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Antioxidant Protection of Eicosapentaenoic Acid and Fish Oil Oxidation by Polyphenolic-Enriched Apple Skin Extract

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Two apple skin extracts (ASE) prepared from "Northern Spy" cultivar were examined for their antioxidant properties to inhibit lipid oxidation in aqueous eicosapentaenoic acid (EPA) emulsions and bulk fish oil. The ASE were effective in reducing the oxidation induced by heat, UV light and peroxyl radical, when the extent of oxidation of the emulsions and bulk oil was measured by using the ferric thiocyanate test, the thiobarbituric acid reactive substances assay and Rancimat. On the basis of total phenolic concentration of extracts, removal of sugars and organic acids (ASE 2) from crude ethanol extract of apple skins (ASE 1) enhanced the antioxidant properties in both the emulsion and bulk fish oil systems. The average induction times of accelerated oxidation at 50 to 80 °C of fish oil incorporated with ASE 2 (400 μg/mL) was similar to that of α-tocopherol (400 μg/mL) and butylated hydroxytoluene (200 μg/mL).

KEYWORDS: Apple; extract; omega-3; fish oil; antioxidant; ferric thiocyanate; TBARS; Rancimat

INTRODUCTION

Dietary lipids and fatty acid profiles, and their balance within the body, have become one of the interesting areas of recent investigations since lower levels of endogenous omega-3 fatty acids have been implicated to several chronic diseases (*1*). Within omega-3 fatty acids, α-linolenic acid (LNA, C18:3 n-3), eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3) are the most important long chain polyunsaturated fatty acids (PUFA) with strong scientific evidence for their potential to reduce the risk of cardiovascular disease and various cancers (*2*). As a result, omega-3 fatty acid containing functional foods and nutraceuticals have been introduced to the market. Fish oil, as an example, is a vital source of EPA and DHA in the human diet.

However, the presence of multiple double bonds in PUFA makes them vulnerable to oxidation, which produces various aldehydes and ketones resulting in unacceptable flavors, odors and colors in PUFA containing foods (*3*). Moreover, the products of lipid oxidation, such as malonaldehyde, can have adverse health effects to the consumer due to their cytotoxic and genotoxic effects (*4*, *5*). The high rate of oxidation of PUFA can be controlled by the addition of synthetically produced antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), *tert*-butyl hydroquinone (TBHQ) and synthetic or naturally sourced α-tocopherol. However, potential carcinogenic properties of the synthetic antioxidants have been reported (*6*) and their use in food has been already limited in some countries. Recently, consumer health consciousness has led to a demand for "natural" alternatives to synthetic food antioxidants such as BHT and BHA.

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Natural plant-based antioxidants such as phenolics derived from fruits, vegetables and many herbal or aromatic plants have received much attention for their antioxidative characteristics. Many phytochemicals are also important dietary antioxidants and cell signaling modulators in preventing oxidative stress mediated degenerative diseases (*7*). Phytochemical rich extracts from food and food ingredient sources such as oregano (*8*), green tea (*9*), rosemary (*10*), grape seed extract (*11*), and garlic (*12*) have been shown to possess the ability of inhibition of lipid oxidation in various model systems. However, though many of the plant extracts exhibit ability to inhibit lipid peroxidation, the characteristic smell and flavor due to their incorporation have raised concerns for their use as alternatives of synthetic antioxidants.

Apple is a great source of natural antioxidants in the North American and European diet and provides about 22% of total dietary phenolics (*13*). Apple skin has 3- to 6-fold higher flavonoid content than apple flesh and has unique flavonoids, such as quercetin glycosides, not found in the flesh (*14*). The apple skin extract (ASE) has been shown to possess powerful antioxidant activity (*15*). It has been estimated that 2−3 million kg of apple skins are generated as a result of apple processing in Nova Scotia, Canada (*16*). However, to our knowledge, omega-3 fatty acid or lipid preserving potential of ASE against oxidation has not been investigated. The goal of the present study was to examine two ASE for their efficacy as food antioxidant compared to the commercial food antioxidants, α-tocopherol, BHT and TBHQ. The ability of ASE to inhibit oxidation of aqueous EPA emulsion and fish oil under three different induction systems, namely, heat, UV light, and peroxyl radical, was studied.

MATERIALS AND METHODS

Chemicals. The omega-3 fatty acid EPA was obtained from Nu-Chek Prep, Inc. (Elysian, MN). The bulk fish oil (Canadian Food Inspection

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Agency [CFIA] registration number 3529) was a gift from Ocean Nutrition Canada, Dartmouth, NS, Canada. The fish oil was devoid of any antioxidants. The composition of the oil was 17.6% monounsaturates and 77.6% polyunsaturates [61% EPA and 4.3% DHA] by weight of total fatty acids. Approximately 51% of EPA and DHA were in the triacylglycerol form. BHT, TBHQ, α-tocopherol, 2,2′-azobis(2-amidinopropane) dihydrochloride, 2,4,6-tripyridyl-*s*-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH•), Folin−Ciocalteu reagent, 2-thiobarbituric acid (TBA), ferrous sulfate ($FeSO_4$), ammonium thiocyanate (NH_4SCN), isooctane and trichloroacetic acid (TCA) were obtained from Sigma-Aldrich Chemical Co. The 96-well microplates were purchased from Fisher Scientific (Ottawa, ON, Canada). All other chemicals and reagents were purchased from the suppliers mentioned above with the highest grade in their purity.

