ANTIOXIDANT ACTIVITY OF PHENOLIC COMPOUNDS ADDED TO A FUNCTIONAL EMULSION CONTAINING OMEGA-3 FATTY ACIDS AND PLANT STEROL ESTERS

Raquel Rainho Espinosa, Raffaella Inchingolo, Severino Matias Alencar, Maria Teresa Rodriguez-Estrada, Inar Alves Castro

Food Chemistry, Impact Factor: 3.259

Article In Press, Accepted Manuscript, Available online 4 March 2015

DOI:10.1016/j. foodchem.2015.02.130

Antioxidant activity of phenolic compounds added to a functional emulsion containing omega-3 fatty acids and plant sterol esters

Raquel Rainho Espinosa[a], Raffaella Inchingolo[b], Severino Matias Alencar[c], Maria Teresa Rodriguez-Estrada[b], Inar Alves Castro[a*]

[a]LADAF, Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of São Paulo, Av. Lineu Prestes, 580, B14, 05508-900, São Paulo, Brazil.

[b]Department of Agricultural and Food Sciences, Alma Mater Studiorum-Università di Bologna, Viale Fanin 40, 40127 Bologna, Italy.

[c]Department of Agri-Food industry, Food and NutritionCollege of Agriculture "Luiz de Queiroz" of University of São Paulo, São Paulo, Brazil.

***Corresponding author.** Address: Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of São Paulo, Av. Lineu Prestes, 580, B14, 05508-900, São Paulo, Brazil. Tel.: +55 (11) 3091-1152; Fax: +55 (11) 3815-4410. E-mail address: inar@usp.br; www.ladaf.com.br (I. Castro).

Short running title: Antioxidant activity of phenolic compounds added to an emulsion

Abstract

The aim of this study was to compare the effect of eleven compounds extracted from red propolis on the oxidative stability of a functional emulsion. Emulsions containing 1.63 g/100 mL of α-linolenic acid (ALA), 0.73 g/100 mL of stearidonic acid (SDA) and 0.65 g/100 mL of plant sterol esters (PSE) were prepared without or with phenolic compounds (vanillic acid, caffeic acid, *trans*-cinnamic acid, 2,4-dihydroxycinnamic acid, *p*-coumaric acid, quercetin, *trans*-ferulic acid, *trans,trans*-farnesol, rutin, gallic acid or sinapic acid). *Tert*-butylhydroquinone and a mixture containing ascorbic acid and $FeSO_4$ were applied as negative and positive controls of the oxidation. Hydroperoxide, thiobarbituric acid reactive substances (TBARs), malondialdehyde, hexanal and phytosterol oxidation products (POPs) were evaluated as oxidative markers. Based on hydroperoxide and TBARs analysis, sinapic acid and rutin (200 ppm) showed the same antioxidant activity than TBHQ, representing a potential alternative as natural antioxidant to be applied in a functional emulsion containing ω-3 FA and PSE.

Keywords: oil-in-water emulsion, omega-3 fatty acids, phenolic compounds, plant sterol esters, antioxidant activity, oxidation, *Echium*, propolis.

1. Introduction

Atherosclerosis is an inflammatory condition associated with the genesis of several cardiovascular diseases (CVDs), including stroke and myocardial infarction (Libby et al., 2011), which constitute the primary cause of mortality in many countries. Although CVD manifests mostly in the adult and elderly population, the atherosclerotic process begins in childhood (Mendis et al., 2005). For this reason, strategies that target the initial prevention are important to reduce further disease progression. Among the factors that can be manipulated, dietary lipids are relevant, due to the influence of low-density lipoprotein cholesterol, triacylglycerols and saturated fat on CVD development (Waqar et al., 2010). Some bioactive lipid compounds, such as omega 3 fatty acids (ω-3 FA) and plant sterol esters (PSE), have been highlighted in the scientific literature as the most effective at improving cardiovascular protection (Garcia-Llatas et al., 2011; Harris et al., 2008).

There are different sources of ω-3 FA that can be added to food formulations. These compounds can be of animal or vegetal origin. Animal oils can be obtained from fish or algae and are rich in eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, whereas vegetable oils such as *Echium* oil is rich in α-linolenic (ALA) and stearidonic acid (SDA) (Whelan, 2009). Biological activity of ALA and SDA most likely relates to their conversion to EPA (Calder, 2012; Whelan, 2009). The action of these specific fatty acids in animal metabolism is still under discussion, but the general mechanism involves a hypolipideamic effect based on the downregulation of liver X receptor (LXRα), with a subsequent inhibition of fatty acids synthesis, associated with the upregulation of peroxisome proliferator-activated receptors (PPARα) which promotes fatty acids β-oxidation (Adkins et al., 2010; Calder, 2012). Besides reducing very low density lipoprotein and triacylglycerol (TAG), ω-3 FA show a relevant anti-inflammatory effect, due to the replacement of arachidonic acid as substrate for enzymatic oxidation mediated by lipoxygenase and cyclooxygenase, resulting in eicosanoids with milder inflammatory action (Calder, 2012). Plant sterols are compounds with a molecular structure similar to that of cholesterol, found in seeds,

vegetable oils and cereals. These molecules are able to displace cholesterol during micelle formation in the intestine due to their higher hydrophobicity, reducing cholesterol absorption. Additionally, plant sterols act to increase the expression of ABCG 5 and ABCG 8, carriers involved in the reverse transport of cholesterol from enterocyte to intestinal lumen. PSE also reduce the activity of acetyl-coenzyme A acetyltransferase (ACAT), an enzyme that re-esterifies cholesterol, which is a necessary step for its incorporation into chylomicrons (Garcia-Llatas et al., 2011).

Therefore, the combination of the anti-inflammatory and hypotriglyceroleamic effects promoted by ω-3 FA with the hypocholesteroleamic effect of PSE in a food emulsion, could be an interesting strategy to reduce the risk of CVD. In addition, as ω-3 FA can increase total cholesterol, their combination with PSE could also contribute to reduce this effect (Castro et al., 2005). However, the susceptibility to oxidation increases according to the fatty acids unsaturation degree (Decker et al., 2010), becoming the ω-3 highly polyunsaturated fatty acids (such as EPA, DHA and SDA) prone to oxidise when added to an emulsion. Although *Echium* oil has been reported as a potential source of ω-3 FA to be used in functional foods, information about its oxidative stability as bulk oil or as part of an emulsion is scarce. Oxidized *Echium* oil presents a strong fishy odour that makes completely unfeasible its application in food systems. Gray et al. (2010) observed that *Echium* bulk oil oxidizes relatively fast, forming thiobarbituric acid reactive substances (TBARs) and 2,4-heptadienal after 2 days of storage at 40^{o}C, while lipid hydroperoxides increased since from the first day. Plant sterols are also susceptible to oxidation after heat treatment, contact with oxygen or exposure to sunlight, forming phytosterol oxidation products (POPs) (Garcia-Llatas et al., 2011; Otaegui-Arrazola et al., 2010). For this reason, it is necessary the use of antioxidant compounds able to delay lipid oxidation. The most efficient antioxidants are synthetic and controlled compounds that have been shown to be toxic and mutagenic at high dosages (Giraldo et al., 2007). Thus, the application of these artificial antioxidants in a functional food is contrary to the concept of healthy and should be discouraged. In this case, natural antioxidants could be applied instead of

artificial ones. Several natural compounds obtained from vegetal sources or from their processing by-products or waste products may represent interesting alternatives to replace artificial antioxidants in food emulsions (Capitani et al., 2009). Among these compounds, the cinnamic and benzoic acid derivatives, as well as flavonoids, are well known for their antioxidant properties (Natella et al., 1999). Phenolic compounds act as antioxidants due to their capacity of transferring single-electron and/or hydrogen-atom to free radicals, and also due to their ability to bind potentially pro-oxidant metal ions, resulting in a stable phenoxyl radical (Craft et al., 2012). However, besides the bond dissociation energy (BDE) and ionization potential (IP), the action of these natural polyphenols as antioxidants in an oil-in-water (O/W) emulsion depends on several factors, including their concentration, physical location, chemical structure, steric issues, nature of the lipid system, interaction with other compounds and relative polarity to the type of lipids present in the emulsion (Sørensen et al., 2011; Shahidi et al., 2011; Jayasinghe et al., 2013). Considering the necessity of replacing artificial by natural antioxidants in functional foods formulation, the objective of this study was to evaluate the antioxidant action of eleven phenolic compounds extracted from red propolis on the oxidative stability of an O/W emulsion containing both ω-3 FA (from *Echium* oil) and PSE.

