Food Chemistry 182 (2015) 95–104

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

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

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ARTICLE INFO

Article history:
Received 8 September 2014
Received in revised form 20 February 2015
Accepted 25 February 2015
Available online 4 March 2015

Keywords:
Oil-in-water emulsion
Omega-3 fatty acids
Phenolic compounds
Plant sterol esters
Antioxidant activity
Oxidation
Echium
Propolis

ABSTRACT

The effect of eleven compounds extracted from red propolis on the oxidative stability of a functional emulsion was evaluated. Emulsions prepared with *Echium* oil as omega 3 (ω-3 FA) source, 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 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.

1. Introduction

Atherosclerosis is an inflammatory condition associated with the genesis of several cardiovascular diseases (CVDs), including stroke and myocardial infarction (Libby, Ridker, & Hansson, 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, Nordet, Fernandez-Britto, & Sternby, 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 & Rodriguez-Estrada, 2011; Harris, Miller, Tighe, Davidson, & Schaefer, 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 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 hypolipidemic 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 & Kelley, 2010; Calder, 2012). Besides reducing very low density lipoprotein and triacylglycerol (TG), 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

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http://dx.doi.org/10.1016/j.foodchem.2015.02.130
0308-8146/

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 & Rodriguez-Estrada, 2011).

Therefore, the combination of the anti-inflammatory and hypotriglyceridemic effects promoted by ω-3 FA with the hypocholesterolemic effect of PSE in a food emulsion, could be an interesting strategy to reduce the risk of CVD. As ω-3 FA can increase cholesterol concentration in plasma, the combination of ω-3 FA with PSE could also contribute to annul the increase of cholesterol caused by ω-3 FA, since PSE reduce intestinal cholesterol absorption, inducing a higher uptake by the cholesterol receptors in the liver (Castro, Sinnecker, & Barroso, 2005; De Smet, Mensink, & Plat, 2012). However, the susceptibility to oxidation increases according to the fatty acids unsaturation degree (Decker, Alamed, & Castro, 2010), becoming the ω-3 highly polyunsaturated fatty acids (such as EPA, DHA and SDA) prone to oxidation 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. Oxidised *Echium* oil presents a strong fishy odour that makes completely unfeasible its application in food systems. Gray, Payne, McClements, Decker, and Lad (2010) observed that *Echium* bulk oil oxidises relatively fast, forming thiobarbituric acid reactive substances (TBARS) and 2,4-heptadienal after 2 days of storage at 40 °C, while lipid hydroperoxides increased since 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 & Rodriguez-Estrada, 2011; Otaegui-Arrazola, Menéndez-Carreño, Ansorena, & Astiasarán, 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, Carvalho, Rivelli, Barros, & Castro, 2009). Among these compounds, the cinnamic and benzoic acid derivatives, as well as flavonoids, are well known for their antioxidant properties (Natella, Nardini, Di Felice, & Scaccini, 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 prooxidant metal ions, resulting in a stable phenoxyl radical (Craft, Kerrihard, Amarowicz, & Pegg, 2012). However, besides the bond dissociation energy (BDE) and ionisation 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 (Jayasinghe, Gotoh, & Wada, 2013; Shahidi & Zhong, 2011; Sørensen et al., 2011). 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. Materials

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, Suzuki, and Wada (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 prooxidants (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 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