Preparation of Apple Skin Powder. The apple skins of the apple cultivar "Northern Spy" were collected from a commercial pie manufacturer, Apple Valley Foods Inc., Kentville, NS, Canada, in 2006. Immediately after peeling, the skins were blanched with 2% $CaCl_2$ in water (w/v) at 55 ± 5 °C for 10 min to prevent oxidation of phenolic compounds. After draining of the excess water and within 3 h of blanching treatment, the blanched apple skins were transported in plastic containers to the Nova Scotia Agricultural College (NSAC). The apple skins were dried in clean plastic trays at 60 ± 2 °C for 48 h using a convection oven with air circulation (Milner Agincourt, ON, Canada). The dried skins were ground into a fine powder using a Willey mill with 1 mm sieve screen (Model Laboratory Heavy Duty, Arthur Thomas Co., Philadelphia, PA) and kept in a freezer (−80 °C) for later use.

Preparation of ASE. *ASE 1.* Twenty grams of apple skin powder was weighed into a flask and sonicated using 200 mL of 95% ethanol two times for 15 min with a 10 min interval. The suspension was then transferred into 50 mL Corning tubes for centrifugation at 3000 rpm for 15 min. The supernatants (ASE 1) were collected in an amber vial and stored at −80 °C until use.

ASE 2. For the removal of sugars from ASE 1, flash chromatography using a sorbent (Sorbent SP207-05 Sepabeads resin brominated styrenic adsorbent; particle size 250 μm, surface area 630 m^2/g; Sorbent Techologies, Atlanta, GA) was used. The chromatography column (3.8 × 45 cm, Sati International Scientific Inc., Dorval, QC, Canada) containing the adsorbent was conditioned with deionized water and loaded with ASE 1 at the top of the column. The column was immediately washed with water by sending 2 to 3 times of bed volume of water through it. The removal of sugar was monitored by measuring the Brix value of wash water using a refractometer. Once the Brix value was less than 1%, washing step was terminated. The phenolic compounds retained in the column were eluted using 100% ethanol, and the eluate was concentrated using a rotary evaporator (Rotavapor R-200, Buchi, Flawil, Switzerland) at 45 °C. The sugar-removed concentrated ASE was freeze-dried (SuperModulo freeze-dryer, Thermo Electron Corporation, NY) to produce a powder, which was dissolved in 95% ethanol at a ratio of 1 g: 2 mL to obtain ASE 2. The yield of ASE 2 was approximately 1.26% (w/w) on dry weight basis of ASP.

Preparation of Aqueous EPA Emulsion and Bulk Fish Oil Containing Antioxidants. To prepare an aqueous emulsion of EPA, a modified method was followed based on the procedures described by Okuda et al. (*17*) and Boadi et al. (*18*). Briefly, the emulsion was prepared at a concentration of 1.5 mg of EPA per mL of buffer (pH 7) composed of 0.05 M Tris-HCl, 0.15 M KCl and 1% Tween 20 as an emulsifier at room temperature. The sample was homogenized using a Polytron homogenizer (model PCU, Drehzahlregler, Switzerland) at 4.5 speed for 30 s. The ASE or pure antioxidant compounds were added to emulsions by placing specific volumes of stock solutions (ASE 1 and ASE 2) to obtain various final concentrations in each test tube. The solvent (ethanol) of the added extracts or antioxidant compounds were removed completely under a nitrogen flow and then mixed with 0.8 mL (for peroxyl radical-induced oxidation) or 0.9 mL (for heat- and UV-induced oxidation) of the emulsion in disposable borosilicate glass tubes (13 mm × 100 mm) with breathable caps. The resulting emulsions were also made to contain 0.1 mL of ethanol per 1 mL of emulsion (or 10%, v/v ethanol) in order to ensure the complete dissolution of the extracts.

To prepare bulk fish oil samples containing different concentrations of ASE 1, ASE 2 and commercial food antioxidants, the antioxidative additives were incorporated by placing desirable volumes of stock solutions in each test tube and drying the solvent completely under a nitrogen flow. Eighty microliters of fish oil was then added to the test tubes. To ensure complete dissolution of extracts and antioxidants in bulk oil, 20 μL of ethanol was also added. All the emulsion and bulk oil samples were vortexed for 15 s to obtain homogeneous dispersions.

LC−MS/MS Analysis of Phenolics in Apple Skin Extracts. Analyses of major individual phenolic compounds present in apple skin extracts were performed according to the procedure reported by Rupasinghe et al. (*16*). All analyses were performed using a Waters Alliance 2695 separations module (Waters, Milford, MA) coupled with a Micromass Quattro *micro* API MS/MS system and controlled with Masslynx V4.0 data analysis system (Micromass, Cary, NC). The column used was a Phenomenex Luna C_{18} (150 mm × 2.1 mm, 5 μm) with a Waters X-Terra MS C_{18} guard column. For the separation of the flavonol, flavan-3-ol, phenolic acid and dihydrochalcone compounds, a gradient elution was carried out with 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a flow rate of 0.35 mL/min. A linear gradient profile was used with the following proportions of solvent A applied at time *t* (min) (*t*, A%): (0, 94%), (9, 83.5%), (11.5, 83%), (14, 82.5%), (16, 82.5%), (18, 81.5%), (21, 80%), (29, 0%), (31, 94%), (40, 94%). The analysis of cyanidin-3-*O*-galactoside was carried out using the mobile phases of 5% formic acid in water (solvent A) and 5% formic acid in methanol (solvent B) at a flow rate of 0.35 mL/min. The linear gradient profile used was as follows (t, A%): (0, 90%), (10, 70%), (17, 60%), (21, 48.8%), (26, 36%), (30, 10%), (31, 90%), (37, 90%).