2. Materials and methods

2.1 Reagents and solvents

Echium oil (NEWmega™ Echium Oil, Ref.15200) was purchased from De Wit Speciality Oils (De Waal, Tescel, The Netherlands). It was a refined, bleached, deodorised and winterised oil obtained from *Echium* seeds (*Echium Plantagineum* species). The oil presented the following major fatty acids as measured by gas chromatography according to Shirai et al. (2005): C16:00 – 7%, C18:00 – 3%, C18:1 ω9 – 15%, C18:2 ω6 – 15%, C18:3 ω-3 – 32%, C18:3 ω6 – 11% and C18:4 ω-3 – 14%. Plant sterol esters (CardioAid-S WD ™) was a mixture containing 41% of total sterols (β-

sitosterol: 47.3%, campesterol: 25.3%, stigmasterol: 17.2%, β-sitostanol: 1.1%, campestanol: 0.6%, brassicasterol: 3.6% and other sterols: 4.9%) supplied by Archer Daniels Midland Company - ADM ® (Decatur, IL, USA), as previously reported by Botelho et al. (2014). Phenolic compounds were provided by the College of Agriculture "Luiz de Queiroz" of University of São Paulo (São Paulo, Brazil), which were obtained from an ethanolic extract of Brazilian red propolis, fractioned by liquid-liquid extraction with hexane and chloroform and purified by semi-preparative reverse-phase HPLC (Oldoni et al., 2011). Analytical and HPLC-grade solvents were purchased from Merck & Co. (Whitehouse Station, NJ, USA). Reagents and phenol standards were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Silica solid-phase extraction (SPE) cartridges (Strata, 70A, 500 mg/3 mL) from Phenomenex (Torrence, CA, USA) were utilised for POPs purification. Standards of sterols and cholesterol oxidation products were purchased from Steraloids (Newport, RI, USA).

2.2 Experimental design

This study was carried out in three steps. Firstly, it was evaluated the effect of some known artificial and natural antioxidants (tocopherol, Trolox, TBHQ and ascorbic acid) and pro-oxidants (iron and iron + ascorbic acid) on emulsions containing *Echium* oil and PSE, aiming to establish reference values for chemical markers applied to monitor the lipid oxidation. Samples were analysed at the beginning (T_0), after heating (T_H) and also after 30 days of storage at room temperature (T_{30}). From this first step the positive and negative controls of the reaction were selected. In a second step, 11 phenolic compounds (vanillic acid, caffeic acid, *trans*-cinnamic acid, 2,4-dihydroxycinnamic acid, *p*-coumaric acid, quercetin, *trans*-ferulic acid, *trans*,*trans*-farnesol, rutin hydrate, gallic acid and sinapic acid) were added to the emulsion and the oxidative stability was evaluated. Samples were analysed at the beginning (T_0), after heating (T_H) and also after 14 days of storage at room temperature (T_{14}). Phenolic compounds that showed better performance in

the second step, were also evaluated in terms of quantification of hexanal, malondialdehyde (MDA) by HPLC, POPs and fatty acids composition. Afterward, the *in vitro* antioxidant activity of the compounds extracted from red propolis was compared with those commercially synthesised, using ORAC methodology. All assays were performed in duplicate.

2.3 Emulsion preparation

Oil-in-water emulsions were prepared using a sodium acetate-imidazole buffered solution (10 mmol/L each, pH 7.0) containing 0.6% Tween 20. The emulsions were prepared by mixing the *Echium* oil (5.0 g/100 mL), CardioAid (1.6 g/100 mL) with water, using a high-pressure homogenizer (Homolab mod A-10, Alitec, São Paulo, Brazil) at a pressure of 500 bar. During each step of the emulsion preparation, the samples were covered as much as possible to reduce light exposure and were kept in an ice bath. At the first step seven emulsions were prepared. Emulsions were added with no antioxidants (CONT), with antioxidants: tocopherol (TOC), ascorbic acid (ASC), *Tert*-butylhydroquinone (TBHQ) and Trolox (TLX), or with pro-oxidants: ascorbic acid (5.35 mg/g oil) + $FeSO_4.7\ H_2O$ (4.42 mg/g oil) (ASCIR),or 4.96 mg $FeSO_4.7\ H_2O$/g oil (IRON), heated at 84 ^oC/90 min and stored at room temperature for 30 days. The proportion between ascorbic acid and $FeSO_4.7\ H_2O$ used to prepare the pro-oxidant mixture was based on a previous study (Branco et al., 2011) . In the second step, emulsions containing the phenolic compounds (200 ppm) were evaluated. The heat treatment was carried out at 90^oC for 45 min and the emulsions were stored at room temperature for 14 days, covered with aluminium foil. Selected phenolics (quercetin, rutin and sinapic acid) were also evaluated at 500 and 1000 ppm. Sampling was performed from the individual sealed screw-cap vials, keeping the same headspace in the samples during all assay.

2.4 Fatty acid composition

Lipids from the *Echium* oil and emulsions were derivatised by direct esterification (Shirai et al., 2005) and its composition was determined by gas chromatography (GC) (Agilent 7890 A GC

System, Agilent Technologies Inc., Santa Clara, CA, USA). A polyimide coated fused silica capillary column (J&W DB-23 Agilent 122-236; 60 m x 250 mm inner diameter, 0.15 mm film thickness) was used. Oven temperature was programmed from 80 to 175°C at 5°C/min, and from 175 to 230°C at 3°C/min; the final temperature was kept for 5 min. The injector and detector temperatures were set at 250 and 280 ^{o}C, respectively. High-purity helium was used as carrier gas at a flow rate of 1 mL/min, with a split ratio of 1:50. The fatty acids were identified by comparing their retention times with those of four purified standard mixtures of fatty acid methyl esters: 4-7801; 47085-U; 49453-U and 47885-U (Sigma Chemical Co.; St. Louis, MO, USA). Results were expressed as g/100 g of total fatty acids. Two independent replicates were run per sample.

2.5 Lipid hydroperoxide and TBARs analysis

Lipid hydroperoxide concentrations were determined according to the procedures described by Shantha and Decker (1994). Emulsion samples (300 µL) were mixed with 1.5 mL of an isooctane/2-propanol solution (3:1, v/v), resulting in a final volume of 1.80 mL. The mixture was vortexed three times for 10 s, and 200 µL of the mixture was added to a 2.8 mL solution of methanol/1-butanol (2:1, v/v). A thiocyanate/ferrous solution was prepared by mixing 500 µL of 3.94 M thiocyanate solution with 500 µL of 0.072 M Fe^{+2} solution. The 0.072 M Fe^{+2} solution was obtained from the supernatant of a mixture of 1.5 mL of 0.144 M $FeSO_4$ and 1.5 mL of 0.132 M $BaCl_2$ in 0.4 M HCl. 30 µL of thiocyanate/ferrous solution were added to 3.0 mL of methanol/1-butanol mixture, vortexed and incubated at room temperature for 20 min. Following the incubation period, the samples' absorbance readings were measured at 510 nm using a UV–Vis mini 1240 spectrophotometer (Shimadzu, Kyoto, Japan). The hydroperoxide content was determined using a standard curve prepared with known concentrations of cumene hydroperoxide. Concentrations were expressed as meq/kg of oil. The amount of thiobarbituric-acid-reactive substances (TBARs) was determined according to the method proposed by McDonald et al. (1987). Measurements were

taken in duplicate, and the values were expressed as μmol/kg of oil. Two independent replicates were run per sample.

2.6 Oxygen radical absorbance capacity (ORAC)

The ORAC method is based on the inhibition by antioxidants of the peroxyl-radical-induced oxidation of fluorescein, initiated by thermally induced decomposition of the azo-compound 2,2‘-azobis-2-amidino-propane-dihydrochloride (AAPH) (Prior et al., 2003). Phenolic solutions (5 g/L in methanol) were diluted to 2 mg/L with 75 mM phosphate buffer (pH 7.1). The samples and blank (25 μL) were incubated with 40 nM fluorescein (150 μL) in a 96-well microplate at 37°C for 30 min. Thereafter, 153 mM AAPH (25 μL) was added to the reaction media and the fluorescence (485 nm excitation and 525 nm emission) was monitored for 1 h by a multi-detection microplate reader (Synergy HT, BioTek Instruments Inc., Winooski, VT, USA). The difference between the integrals of the decay curve for samples and blank was calculated using a Gen 5 software (version 1.06.10, BioTek Instruments Inc., Winooski, VT, USA) and the concentration equivalent of Trolox was calculated by linear regression (6.25-100.00 μM). Results were expressed as μM of Trolox equivalent (μM TE)/mg of phenolic compound. Two independent replicates were run per sample.

2.7 Phytosterols oxidation products (POPs)

Phytosterols oxidation products (POPs) were determined according to a modified version of the methods described by Cercaci et al. (2007) and Alemany-Costa et al. (2012). Briefly, emulsions were subjected to a direct cold saponification, and the unsaponifiable matter was fractionated by SPE, to obtain the purified and enriched POPs fraction. The identification and quantification of POPs were carried out using fast gas chromatography-mass spectrometry (GC-MS) according to Botelho et al. (2014) with minor modifications. In particular, the oven temperature was set from 210°C to 325°C at the rate of 15°C/min. The injector, transfer line, ion source and interface temperatures were set at 325, 280, 200 and 330°C, respectively. The acquisition and integration

modes were total ion current (TIC) and single ion monitoring (SIM), respectively. Identification of POPs was performed by comparing the retention time and mass spectra with those reported in our previous works and literature. POPs were recognised and quantified by their corresponding characteristic ions that show a high abundance by SIM mode (*m/z*): IS (19-hydroxycholesterol) (353), 7α-hydroxycampesterol (470), 7α-hydroxystigmasterol (482), 7α-hydroxysitosterol (484), 7β-hydroxystigmasterol (482), 7β-hydroxysitosterol (484), α-epoxysitosterol (412), 7-ketocampesterol (486), 6β-hydroxycampesterol (417), stigmastentriol (429), sitostanetriol (431), 6-ketositostanol (473), 7-ketositosterol (500). Considering that POP standards are not commercially available and that POPs fragmentation is similar to that of cholesterol oxidation products (COPs), POPs quantification was performed using the calibration curves obtained for cholesterol oxides in the SIM mode (Cardenia et al., 2012). Two independent replicates were run per sample.

