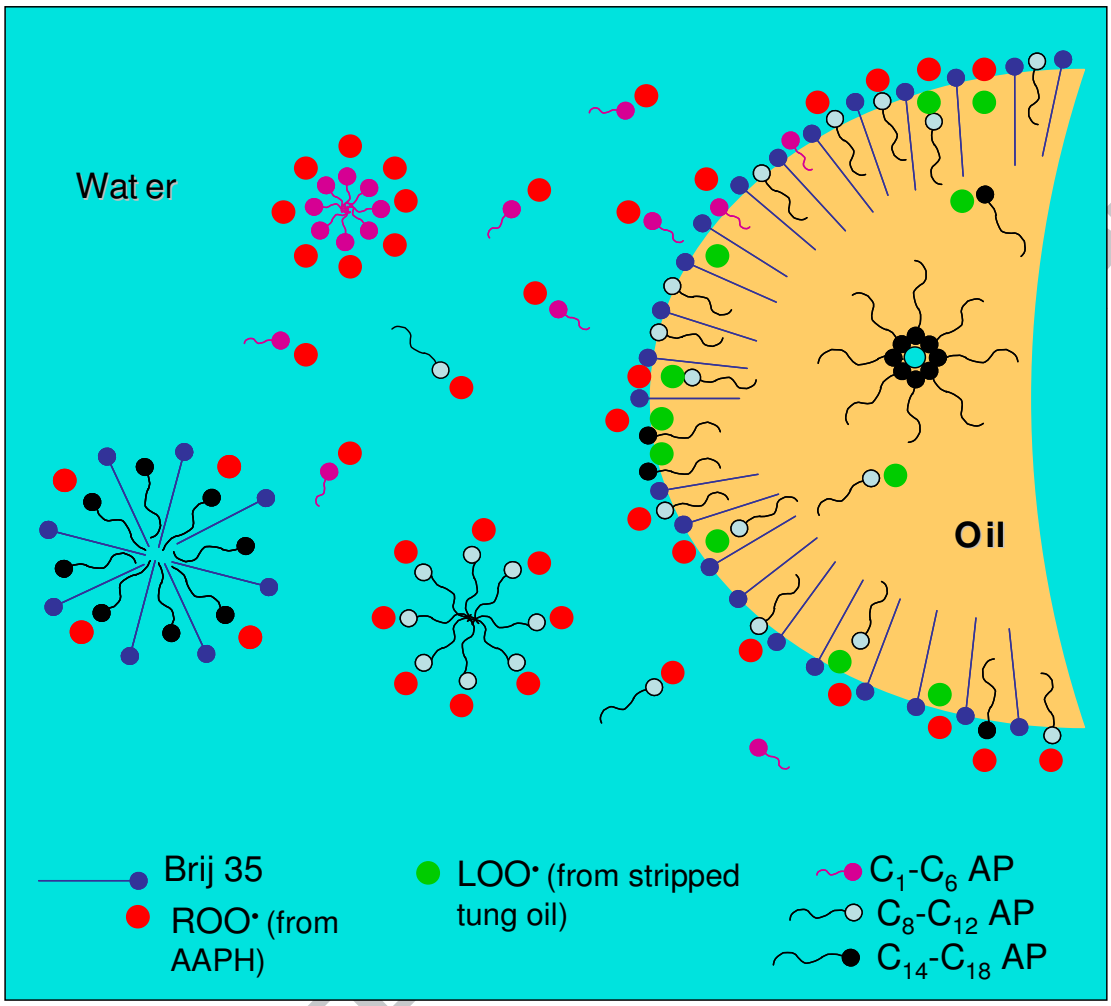

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664 **HIGHLIGHTS**

665 - Protocatechuic acid (PA) was successfully lipophilized using alcohols from C_1-C_{18}.

666 - Lipophilization could improve PA antioxidant capacity in heterogeneous media.

667 - Antioxidant activity is related to compound polarity and method of evaluation.

668 - Protocatechuates could be considered as potential preservatives in food.

669 - An innovative way to add-value to phenolic-rich vegetal extracts is proposed.

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