Electrospray ionization in negative ion mode (ESI−) was used for the analysis of the flavonol, flavan-3-ol, phenolic acid and dihydrochalcone compounds. The following conditions were used: capillary voltage −3000 V, nebulizer gas (N_2) temperature 375 °C at a flow rate of 0.35 mL/min. For the analysis of cyanidin-3-*O*-galactoside, electrospray ionization in positive ion mode (ESI+) was used. The settings for the positive ion experiments were as follows: capillary voltage 3500 V, nebulizer gas 375 °C at a flow rate of 0.35 mL/min. The cone voltage (25 to 50 V) was optimized for each individual compound. Multiple reaction-monitoring (MRM) mode using specific precursor/product ion transitions was employed for quantification in comparison with standards: m/z 301 → 105 for quercetin (Q), m/z 609 → 301 for Q-3-*O*-rutinoside, m/z 463 → 301 for Q-3-*O*-glucoside and Q-3-*O*-galactoside, m/z 448 → 301 for Q-3-*O*-rhamnoside, m/z 595 → 301 for Q-3-*O*-peltoside, m/z 273 → 167 for phloritin, m/z 435 → 273 for phloridzin, m/z 353 → 191 for chlorogenic acid, m/z 179 → 135 for cafeic acid, m/z 193 → 134 for ferulic acid and isoferulic acid, m/z 449 → 287 for cyanidin-3-*O*-galactoside, m/z 289 → 109 for catechin, m/z 290 → 109 for epicatechin, and m/z 305 → 125 for epigalocatechin. In MRM experiments, both quadrupoles were operated at unit resolution.

Antioxidant Capacity Assays. Determinations for total phenolic content (TPC), oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) were performed according to the procedures described by Rupasinghe et al. (*16*) based on the studies of Singleton et al. (*19*), Prior et al. (*20*) and Benzie and Strain (*21*), respectively. The results for TPC assay were reported as mg gallic acid equivalents (GAE) per L of stock extract solution (mg GAE/L). Similarly, the results for ORAC and FRAP assays were given as mg Trolox equivalents per L of stock extract solution (mg Trolox/L). DPPH• radical scavenging assay was carried out as described by Blois (*22*) with some modifications as follows: Various dilutions of the test materials (1.5 mL of pure antioxidants or ASE) were mixed with 1.5 mL of a 0.2 mM ethanolic DPPH• solution. After an incubation period of 30 min at 25 °C, the absorbance at 520 nm was recorded as A_{sample} using 96-well microplates and a FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC). The outer wells of the microplates were not used to ensure temperature uniformity in all wells. The same procedure was carried out for the solution without the test material and its absorbance was recorded as $A_{control}$. The free radical scavenging activity of each solution was then calculated as percent inhibition according to the following equation:

$$\%\ \text{inhibition} = 100\,(A_{control} - A_{sample})/A_{control} \quad (1)$$

Antioxidant activities of test compounds or extracts were expressed as IC_{50}, defined as the concentration of the test material required to cause a 50% decrease in initial DPPH• concentration. Trolox was used for comparing the radical scavenging activity with those of ASE 1 and ASE 2.

Each data point in the calibration curves is the mean of three independent measurements (triplicate).

Induction of Oxidation. Oxidation conditions for emulsion and bulk oil samples were optimized separately to provide maximum hydroperoxide formation determined by ferric thiocyanate (FTC) test and maximum secondary oxidation product formation determined by thiobarbituric acid reactive substances (TBARS) assay. For followup of primary oxidation products of EPA emulsions by FTC test, oxidation was induced by (i) heating at 50 °C for 2 min in a shaking water bath, (ii) exposing to UV light (one full spectrum terrarium lamp at 18 cm distance, Repti Glo 2.0uvB; 800 lm, 13 W, HAGEN, China) at room temperature for 20 min using a horizontally rotating shaker at 150 rpm and (iii) adding peroxyl radical generator, AAPH (100 μL of 100 mM) and incubating at room temperature for 40 min using a horizontally rotating shaker at 150 rpm. For the FTC assay of bulk fish oil samples, heating at 70 °C for 10 min, exposure to UV light at room temperature for 1 h and incubating at room temperature for 60 min with the addition of 10 μL of 0.1 M AAPH were the optimum conditions for peroxide formation. For following TBARS formation as secondary products in EPA emulsions with or without ASE or pure antioxidants, inductions of oxidation were performed as (i) heating at 70 °C for 3 h (ii) exposing to UV light at room temperature for 24 h and (iii) adding AAPH (100 μL of 100 mM) and incubating at room temperature for 20 h. For the TBARS measurements of bulk fish oil samples, the induction conditions were the same as the conditions mentioned for emulsions. At the end of all oxidations, 100 and 10 μL of 1000 ppm BHT in ethanol were added to emulsion and bulk oil samples, respectively, to stop oxidation immediately. Triplicate samples at each concentration of antioxidants or ASE were subjected to oxidation and analyzed immediately with corresponding controls.