2.8 Malondialdehyde (MDA) analysis

MDA concentration was determined by reverse phase high-performance liquid chromatography (HPLC) (Hong et al., 2000). Emulsion (0.05 mL) was submitted to alkaline hydrolysis with 12.5 µL of 0.2% butylated hydroxytoluene in ethanol and 6.25 µL of a 10 M sodium hydroxide aqueous solution. This mixture was incubated at 60°C for 30 min and 750 µL of 7.2% TCA aqueous solution containing 1% KI were added. The samples were kept on ice for 10 min and centrifuged at 13,000 x g for 10 min. The supernatant (500 µL) was mixed with 250 µL of 0.6% TBA and heated at 95°C for 30 min. After cooling, the MDA was extracted from the solution with 750 µL of *n*-butanol, and 50 µL were analysed by HPLC (Agilent Technologies 1200 series; Santa Clara, CA, USA). The TBA-MDA conjugate derivative was injected into a Phenomenex reverse-phase C18 analytical column (250 mm x 4.6 mm; 5 µm, Phenomenex, Torrance, CA, USA) with a LC8-D8 pre-column (Phenomenex AJ0-1287) and was fluorometrically quantified at an excitation and emission wavelengths of 515 nm and of 553 nm, respectively. The analysis were run

under isocratic conditions, using a mobile phase of 60% phosphate buffered saline (PBS) (50 mmol/L, pH 7.1) + 40% methanol at a flow rate of 1.0 mL/min. A standard curve (0.5-20 μmol/L, r = 0.995) was prepared using 1,1,3,3-tetraethoxypropane. Four independent replicates were run per sample.

2.9 Hexanal by headspace solid-phase microextraction (HS-SPME) coupled with gas chromatography–mass spectrometry (GC–MS)

Hexanal content of the samples was determined according to Garcia-Llatas et al. (2007) after some modifications. In this study, hexanal was analysed as an oxidative marker of ω-6 fatty acid (γ-linoleic acid) present in the *Echium* oil. Emulsions (990 μL) were added with 10 μL of internal standard (1 μL of MBIK/mL in methanol) and hermetically sealed in a 20 mL headspace glass vial with a polypropylene hole cap and PTFE/silicone septum (Supelco, Bellefonte, PA, USA). A Combi PAL autosampler was used for automated HS-SPME analysis. The vials were agitated (400 rpm) at 40^{o}C for 15 min. After this period, a conditioned (at 300^{o}C/2 h) fibre (carboxen/polydimethylsiloxane (CAR/PDMS); StableFlex fibres; 85 μm; Supelco 57295-U, Bellefonte, USA) was exposed to the headspace of the emulsions for 45 min under agitation at 40^{o}C. Then, the fibre was introduced into the GC-MS injector at 250^{o}C for 5 min under a split ratio of 1:10. During the GC analysis, the fibre was thermally desorbed at 250^{o}C for 3 min. Vials containing only water were inserted after each 5 samples to prevent residual peaks from the fibre. The analysis was carried out in an Agilent 7890 A GC-MS (Palo Alto, CA, USA). The stationary phase was a ZB-5 MS capillary column (5% polysilarylene/95% polydimethylsiloxane; 30 m × 0.32 mm; 1 μm film thickness; Phenomenex®; Torrance, USA). The ion source and quadrupole temperatures were set at 230 and 150°C, respectively. Ultra pure helium was the carrier gas operated at a constant flow of 3.0 mL/min. Oven temperature was kept at 40 ^{o}C for 5 min, increased until 100^{o}C at 4^{o}C/min, and then taken to 220^{o}C at 17 ^{o}C/min; the final temperature was kept for 10

min. All mass spectra were acquired in electron-impact (EI) mode with an ionization voltage of 70 eV, adopting a mass range of 35–300 *m/z*. TIC and SIM were used as data acquisition mode using the NIST library. The following retention times and quantification ions were used: IS 8.7 min (43, 58 and 100 *m/z*) and hexanal 11.7 min (44, 56 and 72 *m/z*). All quantification was based on the peak area ratio of the signal of the analyte and the IS signal. A standard curve (μg/mL hexanal = 39.112 hexanal/IS area ratio; r = 0.995) was prepared with five concentrations of hexanal (0-0.08 μg/mL of fresh emulsion), keeping the same amount of IS. Results were expressed as μg hexanal/mL of emulsion. Two independent replicates were run per sample.

2.10 Statistical analysis

Values were expressed as mean ± standard error of mean (SEM). Variance homogeneity and normality were previously evaluated for all variables. Differences among the samples of the experiment were evaluated by Repeated Measures ANOVA, followed by Tukey's HSD or Dunnett's test (vs CONT) in each time interval. Equivalent non-parametric tests were applied when the distribution normality was not verified. Significance was set at *p*-values less of 0.05. All analyses were performed with the software STATISTICA version 9.0 (StatSoft, Inc., Tulsa, Ok, USA).

3. Results

Figure 1 presents the LOOH and TBARs values of the control sample (CONT), the emulsions containing antioxidants (TOC, ASC, TBHQ and TLX) and the emulsions added with pro-oxidants (ASCIR and IRON), at the beginning (T_0), after heating (T_H) and after 30 days (T_{30}) of storage at room temperature. No significant differences in the hydroperoxide concentration were observed among samples at T_0 and T_H (Figure 1A). After 30 days of storage, emulsions containing tocopherol (TOC) and the mixture of ascorbic acid + Fe^{2+} (ASCIR) showed the highest LOOH values. No

significant differences in the TBARs concentration were detected among the samples during the assay (Figure 1B). Based on these results, TBHQ and ASCIR were selected as negative and positive controls, respectively, in this study.

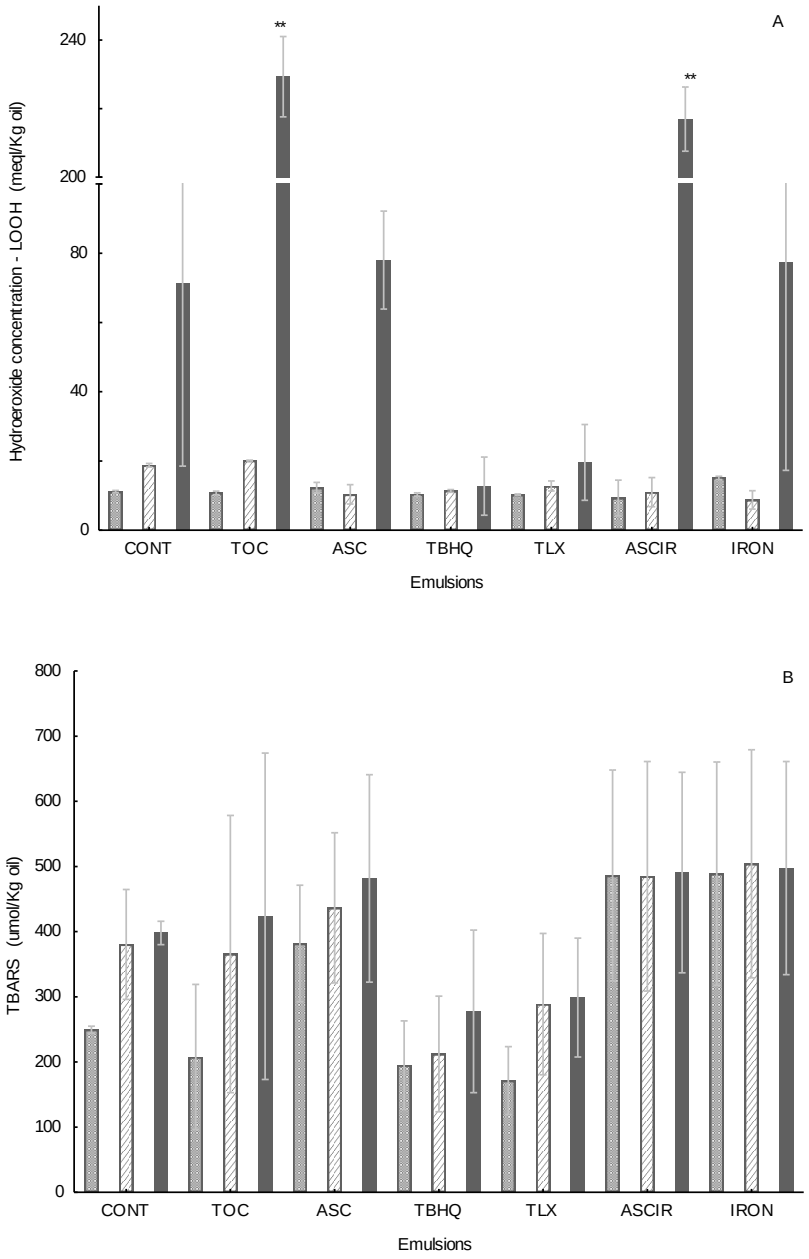
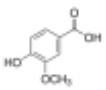
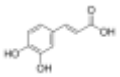
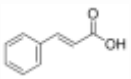
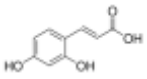
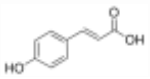
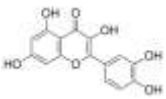
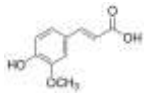
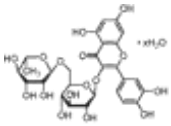
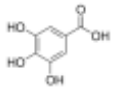
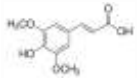
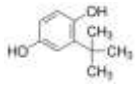


Figure 1. Hydroperoxides (meq/kg oil) and TBARs concentration (μmol/Kg oil) in functional O/W emulsions containing both ω-3 FA and PSE. Samples without antioxidants (CONT), with antioxidants (tocopherol (TOC), ascorbic acid (ASC), TBHQ (TBHQ) and Trolox (TLX) at 1.75 mg/g oil), and with prooxidants (ascorbic acid (5.26 mg/g oil) + $FeSO_4.7\ H_2O$ (4.38 mg/g oil) (ASCIR), and iron (48.8 mg $FeSO_4.7\ H_2O$/g oil) (IRON)), analyzed at the beginning (▪ T_0), after heating (⌜ T_H) and after 30 days (■ T_{30}) of storage at room temperature. Figure 1A: LOOH concentration, *p* (samples) = 0.008 and *p* (time) < 0.001. Figure 1B: TBARs concentration, *p* (samples) = 0.689 and *p* (time) < 0.001. Error bars are SEM of the values for each emulsion (n=2); ***P* < 0.01.

At the second step, emulsions prepared with 11 phenolic compounds at 200 ppm were compared with emulsions prepared with TBHQ and ASCIR, before and after heating, and after 14 days of storage at room temperature (Table 1). Regarding LOOH values, samples added with sinapic acid, rutin, quercetin, TBHQ and ASCIR showed lower LOOH values than CONT. The lower LOOH concentration observed in the ASCIR samples was due to the higher LOOH degradation, as evidenced by the highest concentration of TBARs in the ASCIR samples (Table 1).

Table 1. Hydroperoxides (LOOH) and TBARs concentration of the emulsions prepared with eleven natural compounds (200 ppm) compared with emulsions prepared with TBHQ, ASCIR and CONT, before (T0), after heating(TH) and after 14 days of storage (T14).

			LOOH (meq/kg oil)			TBARs (µmol/kg oil)		
Compound[a] and Molecular structure	**MW (g)[b]**	**µMol[c]**	**T_0**	**T_H**	**T_{14}**	**T_0**	**T_H**	**T_{14}**
Vanillic acid 	168.15	67.8	9.2±0.4	25.1±5.9	41.4±1.3	45.1±1.6	149.2±13.6	257.1±10.4
Caffeic acid 	180.16	63.3	8.1±0.3	23.6±4.9	30.7±0.8	41.3 ±0.6	117.8 ±8.9	170.1±11.2
trans-Cinnamic acid 	148.16	76.9	7.3±1.7	25.8±6.1	38.5±0.9	45.0 ± 6.5	135.6±27.3	259.1±30.9
2,4-Dihydroxycinnamic acid 	180.16	63.3	6.9±2.5	27.0±5.1	45.5±5.0	44.2±3.3	140.2±14.7	266.1±13.3
p-Coumaric acid 	164.16	69.4	9.0±1.3	27.1±8.1	40.5±0.6	50.4±5.1	153.9±22.5	273.1±19.1
Quercetin 	338.27	33.7	5.0±3.7	12.5±0.4	26.5±0.8*	40.0±0.0	101.8±6.9	176.0±6.1
trans-Ferulic acid	194.18	58.7	9.9 ± 0.5	18.3±0.6	39.6 ± 0.5	50.5 ± 9.5	142.3 ±20.8	231.3 ± 10.4

 Rutin hydrate	610.52	18.7	7.2±0.0	13.5±0.3	20.5±0.1*	45.0±12.7	99.4±15.3	134.2±3.4*
 Gallic acid	170.12	67.0	10.1±0.0	18.3±0.9	33.1±2.1	39.8±3.1	139.9±16.6	210.1±28.5
 Sinapic acid	224.21	50.8	8.6±0.1	13.4±0.9	14.6±0.7*	44.7±10.4	93.6±13.4	93.9±1.0*
 trans,trans-Farnesol	222.37	51.3	8.0±0.8	18.1±0.6	37.6±2.6	44.0±2.6	140.5±23.9	211.2±5.9
 TBHQ (CONT-)	166.22	68.6	8.5±0.6	11.5±0.4	11.2±0.3*	37.3±4.1	68.3±11.9	90.3±3.7*
CONT	-	-	7.1±2.3	22.9±3.7	38.5±3.3	43.0±3.3	139.6±19.4	230.9±32.1
ASCIR (CONT+)	-	-	4.3±1.1	7.4±2.6	16.2±2.4*	700.9±12.0*	1655.3±32.5*	1698.2±8.8*
P[d]	-	-	0.367	0.034	<0.001	<0.001	<0.001	<0.001

[a]Values are mean ± SEM (n= 2).
[b] Molecular weight (g).
[c]Concentration expressed as μMol/L emulsion equivalent to 200 ppm (based in oil content).
[d]Probability value obtained by ANOVA.*p<0.05) from CONT, as evaluated by Dunnet test (2-tailed).

Still regarding TBARs, samples containing sinapic acid, rutin and TBHQ showed lower values than CONT. Taking these data into account, sinapic acid, rutin and quercetin were chosen for the next step of the study. The selected phenolic compounds concentration effect on oxidative stability of the emulsions was also evaluated (Figure 2). The compounds concentration did not influence the antioxidant activity of the sinapic acid, rutin and TBHQ as measured by LOOH (Figure 2A), but quercetin at 200 ppm showed lower antioxidant activity than at 500 and 1000 ppm. No difference was verified in TBARs concentration by increasing the phenolics concentration from 200 to 1000 ppm (Figure 2B).

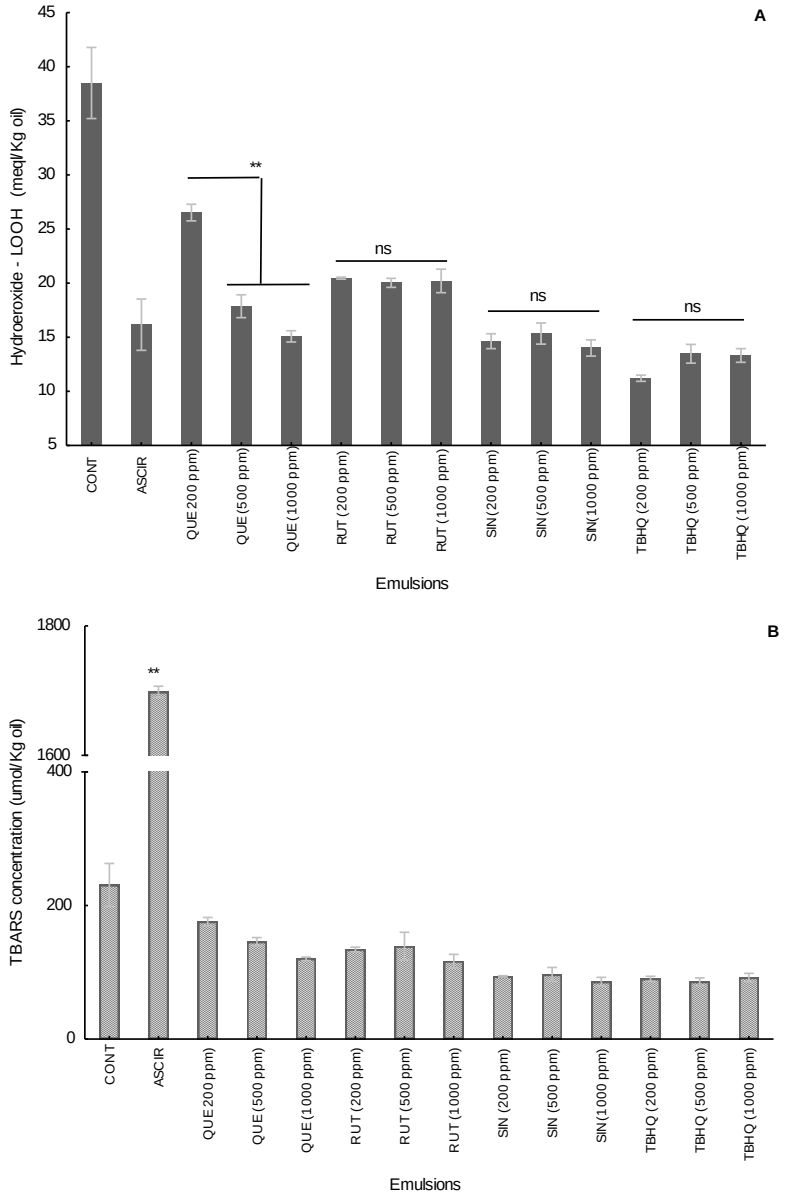


Figure 2. Hydroperoxides (LOOH) and TBARs concentration in the functional emulsions containing n-3 FA and PSE. Samples without antioxidants (CONT), with pro-oxidant ascorbic acid (5.26 mg/g oil)+ FeSO4.7 H2O (4.38 mg/g oil) (ASCIR), and with phenolic compounds: quercetin (QUE), rutin (RUT) sinapic acid (SIN) and TBHQ at 200, 500 and 1000 ppm, measured after 14 days of storage at room temperature. Error bars are SEM of the values for each emulsion (n=2); $^{**}p < 0.01$.