Ferric Thiocyanate (FTC) Test. The procedure was adapted from Osawa and Namiki (*23*) to perform the test in 96-well microplates using a FLUOstar OPTIMA microplate reader (BMG Labtech, Durham, NC) as follows: At the end of the incubations of emulsion or bulk oil samples, a 30 μL aliquot was taken from the mixture and diluted with 210 μL of 75% ethanol, followed by the addition of 30 μL of 3% ammonium thiocyanate. Precisely 3 min after addition of 30 μL of 2 mM ferric chloride in 3.5% HCl, the absorbance for the red color was measured at 500 nm. The level of lipid oxidation in all oxidized samples was calculated as percent inhibition according to eq **1** wherein A_{sample} represents the absorbance for the sample containing the antioxidant and $A_{control}$ represents the absorbance for the sample that does not contain any antioxidant.

Thiobarbituric Acid Reactive Substances (TBARS) Assay. After the completion of the oxidation treatments, TBARS assay was followed by a modified method reported by Okuda et al. (*17*) and Boadi et al. (*18*), as follows: The TBA reagent (1 mL of 15% (w/v) TCA and 0.375% (w/v) TBA in 0.25 M HCl) was added to all of the oxidized emulsion and bulk fish oil samples and vortexed. The reaction mixtures were then placed in a water bath at 80 °C for 15 min. At the end of this time, the samples were cooled to room temperature immediately and centrifuged at 2000 rpm for 10 min (model Durafuge 300, Precision Scientific, Asheville, NC). The absorbances of the supernatants were measured at 532 nm using 96-well microplates in the FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC). The results were given as percent inhibition of oxidation experienced by the sample containing the antioxidative additive calculated as in eq **1**.

Oxidative Stability under Accelerated Conditions Using Rancimat. The resistance of bulk fish oil samples containing ASE 1, ASE 2 or pure antioxidant compounds to auto-oxidation was measured at 50, 60, 70, and 80 °C using the Rancimat 743 (Metrohm AG, Herisau, Switzerland). Oil samples without any antioxidants (control) were also analyzed under the same conditions. The time taken until there is a sharp increase of conductivity measured by the instrument is termed as the induction time (IT) that is expressed in hours (h). IT was determined by the intersection of the baseline with the tangent to the conductivity curve. The desirable concentrations of the antioxidant substances (extracts or pure compounds) in oil samples were ensured by placing calculated volumes of stock solutions into the Rancimat vessels, drying the solvent (ethanol) and then by adding 3.0 ± 0.05 g of fish oil. To provide homogeneous dispersion of the antioxidant materials in oil samples, 500 μL of ethanol was added to each vessel and the mixtures were vortexed for 5 min. All the samples were prepared and analyzed in triplicate.

Table 1. The Concentrations of Polyphenolic Compounds of the Two Apple Skin Extracts Prepared from "Northern Spy" Apples

polyphenolic subclass	compound	polyphenolic content[a] (μg/mL) ASE 1	ASE 2
flavonols	quercetin-3-*O*-peltoside	nd	73.5 ± 4.1
	quercetin-3-*O*-galactoside	133 ± 6.9	11774 ± 518
	quercetin-3-*O*-glucoside	18.8 ± 1.2	3904 ± 156
	quercetin-3-*O*-rhamnoside	53.0 ± 2.7	10440 ± 417
	quercetin-3-*O*-rutinoside	5.1 ± 0.4	2748 ± 104
	quercetin	2.2 ± 0.9	284 ± 11.4
dihydrochalcones	phloridzin	61.4 ± 2.9	1827 ± 71
	phloretin	1.1 ± 0.1	9.5 ± 0.4
phenolic acids	chlorogenic acid	34.8 ± 2.4	4087 ± 204
	cafeic acid	0.8 ± 0.08	36.5 ± 1.8
	ferulic acid	1.1 ± 0.1	23.0 ± 0.9
	isoferulic acid	nd	27.5 ± 1.1
anthocyanins	cyanidin-3-*O*-galactoside	5.3 ± 0.3	1800 ± 72
flavan-3-ols	(+)-catechin	11.5 ± 0.9	359 ± 14.3
	(−)-epicatechin	71.0 ± 4.2	4627 ± 185
	epigalocatechin	nd	5.5 ± 0.2
total phenolics detected by LC−MS/MS		399.1	42025.5

[a] Mean ± standard deviation of three replicates; nd, not detected.

Statistical Analysis. All measurements were done in triplicate, and the values were reported as mean ± standard deviation (SD). All the experiments except for Rancimat were conducted on two different dates within a week (two independent experiments). The design for all the parameters (except for Rancimat) was randomized blocks design (RBD) with experimental run as the blocking factor, and antioxidant source and concenration in combination as the factor of interest. For IT, a completely randomized design (CRD) was selected. The assumptions of normality of error terms were tested using the Anderson−Darling test. Assumptions of constant variance were checked by plotting residual versus fits scatter diagram. The data were analyzed using the general linear model (GLM) procedure of the SAS Institute, Inc. Significant differences among means were determined by the Tukey's Studentized range test at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Phenolic Constituents in ASE. The total of major phenolics present in ASE 1 and ASE 2 was determined by LC−MS/MS to be 399.1 μg/mL and 42025.5 μg/mL, respectively (**Table 1**). The major polyphenolic compounds detected in the ethanolic ASE belong to subclasses of flavonols, dihydrochalcones, flavan-3-ols, phenolic acids, and anthocyanins and were similar to those reported by other investigations of apple skins (*24*, *25*). The most abundant phenolics in ASE 1 and ASE 2 were quercetin-3-*O*-galactoside, quercetin-3-*O*-rhamnoside, and (−)-epicatechin. The concentration of phenolics found for ASE 2 was 105-fold higher than that of ASE 1.