In the third step of this study, the oxidative stability of the functional oils (ω-3 FA and PSE) present in the emulsions added with sinapic acid (200 ppm), rutin (200 ppm) and quercetin (500 ppm) was evaluated after 14 days of storage at room temperature, and compared with ASCIR and TBHQ (200 ppm). The amount of the two ω-3 FA present in the *Echium* oil composition, α-linolenic (18:3 Δ9,12,15) and stearidonic (18:4 Δ6,9,12,15) acids, did not change among the samples. Secondary products of oxidation are shown in Figure 3. Hexanal content was measured by HS-SPME-GC-MS, and just the samples containing the pro-oxidant ASCIR showed higher values than CONT (Figure 3A). As the TBARs determination is a non-specific methodology for MDA quantification, a HPLC analysis of the latter was also carried out with these emulsions. Data present in Figure 3B also confirmed that only samples containing the pro-oxidant ASCIR showed higher values than CONT.

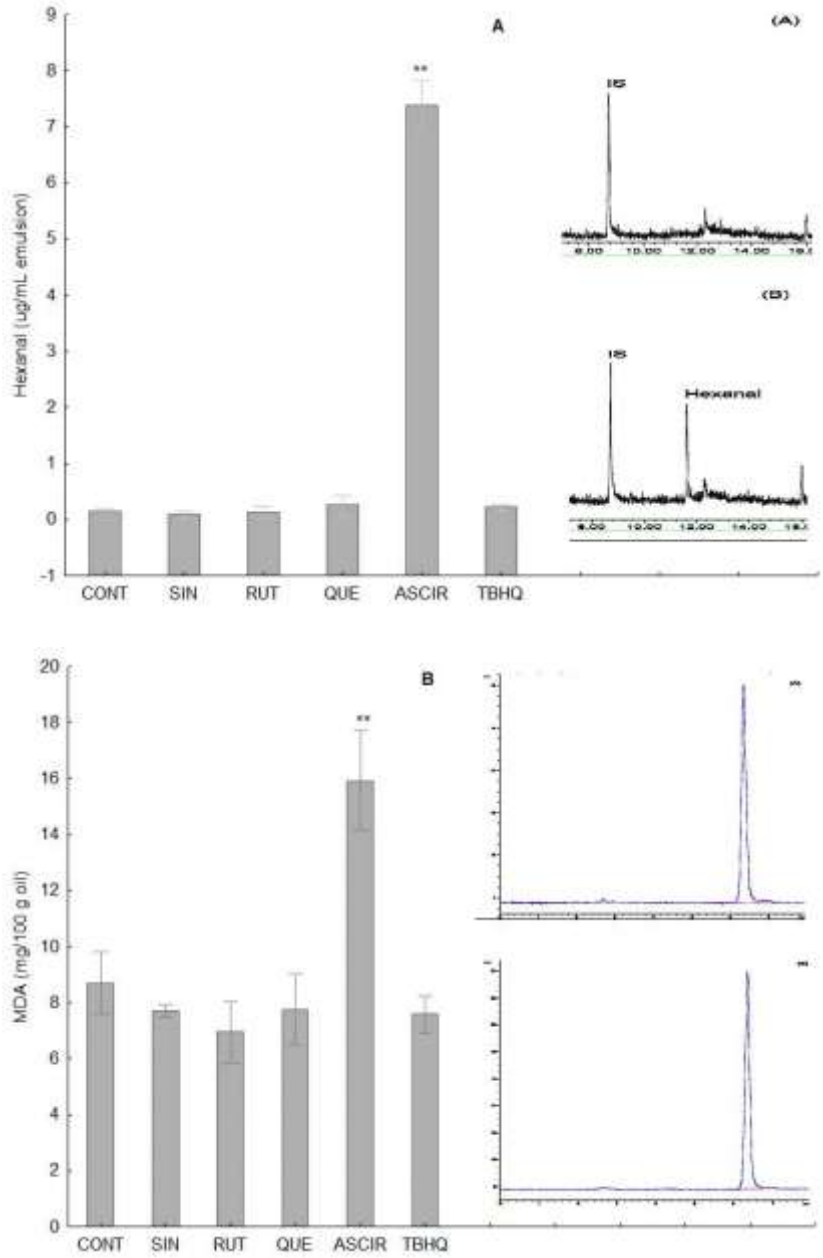


Figure 3. Secondary products of oxidation measured in O/W emulsions without antioxidants (CONT), with pro-oxidant (ASCIR), and with phenolic compounds (rutin (RUT), sinapic acid (SIN) and TBHQ at 200 ppm, and quercetin (QUE) at 500 ppm), after 14 days of storage at room temperature. **Figure 3A.** Hexanal (mg/mL emulsion). Error bars are SEM of the values for each emulsion (n=2); $^{**}p<0.01$. Chromatograms (A): TBHQ and (B): ASCIR. **Figure 3B.** Malonaldehyde (mg/100 g oil). Error bars are SEM of the values for each emulsion (n=4); $^{**}P < 0.01$. Chromatograms (A): TBHQ and (B): ASCIR.

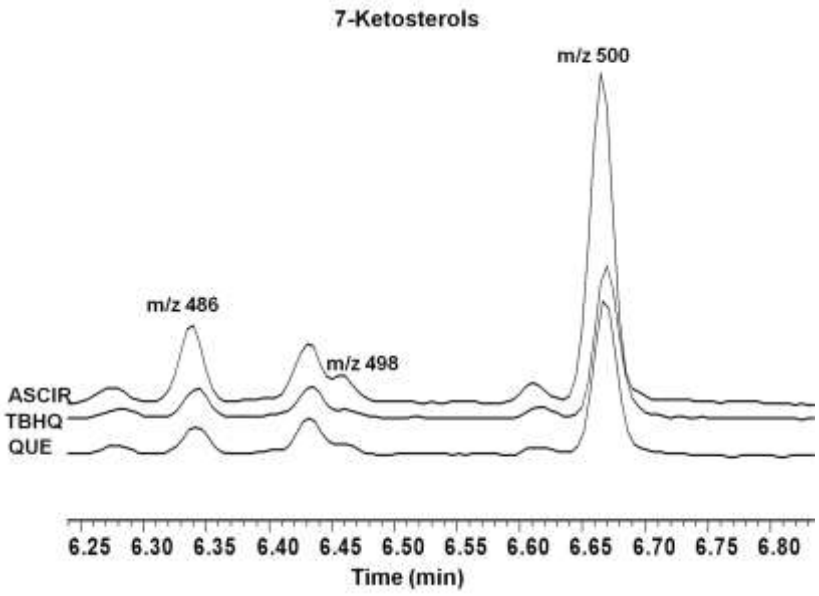
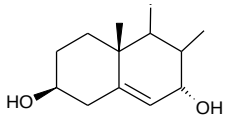
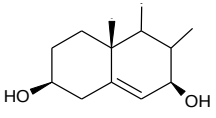
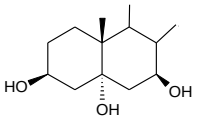
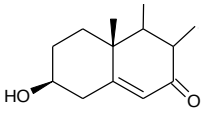
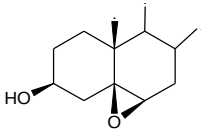
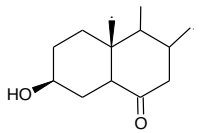


Figure 3C. Peaks relative to 7-Keto derivatives (7-Keto-campesterol, 7-Keto-stigmasterol and 7-Keto-sitosterol).

Finally, the major POPs were analysed in the emulsions after 14 days of storage (Table 2). Among the 16 POPs identified in the samples, 7-Keto and triol derivatives were the most abundant. No significant difference was found in triol-derivates among samples, while 7-Ketocampesterol ($p=0.011$) and 7-Ketositosterol differed (p=0.009) among the emulsions tested.

Table 2. POPs concentration of the emulsions without antioxidants (CONT), with pro-oxidant (ASCIR), and with phenolic compounds: rutin (RUT), sinapic acid (SIN) and TBHQ at 200 ppm, and quercetin (QUE) at 500 ppm, after 14 days of storage at room temperature.