Antioxidant Capacity Assays. The antioxidant capacity of ASE 1 and ASE 2 measured by FRAP, ORAC, and DPPH• assays indicated that ASE 2 has greater antioxidant capacity than ASE 1 (**Table 2**). The results obtained from the FRAP assay showed that both extracts have ferric ion reducing activity with ASE 2 having 317 times higher activity than ASE 1. Similarly, ORAC values, which indicate the protection from fluorescence decaying during peroxyl radical-induced oxidation is followed in the presence of antioxidative substances, showed that ASE 2 provides 134-fold higher activity than ASE 1. The IC_{50} values obtained from DPPH• radical scavenging assay indicated that the ASE 2 has the greatest radical scavenging capacity, followed by ASE 1 and Trolox. Both extracts showed higher antioxidant activity by donating electrons to the synthetic DPPH• radicals than Trolox, the water-soluble

Table 2. Antioxidant Activities of the Apple Skin Extracts Determined by Various Antioxidant Assays

antioxidant activity assay	ASE 1	ASE 2
TPC (mg gallic acid equivalents per L of extract solution) ($R^2 = 0.99$)[a]	6.7 ± 0.6	1,990 ± 74
FRAP (mg Trolox equivalents per L of extract soltion) ($R^2 = 0.99$)	728 ± 32	230,773 ± 6,100
ORAC (mg Trolox equivalents per L of extract solution) ($R^2 = 0.97$)	147 ± 0.7	19,702 ± 150
DPPH (IC_{50}, μg/mL extract solution; IC_{50} for Trolox = 178 μg/mL extract solution) ($R^2 > 0.96$)	129 ± 0.6	36.3 ± 0.3

[a] R^2, correlation coefficient of the calibration curve of the standard used for each assay.

Table 3. Percent Inhibition of Heat-, UV- and AAPH-Induced Oxidation of EPA Emulsion and Bulk Fish Oil Samples by Apple Skin Extracts (ASE 1 and ASE 2) with Comparison to α-Tocopherol, BHT and TBHQ Determined by FTC Test[a]

	EPA emulsion[b]				bulk fish oil[c]			
		% inhibn of oxidn (%)				% inhibn of oxidn (%)		
antioxidant source	concn (μg/mL)	heat	UV light	AAPH	concn (μg/mL)	heat	UV light	AAPH
ASE 1	200	53.1 ± 2.8 gh	40.9 ± 4.1 h	54.5 ± 3.0 h	2000	25.3 ± 2.5 g	34.7 ± 0.4 g	65.9 ± 1.7 i
	400	55.2 ± 1.7 fgh	49.3 ± 1.7 g	72.1 ± 1.5 bc	4000	42.5 ± 1.2 e	35.5 ± 0.9 fg	79.3 ± 1.7 gh
	2000	63.0 ± 1.7 de	57.1 ± 1.7 f	69.6 ± 3.4 cd	20000	60.2 ± 3.3 c	44.1 ± 0.8 e	77.1 ± 2.3 h
ASE 2	4	59.7 ± 4.2 ef	64.0 ± 1.9 e	60.5 ± 2.3 fg	40	35.6 ± 1.5 f	42.2 ± 3.6 ef	80.5 ± 2.4 hfg
	20	66.8 ± 3.1 cd	65.8 ± 2.3 de	76.4 ± 2.0 b	200	43.8 ± 1.5 e	55.7 ± 0.5 d	83.7 ± 2.1 ef
	40	68.8 ± 5.2 c	73.3 ± 2.9 b	92.8 ± 2.2 a	400	51.0 ± 1.7 d	60.9 ± 0.2 cd	97.0 ± 2.1 a
α-tocopherol	20	2.4 ± 1.6 k	3.1 ± 1.1 j	2.7 ± 2.2 j	20	20.9 ± 2.2 h	35.5 ± 2.5 fg	40.3 ± 2.5 j
	200	13.1 ± 2.5 j	35.0 ± 1.9 i	32.7 ± 2.9 i	200	33.1 ± 3.3 f	57.9 ± 1.1 cd	81.7 ± 1.7 efg
	400	17.3 ± 2.4 j	56.1 ± 1.5 f	30.0 ± 2.7 i	400	89.6 ± 1.3 a	91.0 ± 2.6 a	90.9 ± 1.5 bc
BHT	20	81.6 ± 2.5 a	70.2 ± 2.0 bc	68.0 ± 2.2 cde	20	86.8 ± 0.8 ab	62.5 ± 0.6 c	89.1 ± 2.0 bc
	200	50.7 ± 1.3 h	63.2 ± 0.7 e	64.1 ± 1.6 ef	200	23.4 ± 0.8 gh	64.2 ± 7.6 c	84.3 ± 2.2 de
	400	40.7 ± 2.4 i	53.9 ± 0.6 f	66.6 ± 1.9 de	400	46.1 ± 1.3 e	58.9 ± 3.4 cd	87.7 ± 1.8 cd
TBHQ	20	74.5 ± 1.8 b	81.7 ± 2.3 a	57.9 ± 2.6 gh	20	84.2 ± 1.8 b	79.1 ± 0.4 b	92.1 ± 2.3 b
	200	57.5 ± 1.3 fg	68.4 ± 1.6 cd	67.7 ± 2.9 cde	200	34.1 ± 1.3 f	40.5 ± 7.0 ef	90.3 ± 1.7 bc
	400	53.7 ± 0.7 gh	57.8 ± 2.9 f	65.8 ± 2.3 de	400	36.3 ± 0.8 f	39.4 ± 3.5 efg	91.6 ± 2.4 b
$Tukey_{(0.05)}$		<0.0001	<0.0001	<0.0001		<0.0001	<0.0001	<0.0001

[a] Means followed by the same letter within each row are not significantly different [Tukey's Studentized range test ($P < 0.05$)]. Blocking was done by independent experiments conducted at two different times. [b] Significant effect of experimental run observed for UV light in EPA emulsion. [c] Significant effect of experimental run observed for heat, UV light, AAPH in bulk fish oil.

form of α-tocopherol. The magnitude of the differences obtained from all three antioxidant capacity assays seems to be dependent on the mode of action of the assays. However, the content of total phenolics present in the extracts was directly proportionate to the antioxidant capacity in the extracts.

Inhibition of Formation of Primary Oxidation Products (FTC Test). The ferric thiocyanate test is based on the complexation of ferric ion which is generated from a ferrous ion after donation of an electron to an unstable lipid hydroperoxide molecule for homolytic cleavage. In the presence of an antioxidant, hydroperoxides will be formed in lower amounts; thus, oxidation of ferrous ions will occur in lower rates. Generally, a concentration-dependent increase in % inhibition of hydroperoxide formation was observed for ASE 1, ASE 2 and α-tocopherol containing EPA emulsions oxidized by heat induction, UV light induction, and peroxyl radical induction (**Table 3**). Synthetic antioxidants, BHT and TBHQ, at the lowest tested concentration (20 μg/mL), caused higher inhibition of heat- and UV-induced oxidation than the concentrations of 200 and 400 μg/mL ($p < 0.05$) (**Table 3**) possibly due to prooxidant properties of BHT and TBHQ at higher concentrations. Overall, ASE 1 at 2000 μg/mL, ASE 2 at 40 μg/mL, α-tocopherol at 400 μg/mL and BHT and TBHQ at 20 μg/mL seem to possess the most preserving effect against hydroperoxide formation in the EPA emulsion system.

Higher concentrations of ASE 1 and ASE 2 were added to bulk fish oil samples than were added to emulsion samples (**Table 3**) due to the fact that the bulk oil system contains a greater amount of PUFA. ASE 1 and ASE 2 in the bulk oil showed concentration dependent protection for heat-, UV-, and AAPH-induced oxidation. α-Tocopherol in bulk oil system was found to provide greater inhibition of peroxide formation when compared to that in EPA emulsions (**Table 3**). The reason for this may be attributed to higher solubility or dispersion of α-tocopherol in a bulk oil system because of its higher hydrophobic characteristics due to higher number of carbon atoms and aliphatic alkyl side chain. Again, BHT and TBHQ were the most effective at 20 μg/mL in bulk oil ($p < 0.05$) compared to higher concentrations tested. Overall, ASE 1 (20,000 μg/mL) and ASE 2 (400 μg/mL) in bulk oil seem to exert significant protection against lipid peroxidation under the three induction systems.

Inhibition of Formation of Secondary Oxidation Products (TBARS Assay). One of the secondary products of lipid oxidation, malonaldehyde (MDA), is formed by β-scission of peroxidized PUFA and is commonly measured by derivatization with TBA to yield a red chromophore. In general, concentration-dependent inhibition of formation of MDA was observed for ASE 1 and ASE 2 under all three oxidation-induction systems (**Table 4**). It was interesting to note that α-tocopherol was not effective in EPA emulsion system studied when compared to ASE, BHT and TBHQ (**Table 4**), which is similar to the finding from the FTC test (**Table 3**). Again, the antioxidant potency of ASE 2 was found to be greater than that of ASE 1 by TBARS test for emulsions.

Incorporation of ASE in bulk fish oil exhibited a concentration-dependent antioxidant activity. Interestingly, under the experimental conditions studied, nearly complete inhibition of heat-, AAPH- and UV-induced oxidation of bulk fish oil was observed when ASE 2 was incorporated in fish oil at a concentration of 400 μg total phenolics/mL (**Table 4**). The antioxidant activity of ASE 2 at this concentration seems to be comparable to

Table 4. Percent Inhibition of Heat-, UV- and AAPH-Induced Oxidation of EPA Emulsion and Bulk Fish Oil Samples by Apple Skin Extracts (ASE 1 and ASE 2) with Comparison to α-Tocopherol, BHT and TBHQ Determined by TBARS Assay[a]