	7-α- hydroxy			**7-β hydroxy**			**Tri**			**7-keto**			**Epoxi**[c]		**6-keto**
															
Sample	**Campe-**	**Stigma-**	**Sito-**	**Campe-**	**Stigma-**	**Sito-**	**Campe-**	**Stigma-**	**Sito-**	**Campe-**	**Stigma-**	**Sito-**	**5α,6α-**	**5β,6β-**	**Sito-**
CONT	2.26	1.99	3.13	1.12	0.82	1.96	0.37	1.43	14.60	1.06	2.61	12.83	1.53	1.85	1.60
SIN	2.46	2.11	3.72	1.29	1.08	2.59	0.45	1.38	13.74	1.04	3.24	13.45	2.19	2.36	1.25
TBHQ	2.39	2.02	3.59	1.16	0.86	2.23	0.40	1.21	10.11	1.11	3.38	12.48	2.05	1.45	0.10
ASCIR	2.30	1.96	2.91	1.58	1.38	3.33	0.55	1.35	10.39	2.80*	6.09	28.27**	7.88	3.74	2.05
RUT	2.23	2.00	3.28	1.11	0.96	2.15	0.37	1.36	15.16	0.50	2.26	12.29	2.16	3.38	1.73
QUE	2.20	1.96	3.02	1.07	0.67	2.07	0.36	1.12	10.09	0.95	2.47	8.92	1.62	2.42	0.13
SEM[a]	0.05	0.04	0.15	0.08	0.09	0.24	0.02	0.04	1.04	0.23	0.50	2.00	0.81	0.31	0.35
p^b	0.834	0.939	0.643	0.576	0.247	0.710	0.109	0.357	0.583	0.011	0.229	0.009	0.132	0.204	0.514

[a]Pooled Standard Error of Mean

[b]Probability value obtianed by ANOVA.*p<0.05; ** p<0.001 as evaluated by Tukey post-hoc test.

[c]Structural configuration of 5α,6α

Likewise the results shown by other markers, ASCIR emulsions containing ascorbic acid and iron presented higher values of 7-ketosterols than the other emulsions. Regarding the 7-ketositosterol (Figure 3C), ASCIR emulsions showed higher values than the emulsions containing TBHQ and quercetin (p=0.031).

As the phenolic compounds applied in this study were obtained from red propolis, the antioxidant activity of these molecules was compared with the synthetic ones. No difference was observed to antioxidant activity between the molecules obtained by extraction. This result was found when the samples were analysed by ORAC methodology adapted for hydrophilic (Figure 4A) and lipophilic substrates (Figure 4B). In fact, a high correlation between the antioxidant activity of the hydrophilic and lipophilic fractions was noted (r= 0.839; p<0.001), while no correlation was found between the *in vitro* antioxidant capacity (ORAC) and chemical oxidation markers (LOOH and TBARs).

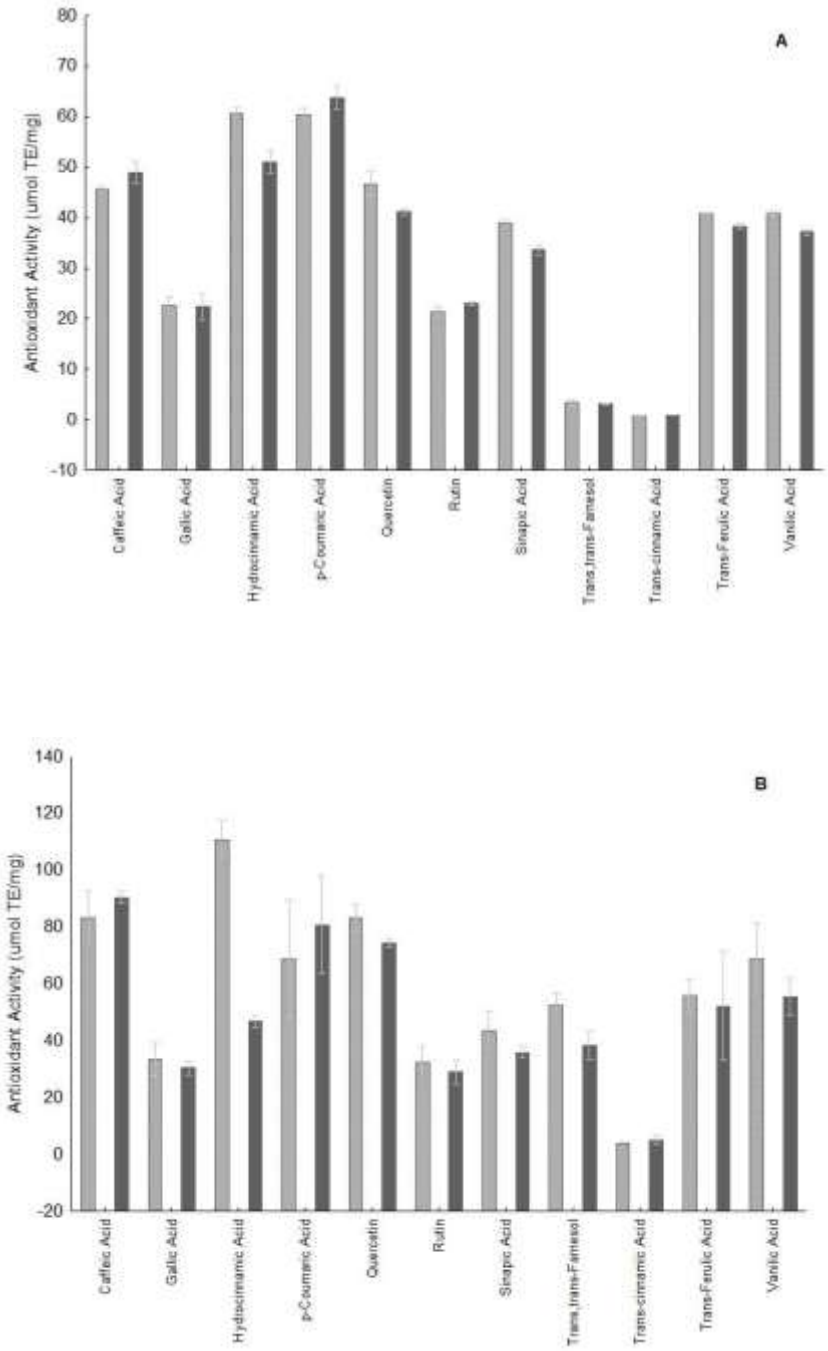


Figure 4. Antioxidant activity of the phenolic compounds extracted from red propolis and standards measured by ORAC for both hydrophilic (4A) and lipophilic (4B) fractions obtained by extraction or synthesis. Error bars are SEM of the values for each emulsion (n=2).

4. Discussion

The present data show that emulsions prepared with *Echium* oil and PSE are prone to oxidation after heat treatment, forming hydroperoxides and other secondary lipid oxidation products. The oxidative damage observed in the functional emulsion was higher when ascorbic acid and iron were present, regardless of the temperature. Even before the heat treatment, the energy generated during homogenization under high pressure was enough to initiate oxidation of samples containing the pro-oxidant (ASCIR). Pro-oxidant effect of ascorbic acid alone or combined with metals in emulsions is well known (Jayasinghe et al., 2013; Sørensen et al., 2011). In a previous study, Branco et al. (2011) showed that when pH increases from 3.0 to 7.0 and the ascorbic acid content was greater than 1.0 mmol/L, TBARs values increased from 3.0 to 12.0 mmol/L, regardless of the iron concentration. Similar results were also observed in ω-3 FA-enriched O/W emulsions added with ascorbic acid and kept at 35^{o}C/48h (Jayasinghe et al., 2013). In fact, as found in our model, Jayasinghe et al. (2013) observed oxidative instability in the initial stage of the storage period. Pro-oxidant effect of ascorbic acid in emulsions is due to its capacity to reduce Fe^{3+} to Fe^{2+}, thus accelerating lipid oxidation (Frankel et al., 2000). Cuvelier et al. (2000) applied a mixture of ascorbic acid with Fe^{2+} to induce oxidation in emulsions at 30^{o}C. In a study carried out by Sørensen et al. (2011), ascorbic acid (free or conjugated with fatty acids) acted as pro-oxidant at the end of the storage period in O/W emulsions containing ω-3 FA. Therefore, ascorbic acid should not be applied in ω-3 FA O/W emulsions neither as antioxidant nor as source of vitamin C, regardless of the processing treatment or temperature of storage.

Among the eleven phenolic compounds tested, sinapic acid (200 pmm), rutin (200 ppm) and quercetin (500 ppm) inhibited the oxidation of the functional emulsion at the same level as TBHQ (200 ppm). Although the effectiveness of an antioxidant strongly depends on its BDE and IP, other factors such as reaction environment, solubility, concentration and presence/influence of other

compounds contribute to its antioxidant activity (Lengyel et al., 2012; Leopoldini et al., 2011). DBE of the O-H bonds in natural phenols does not present a widely range of variation (Evgeny et al., 2011). Thus, it can be suggested that in emulsions, phenolics antioxidant activity depends on impact of their chemical structures on media characteristics (Evgeny et al., 2011). In the present study, TBHQ, sinapic acid, rutin and quercetin were able to react with part of the radicals and transition metals in the aqueous phase or in the oil-water interface, inhibiting fatty acids and plant sterols oxidation. Several factors can be involved in the higher antioxidant activity displayed by these four molecules, including solubility and chemical structure. Sinapic acid is a phenolic acid derived from the hydroxyl *trans*-cinnamic acid, quercetin and rutin are flavonols, and TBHQ is a hydroquinone. A number of studies have investigated the phenolics characteristics associated to their higher capacity of transferring a hydrogen atom (HAT) or a single electron (SET) to a radical molecule (Capitani et al., 2009; Craft et al., 2012). Zhiyong et al. (2003) suggested that the best phenolic antioxidants are compounds that contain an electron donor group directly attached to an aromatic ring. It has also been reported that the antioxidant activity rises with increasing number of phenolic rings, occurrence of multiple OH groups attached to the ring (preferentially in the ortho-dihydroxy configuration), planar structure and presence of additional functional C=C and C=O (Kim et al., 2004; Leopoldini et al., 2011). However, in the emulsion model used in our study, none of these statements was conjointly present and could justify the higher antioxidant activity observed in samples containing these four compounds.