	EPA emulsion[b]				bulk fish oil[c]			
		% inhibn of oxidn (%)				% inhibn of oxidn (%)		
antioxidant source	concn (μg/mL)	heat	UV light	AAPH	concn (μg/mL)	heat	UV light	AAPH
ASE 1	200	57.6 ± 4.3 e	21.6 ± 4.0 d	60.1 ± 4.1 d	2000	10.7 ± 2.3 h	18.5 ± 2.4 f	15.3 ± 2.3 h
	400	68.7 ± 4.0 d	33.0 ± 3.1 c	71.9 ± 4.4 c	4000	42.8 ± 3.7 g	42.8 ± 2.9 e	32.2 ± 2.8 g
	2000	73.8 ± 3.8 cd	41.0 ± 2.8 b	87.8 ± 5.4 a	20000	61.8 ± 4.3 d	77.3 ± 3.2 d	57.7 ± 4.2 f
ASE 2	4	77.2 ± 3.5 c	20.0 ± 2.1 d	48.0 ± 3.9 e	40	54.1 ± 4.0 e	96.1 ± 3.4 a	72.2 ± 3.7 e
	20	80.3 ± 2.9 ab	29.7 ± 2.6 c	71.1 ± 3.9 c	200	77.2 ± 5.2 c	98.1 ± 1.6 a	90.1 ± 4.6 c
	40	84.3 ± 3.2 a	40.6 ± 3.7 b	85.21 ± 5.7 a	400	95.7 ± 3.1 a	98.6 ± 0.9 a	95.7 ± 3.3 ab
α-tocopherol	20	0.0 ± 0.0 i	0.0 ± 0.0 f	0.0 ± 0.0 f	200	82.7 ± 5.3 b	85.8 ± 3.3 c	79.7 ± 4.1 d
	400	2.9 ± 1.6 i	6.8 ± 2.4 e	3.5 ± 3.1 f	400	92.2 ± 5.6 a	90.6 ± 4.2 b	91.8 ± 4.6 bc
BHT	20	34.7 ± 2.4 g	23.7 ± 3.4 d	69.3 ± 4.4 c	20	49.2 ± 3.6 f	90.5 ± 3.2 b	91.9 ± 4.6 bc
	400	43.1 ± 1.8 f	39.7 ± 1.6 b	63.2 ± 3. 9 d	200	95.3 ± 2.8 a	96.7 ± 2.9 a	97.7 ± 1.7 a
TBHQ	20	53.0 ± 3.6 e	77.3 ± 4.1 a	77.8 ± 4.9 b	nd			
	400	16.2 ± 1.4 h	24.2 ± 2.2 d	60.6 ± 3.6 d	nd			
$Tukey_{(0.05)}$		<0.0001	<0.0001	<0.0001		<0.0001	<0.0001	<0.0001

[a] Means followed by the same letter within each row are not significantly different [Tukey's Studentized range test ($P < 0.05$)]. Blocking was done by independent experimentals conducted at two different times. [b] Significant effect of experimental run observed for heat, UV light, AAPH in EPA emulsion. [c] Significant effect of experimental run observed for heat, UV light, AAPH in bulk fish oil.

Table 5. Induction Time (IT) Values in Hours at Different Temperatures Determined by Rancimat for Bulk Fish Oil with and without Apple Skin Extracts, α-Tocopherol and BHT[a]

		IT,[b] h				
antioxidant source	concn (μg/mL of bulk oil)	50 °C	60 °C	70 °C	80 °C[b]	av antioxidant effect
control		24.3 ± 0.2 eA	10.8 ± 0.6 cB	1.1 ± 0.1 bC	0.6 ± 0.0 cD	9.2 ± 10.1 c
ASE 1	20000	38.3 ± 0.0 dA	13.2 ± 0.7 cB	1.6 ± 0.1 bC	1.1 ± 0.0 bC	13.6 ± 15.8 b
ASE 2	400	58.5 ± 0.7 aA	35.6 ± 1.2 bB	4.3 ± 0.0 aC	2.3 ± 0.2 aD	25.2 ± 24.4 a
α-tocopherol	400	52.3 ± 1.1 cA	38.6 ± 1.9 aB	4.8 ± 0.2 aC	2.4 ± 0.2 aD	24.5 ± 22.5 a
BHT	200	56.0 ± 2.0 bA	39.8 ± 1.1 aB	1.3 ± 0.1 bC	1.0 ± 0.0 bC	24.5 ± 25.1 a
av temp effect		45.9 ± 13.3 A	27.59 ± 13.3 B	2.6 ± 1.7 C	1.5 ± 0.8 D	

[a] Means followed by the same lowercase letter within each column and the same uppercase letter within each row are not significantly different [Tukey's Studentized range test ($P < 0.05$)]. Significant interaction effect of concentration and induction time was observed (concn × IT < 0.0001). [b] Induction time values were transformed (reciprocal) before analysis. Untransformed values are shown in the table.

that of α-tocopherol at 400 μg/mL bulk oil ($p < 0.05$) and BHT at 200 μg/mL bulk oil ($p < 0.05$). Thus, based on TBARS results, approximately 400 μg/mL of ASE 2 could be the optimal concentration against formation of secondary oxidation products in bulk fish oil.

Stability of Fish Oil Evaluated by Rancimat. In the lipid oxidation process, there is usually an IT before massive oxidation occurs. The IT is measured as the time required for a sudden and rapid change in the rate of the oxidation process, and this can be evaluated by subjecting the substrate to accelerated conditions. Usually, temperatures in the range of 100−130 °C and air flow of 20 L/h are used as the accelerated conditions in determining IT of lipid substrates. Lipids highly sensitive to oxidation are often analyzed at lower temperatures, ranging between 60 and 80 °C. For example, fish oil was analyzed at 68 °C (*26*). The rate of oxidation may be limited by the mechanism of degradation because the rate of formation of volatile acids is likely reduced above a certain temperature. At high temperatures, the IT becomes too low for an accurate assessment of antioxidant efficacy due to underestimation and this phenomena was observed during our preliminary Rancimat experiments performed at 100 and 110 °C. Thus, 50, 60, 70, and 80 °C were chosen to perform accelerated oxidation for the EPA-enriched fish oil (**Table 5**).

In the present study, the concentrations of tested antioxidants were chosen based on the results from FTC and TBARS assays performed for bulk oil samples (**Table 5**). The best homogeneous dispersion of the antioxidant extracts and pure compounds was also taken into consideration. Although the high concentration of ASE 1 at 20000 μg/mL fish oil seems excessive as a concentration of a natural fruit extract to be added to an oil sample, it was dispersed in the fish oil homogeneously. For all the antioxidants, the IT decreases as the temperature increases. The IT for ASE 2 were higher than that for ASE 1 ($p < 0.05$) at all temperatures, and the average IT of ASE 2 was 1.8- and 2.7-fold higher than that for ASE 1, and fish oil without any antioxidant, respectively. Interestingly, the average IT of ASE 2 was similar to that of α-tocopherol (at the same concentration) and BHT (at a half concentration of ASE 2) ($p < 0.05$). α-Tocopherol was also found to provide a very good antioxidative protection to bulk fish oil (**Table 5**), which is consistent with the TBARS results obtained for bulk oil samples (**Table 4**). However, α-tocopherol acted as a weak antioxidant when it was tested in PUFA emulsions, according to both FTC and TBARS results.

Oxidation of various lipid components in food reduces the nutritional value and generates rancidity, causing undesirable odors and flavors. The oxidative stability of a food is therefore an important parameter in determining its shelf life. The storage stability at 20 °C of fish oil incorporated with tested antioxidants was also estimated (**Table 6**) based on the IT. The estimated oxidative stability at 20 °C provided by ASE 2 (115 h) was similar to that of BHT (116 h) and α-tocopherol (107 h). ASE 1 also seems to provide considerable protection (69 h) against lipid oxidation of fish oil when compared to control fish oil sample, but

Table 6. The Estimated Induction Time (IT) Values in Hours at 20 °C Determined by Rancimat for Bulk Fish Oil with and without Apple Skin Extracts, α-Tocopherol and BHT

antioxidant source	IT (h) at 20 °C	eq	R^{2a}
control	45.6	$y = -0.81x + 61.7$	0.88
ASE 1	69.0	$y = -1.23x + 93.7$	0.83
ASE 2	115.3	$y = -2.00x + 155.3$	0.92
α-tocopherol	107.1	$y = -1.84x + 143.8$	0.91
BHT	116.0	$y = -2.03x + 156.7$	0.89

[a] R^2, correlation coefficient of the function between temperature and IT.

its impact seems to be lower than that of ASE 2, α-tocopherol and BHT.

Based on the results obtained by FTC, TBARS and Rancimat, it is clear that the polyphenolic-enriched ASE 2 inhibits the formation of primary and secondary oxidation products at significant levels when aqueous EPA emulsion and bulk fish oil are subjected to oxidatation. The ability of ASE 2 to stabilize fish oil is nearly similar to that of the food antioxidants, α-tocopherol and BHT. This high antioxidative potential of ASE 2 can be attributed to its significantly high total phenolic content (42 mg/mL) after removal of sugars, organic acid and other low molecular weight hydrophilic compounds from the crude ethanol extract (ASE 1).

Apple extracts have previously been examined for lipid stabilizing properties using linoleic acid, an omega-6 fatty acid, in emulsion systems of SDS/linoleic acid system, the β-carotene−linoleic acid model system (β-CLAMS), and a micellar system to mimic LDL oxidation (*27*, *28*). Flavan-3-ols were found to have the highest correlation with, or contribution to, antioxidant activity determined by the β-CLAMS assay and the micellar system, and epicatechin was the most effective of those tested in the SDS system (*27*, *28*). Phloretin and its glucoside, phloridzin, could efficiently trap reactive methylglyoxal (MGO) and glyoxal (GO), which are reactive carbonyl species, to form mono- and di-MGO or GO adducts under physiological conditions (pH 7.4, 37 °C) (*28*, *29*). Phloridzin was determined in ASE 2 in considerable amounts (1827 ± 71 μg/mL). Recently, it has been shown that quercetin-3-*O*-glucoside, another major flavonoid of apple skin, exhibits a better antioxidant activity than BHT in bulk fish oil (*30*). However, to our knowledge this is the first study to demonstrate the potential of ASE to prevent oxidation of omega-3 fatty acid-enriched fish oil.

In conclusion, the ethanol-based extracts prepared from apple skins can be recognized as a naturally sourced antioxidant mixture for improving the oxidative stability of fish oil and related PUFA. Novel antioxidative preservatives with different physiochemical properties are needed for diversified food systems because the physical and chemical nature of a selected food system such as an emulsion or bulk oil may require an antioxidant additive with different physiochemical properties. Since epidemiological evidence has demonstrated the dietary intake of phenolic compounds with lower incidence of chronic diseases such as cardiovascular disease and cancer, the use of ASE as a food additive could also provide positive health benefits. Apple skins are waste products of apple pie and sauce manufacturing; therefore, a potential exists to use this underutilized bioresource in the development of natural food antioxidants.

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