Phenolic compounds are predominantly polar and expected to be located in the aqueous phase of an emulsion (Sørensen et al., 2008). Based on the "Polar Paradox" theory, non-polar antioxidants are more effective in media of relatively higher polarity (Porter, 1993). But our results showed that molecules with similar solubility presented opposite performance as antioxidants, reinforcing that other factors besides polarity are involved in the reaction (Shahidi et al., 2011). Regarding the SET mechanism, the IP is the most significant parameter; the lower the IP, the easier

the electron abstraction by the radical molecule (Leopoldini et al., 2011). At neutral pH, pKa of the phenolics could contribute to later their solubility, altering their distribution between oil, interface and aqueous media. Conversely, this condition does not explain the differences observed in our study, since pKa of the phenolic compounds are similar (Costa et al., 2013). Beside HAT or SET mechanisms, molecules containing *o*-diphenol groups are able to chelate metal ions such as iron (Sørensen et al., 2008). This fact explains the results observed in emulsions containing rutin and quercetin, as these molecules offer all possible chelating sites with $Fe^{(II)}$, thus preventing their involvement in the Fenton reaction (Leopoldini et al., 2011). However, this cannot justify the results observed in the emulsions formulated with sinapic acid or TBHQ. Therefore, there is not a common single aspect that justifies and explains the lower lipid oxidation in O/W emulsions prepared with these four phenolic compounds. It is possible that a combination of some of the aforementioned factors has improved their antioxidant activity when compared with the other compounds evaluated in this model.

Although sinapic acid, rutin, quercetin and TBHQ reduced LOOH and TBARs, no difference was observed among these samples and CONT when hexanal and MDA were separately analysed. The unsaturation degree is the major factor responsible for fatty acids susceptibility to oxidation. In *Echium* oil, 47% of the most unsaturated fatty acids (stearidonic and α-linolenic acids) are ω-3 FA, followed by 10% of ω-6 γ-linolenic. Secondary lipid oxidation products measured in our study are mainly formed from the oxidation of fatty acids with more than three double bonds (MDA) or from ω-6 FA (hexanal). This fact justifies the lack of significant differences among the treatments, except for ASCIR samples when MDA and hexanal were individually taken as markers. For instance, Gray et al. (2010) evaluated TBARs and 2,4-heptadienal (*t,t*) in *Echium* oil during storage and observed a higher alteration of 2,4-heptadienal (*t,t*) than TBARs, as 2,4-heptadienal (*t,t*) originates from linolenate hydroperoxide and is a volatile marker of ω-3 FA. Although a significant increase of both LOOH and TBARs had been observed in the emulsions containing the pro-oxidant

mixture (ASCIR), no alterations were found in the ω-3 FA content (ALA and SDA) after 14 days of storage, reinforcing that fatty acids profile cannot be used as marker to monitor earlier stages of lipid oxidation.

In our study, molecules extracted from red propolis showed similar antioxidant activity that their synthesised analogues, suggesting that further practical applications can be performed with either extracted or synthesised molecules. However, antioxidant activity measured by ORAC methodology did not correlate with the results observed in the emulsions. Similar conclusions were reported by Sørensen et al. (2013) when comparing the antioxidant activity of canola phenolic compounds by *in vitro* assays and emulsion markers. In fact, inconsistent results have been obtained for a number of recognised antioxidants depending on the methods used to test their antioxidant activity (Frankel et al., 2000). A weakness of the ORAC method is that the antioxidant protection of the fluorescent probe cannot be extrapolated to biological or food substrates. As already discussed, the antioxidant effectiveness is strongly dependent on the test system, including substrates, positioning, method employed and stages of the oxidation (Frankel et al., 2000). Thus, indirect methods, such as ORAC, should be applied just for initial screening on antioxidant activity of new or modified molecules.

The most commonly consumed plant sterols are Δ^5 ones, which have a double bond between C5 and C6 carbons in the sterol nucleus. During food processing and/or storage, this site is susceptible to oxidation by free radical attack, so the hydrogen abstraction represents the first step of the oxidation (Lengyel et al., 2012). In an emulsion system, Cercaci et al. (2007) suggested that oxidation occurs at the droplet interface. Since plant sterols are surface active compounds, they could be particularly prone to oxidation when they are incorporated in emulsions. This is particularly noticeable in the samples containing water-soluble compounds as acid ascorbic and iron, which showed the highest content of total POPs amount. Among the 16 POPs identified in our samples, only 7-keto derivatives (7-ketocampesterol, 7-ketostigmasterol and 7-ketositosterol)

showed a significant increase due to the oxidation induction. As for cholesterol (Rodriguez-Estrada, et al., 2014), 7-keto-phytosterols can be used as markers to monitor the plant sterols oxidation process in model systems, since they are easily formed and are the most representative ring POPs, as confirmed by the sterol thermoxidation study carried out by Barriuso et al. (2012). In the present work, the most abundant was 7-ketositosterol, followed by 7-keto-stigmasterol and 7-ketocampesterol. While the amount of 7-ketositosterol reflected the relative presence of β-sitosterol in the emulsion system (47.3%), the same cannot be stated for campesterol and stigmasterol. This trend is not in agreement with results reported by Barriuso et al. (2012), as they found that stigmasterol was less prone to oxidation with respect to the other two main plant sterols (campesterol ~ β-sitosterol>> stigmasterol) when heated at 180°C for 360 min, in presence of cholesterol. Such behaviour differences could arise from the diverse mixture sterol composition (with the consequent chemical equilibrium among the various chemical species), the different physico-chemical state of the model system (dried bulk sterols vs. sterol-enriched O/W emulsion), diverse heat transfer modalities and heating temperature/exposure time conditions (180 vs. 90°C) used in both studies.

5. Conclusions

Ascorbic acid combined with iron was able to oxide a functional emulsion containing 1.63 g/100 mL of α-linolenic acid (ALA), 0.73 g/100 mL of stearidonic acid (SDA) from Echium oil and 0.65 g/100 mL of plant sterols at room temperature, resulting in a significant increase of all oxidative markers evaluated in this study. Among 11 natural phenolic compounds extracted from red propolis, sinapic acid and rutin hydrate were the most efficient to delay lipid oxidation. There, these two compounds represent a potential alternative as natural antioxidant to be applied in a functional food emulsion containing ω-3 FA and plant sterols as bioactive lipids.

ACKNOWLEDGEMENTS

This research was supported by FAPESP (12/08058-5 and 12/04063-7).

6. References

Adkins, Y., Kelley, D. (2010). Mechanisms underlying the cardioprotective effects of omega-3 polyunsaturated fatty acids. *Journal of Nutritional Biochemistry, 21*, 781-792.

Alemany-Costa, L., Gonzalez-Larena, M., Garcia-Llatas, G., Alegria, A., Barbera, R., Sanchez-Siles, L., Lagarda, M. (2012). Sterol stability in functional fruit beverages enriched with different plant sterol sources. *Food Research International, 48*, 265-270.

Barriuso, B., Otaegui-Arrazola, A., Menendez-Carreno, M., Astiasaran, I., Ansorena, D. (2012). Sterols heating: Degradation and formation of their ring-structure polar oxidation products. *Food Chemistry, 135*, 706-712.

Botelho, P., Galasso, M., Dias, V., Mandrioli, M., Lobato, L., Rodriguez-Estrada, M. T, Castro, I. (2014). Oxidative stability of functional phytosterol-enriched dark chocolate. *LWT-Food Science and Technology, 55*, 444-451.

Branco, G. F., Rodrigues, M. I., Gioielli, L. A., Castro, I. A. (2011). Effect of the simultaneous interaction among ascorbic acid, iron and pH on the oxidative stability of oil-in-water emulsions. *Journal of Agricultural and Food Chemistry, 59*, 12183-12192.

Calder, P. (2012). Mechanisms of Action of (n-3) Fatty Acids. *Journal of Nutrition, 142*, 592S-599S.

Castro, I. A., Sinnecker, P., Barroso, L. P. (2005). Functional foods for coronary heart disease risk reduction: a meta-analysis using a multivariate approach. *American Journal of Clinical Nutrition, 82*, 32 - 40.

Capitani, C., Carvalho, A., Rivelli, D., Barros, S., Castro, I. (2009). Evaluation of natural and synthetic compounds according to their antioxidant activity using a multivariate approach. *European Journal of Lipid Science and Technology, 111*, 1090-1099.

Cardenia, V., Rodriguez-Estrada, M. T, Baldacci, E., Savioli, S., Lercker, G. (2012). Analysis of cholesterol oxidation products by Fast gas chromatography/mass spectrometry. *Journal of Separation Science, 35*, 424-430.

Cercaci, L., Rodriguez-Estrada, M. T, Lercker, G., Decker, E. (2007). Phytosterol oxidation in oil-in-water emulsions and bulk oil. *Food Chemistry, 102*, 161-167.

Costa, M., Losada-Barreiro, S., Paiva-Martins, F., Bravo-Diaz, C. (2013). Effects of acidity, temperature and emulsifier concentration on the distribution of caffeic acid in stripped corn and olive oil-in-water emulsions. *Journal of the American Oil Chemists' Society, 90*, 1629-1636.

Craft, B., Kerrihard, A., Amarowicz, R., Pegg, R. (2012). Phenol-based antioxidants and the in vitro methods used for their assessment. *Comprehensive Reviews in Food Science and Food Safety, 11*, 148-173.

Cuvelier, M., Bondet, V., Berset, C. (2000). Behavior of phenolic antioxidants in a partitioned medium: Structure-activity relationship. *Journal of the American Oil Chemists' Society, 77*, 819-823.

Decker, E. A., Alamed, J., Castro, I. A. (2010). Interaction between polar components and the degree of unsaturation of fatty acids on the oxidative stability of emulsions. *Journal of the American Oil Chemists' Society, 87*, 771-780.

Evgeny, D., Taisa, D. (2011). Dissociation Energies of O−H Bonds of Phenols and Hydroperoxides. In *: Application of Thermodynamics to Biological and Materials Science*. Institute of Problems of Chemical Physics RAS.

Frankel, E., Meyer, A. (2000). The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *Journal of the Science of Food and Agriculture, 80*, 1925-1941.

Garcia-Llatas, G., Lagarda, M., Romero, F., Abellan, P., Farre, R. (2007). A headspace solid-phase microextraction method of use in monitoring hexanal and pentane during storage: Application to liquid infant foods and powdered infant formulas. *Food Chemistry, 101*, 1078-1086.

Garcia-Llatas, G., Rodriguez-Estrada, M. T. (2011). Current and new insights on phytosterol oxides in plant sterol-enriched food. *Chemistry and Physics of Lipids, 164*, 607-624.

Giraldo, L., Laguerre, M., Lecomte, J., Figueroa-Espinoza, M., Barouh, N., Barea, B., Villeneuve, P. (2007). Lipase-catalyzed synthesis of chlorogenate fatty esters in solvent-free medium. *Enzyme and Microbial Technology, 41*, 721-726.

González-Larena, M., Cilla, A., García-Llatas, G., Barberá, R., Lagarda, M. J. (2012). Plant sterols and antioxidant parameters in enriched beverages: Storage stability. *Journal of Agricultural and Food Chemistry, 60*, 4725-4734.

Gray, D., Payne, G., McClements, D., Decker, E., Lad, M. (2010). Oxidative stability of Echium plantagineum seed oil bodies. *European Journal of Lipid Science and Technology, 112*, 741-749.

Harris, W., Miller, M., Tighe, A., Davidson, M., Schaefer, E. (2008). Omega-3 fatty acids and coronary heart disease risk: Clinical and mechanistic perspectives. *Atherosclerosis, 197*, 12-24.

Hong, Y. L., Yeh, S. L., Chang, C. Y., Hu, M. L. (2000). Total plasma malondialdehyde levels in 16 Taiwanese college students determined by various thiobarbituric acid tests and an improved high-performance liquid chromatography-based method. *Clinical Biochemistry, 33*, 619-625.

Jayasinghe, C., Gotoh, N., Wada, S. (2013). Pro-oxidant/antioxidant behaviours of ascorbic acid, tocopherol, and plant extracts in n-3 highly unsaturated fatty acid rich oil-in-water emulsions. *Food Chemistry, 141*, 3077-3084.

Kim, D., Lee, C. (2004). Comprehensive study an vitamin C equivalent antioxidant capacity (VCEAC) of various polyphenolics in scavenging a free radical and its structural relationship. *Critical Reviews in Food Science and Nutrition, 44*, 253-273.

Lengyel, J., Rimarčík, J., Vagánek, A., Fedor, J., Lukeš, V., Klein, E. (2012). Oxidation of sterols: Energetics of C–H and O–H bond cleavage. *Food Chemistry*, 133, 1435-1440.

Leopoldini, M., Russo, N., Toscano, M. (2011). The molecular basis of working mechanism of natural polyphenolic antioxidants. *Food Chemistry, 125*, 288-306.

Libby, P., Ridker, P. M., Hansson, G. K. (2011). Progress and challenges in translating the biology of atherosclerosis. *Nature, 473*, 317-325.

McDonald, R. E., Hultin, H. O. (1987). Some characteristics of the enzymic lipid Peroxidation system in the microsomal fraction of flounder skeletal muscle. *Journal of Food Science, 52*, 15-21.

Mendis, S., Nordet, P., Fernandez-Britto, J. E., Sternby, N. (2005). Atherosclerosis in children and young adults: An overview of the World Health Organization and International Society and Federation of Cardiology study on pathobiological determinants of atherosclerosis in youth study (1985–1995). *Prevention and Control, 1*, 3-15.

Natella, F., Nardini, M., Di Felice, M., Scaccini, C. (1999). Benzoic and cinnamic acid derivatives as antioxidants: Structure-activity relation. *Journal of Agricultural and Food Chemistry, 47*, 1453-1459.

Oldoni, T .L. C., Cabral, I. S. R., Regitano-d`Arce, M. A. R., Rosalen, P. L., Ikegaki, M., Nascimento, A. M., Alencar, S. M. (2011) Isolation and analysis of bioactive isoflavonoids and chalcone from a new type of Brazilian propolis. S*eparation and Purification Technology, 77*, 208-213.

Otaegui-Arrazola, A., Menéndez-Carreño, M., Ansorena, D., Astiasarán, I. (2010). Oxysterols: A world to explore. *Food and Chemical Toxicology, 48*, 3289-3303.

Porter, W. L. (1993). Paradoxical behavior of antioxidants in food and biological systems. In: *Antioxidants: Chemical, Physiological, Nutritional and Toxicological Aspects*. Princeton Scientific: Princeton, NJ.

Prior, R., Hoang, H., Gu, L., Wu, X., Bacchiocca, M., Howard, L., Hampsch-Woodill, M., Huang, D., Ou, B., Jacob, R. (2003). Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORAC(FL)) of plasma and other biological and food samples. *Journal of Agricultural and Food Chemistry, 51*, 3273-3279.

Rodriguez-Estrada, M. T, Garcia-Llatas, G., Lagarda, M. (2014). 7-Ketocholesterol as marker of cholesterol oxidation in model and food systems: When and how. *Biochemical and Biophysical Research Communications, 446*, 792-797.

Shahidi, F., Zhong, Y. (2011). Revisiting the polar paradox theory: a critical overview. *Journal Agricultural and Food Chemistry, 59*, 3499-3504.

Shantha, N. C., Decker, E. A. (1994). Rapid, sensitive, iron-based spectrophotometric methods for determination of peroxide values of food lipids. *Journal of AOAC International, 77*, 421-424.

Shirai, N., Suzuki, H., Wada, S. (2005). Direct methylation from mouse plasma and from liver and brain homogenates. *Analytical Biochemistry, 343*, 48-53.

Sørensen, A., Friel, J., Winkler-Moser, J., Jacobsen, C., Huidrom, D., Reddy, N., Thiyam-Hollander, U. (2013). Impact of endogenous canola phenolics on the oxidative stability of oil-in-water emulsions. *European Journal of Lipid Science and Technology, 115*, 501-512.

Sørensen, A.-D. M., Nielsen, N. S., Decker, E. A., Bruni Let, M., Xu, X., Jacobsen, C. (2011). The efficacy of compounds with different polarities as antioxidant in emulsions with omega-3 lipids. *Journal of the American Oil Chemists' Society, 88*, 489-502.

Sørensen, A. D., Haahr, A. M., Becker, E. M., Skibsted, L. H., Bergenståhl, B., Nilsson, L., & Jacobsen, C. (2008). Interactions between iron, phenolic compounds, emulsifiers, and pH in omega-3-enriched oil-in-water emulsions. *Journal Agricultural and Food Chemistry, 56*, 1740-1750.

Waqar, A. B., Koike, T., Yu, Y., Inoue, T., Aoki, T., Liu, E., Fan, J. (2010). High-fat diet without excess calories induces metabolic disorders and enhances atherosclerosis in rabbits. *Atherosclerosis, 213*, 148-155.

Whelan, J. (2009). Dietary stearidonic acid is a long chain (n-3) polyunsaturated fatty acid with potential health benefits. *Journal of Nutrition, 139,* 5-10.

Zhiyong, C., Jie, R., Yuanzong, L., Wenbao, C., Zhida, C. (2003). Establishment of a quantitative structure–activity relationship model for evaluating and predicting the protective potentials of phenolic antioxidants on lipid peroxidation. *Journal of Pharmaceutical Sciences, 92,* 475-484.