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Enzymatic Synthesis of Lipophilic Rutin and Vanillyl Esters from Fish Byproducts

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ABSTRACT: Lipase-catalyzed synthesis of lipophilic phenolic antioxidants was carried out with a concentrate of n-3 polyunsaturated fatty acids (PUFAs), recovered from oil extracted from salmon (*Salmon salar*) byproduct. Vanillyl alcohol and rutin were selected for the esterification reaction, and obtained esters yields were 60 and 30%, respectively. The antioxidant activities of the esters were compared with those of commercial butylated hydroxytoluene (BHT) and α-tocopherol using DPPH radical scavenging and thiobarbituric acid assays. In the DPPH assay, rutin esters showed better activity than vanillyl esters, and on the contrary in lipophilic medium, vanillyl esters were found to be superior to rutin esters. In bulk oil system, the antioxidant activities of rutin and vanillyl derivatives were lower than that of BHT and α-tocopherol, but in emulsion, they showed better activity than α-tocopherol. By attaching to natural phenolics, the PUFAs are protected against oxidation, and PUFA improves the hydrophobicity of the phenolic, which could enhance its function in lipid systems.

KEYWORDS: lipophilization, PUFA, phenolics, antioxidant

1. INTRODUCTION

The use of phenolic antioxidants to protect food-based products from oxidation and also to improve the shelf life of lipid-containing products has nutritional and pharmaceutical relevance.[1] The use of natural phenolics as antioxidants has been increasing because the most widely used and commercially available antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and *tert*-butylhydroquinone (TBHQ) are not considered safe due to their suspected role as promoters of carcinogenesis.[2] The other alternative is the use of natural phenolics, which is, however, limited due to their poor solubility in hydrophobic media. To address this, several papers on the lipophilization of natural phenolics to prepare lipophilic antioxidants have been published.[3−6] Natural phenolics, which are abundant in the plant kingdom, are of particular interest because of their potential biological properties, such as antioxidant, chelating, free radical scavenging, anti-inflammatory, antiallergic, antimicrobial, antiviral, and anticarcinogenic.[1]

Fish oils are receiving increased interest due to their high content of omega-3 polyunsaturated fatty acids (PUFAs), which are reported to exert positive effects on human health.[7,8] In addition, PUFAs serve a role as precursors of a wide variety of metabolites (prostaglandins, leukotrienes, and hydroxyl fatty acids) regulating critical biological functions. Because mammals have limited ability to synthesize these unsaturated fatty acids, they must be supplied in the diet. The practical use of omega-3 PUFAs for a preventive purpose is limited because of their high susceptibility to autoxidation, which is responsible for the unfavorable off-flavor in rancid oils. A solution would be to combine PUFA with natural antioxidants such as flavonoids, by forming an ester bond. Viskupicova et al.[9] observed that long-chain fatty acids conjugated to rutin provided improved stabilization of sunflower oil against oxidation compared to conjugates with short-chain fatty acids. On the basis of this observation, and the increasing demand for omega-3 PUFA, it was assumed that esterification of PUFA concentrate with natural phenolic compounds can be helpful in protecting both the PUFAs and the product in which the PUFA−phenolic derivatives are present against oxidation. They could, for example, be useful for protecting various types of fish oil based products. The PUFA−phenolic derivatives prepared would confer the beneficial effects of both the PUFA and the phenolic compounds. In addition to antioxidant activity, products of natural phenolics as esters of PUFA have been reported to show significantly improved anti-inflammatory activities as well antiviral and anticancer activities that were not present in the original phenolic molecule, suggesting that PUFA moieties contribute to the bioactivities of the ester derivatives.[10]

Recently, we reported the utilization of byproduct from fish industries as a source of omega-3 PUFAs.[11,12] To the best of our knowledge, there are no reports on the utilization of PUFA concentrate from fish oil to prepare lipophilic antioxidants and test them on stabilization of fish oils. Two natural phenolics, rutin and vanillyl alcohol, were selected for this study. Enzymatic lipophilization of rutin with individual fatty acids (C4−C18) has been reported.[9,13] Similarly, enzymatic esterification of vanillyl alcohol with individual fatty acids to prepare capsinoid derivatives has been reported.[14,15] The objective of the present study was to prepare PUFA−phenolic esters exemplified by rutin and vanillyl alcohol through enzymatic esterification of PUFA concentrate obtained from salmon byproduct and to employ the products as lipophilic antioxidants in PUFA-enriched oil and aqueous emulsion systems.

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2. MATERIALS AND METHODS

2.1. Materials. Immobilized lipase B from *Candida antarctica* (Novozym 435) was a kind donation from Novozymes A/S, Denmark. Protex 30 L (≥2750 GSU/g) was a gift from Genencor, a division of Danisco A/S, Denmark. Rutin, vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), BHT, thiobarbuturic acid, α-tocopherol, and molecular sieves 4 Å were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Precoated silica gel 60 F_{254} TLC plates and silica gel 60 for column chromatography were purchased from Merck (Darmstadt, Germany). Salmon heads (*Salmo salar*) were a kind donation from Kalles Fisk, Goterborg, Sweden. All solvents and other reagents were of analytical grade or HPLC grade purchased from Merck.

2.2. Preparation of n-3 PUFA Concentrate. The preparation of n-3 PUFA concentrate was achieved in three steps: (a) enzymatic oil extraction, (b) hydrolysis of the obtained oil, and (c) urea complexation for PUFA enrichment. Oil was extracted from salmon heads (*S. salar*) using 0.15% v/w Protex 30 L.[11] The recovered oil was hydrolyzed to free fatty acids (FFAs) using a method described by Haraldsson and Kristinsson[16] with slight modifications. Crude oil (100 mL) was mixed with 250 mL of 90% ethanol containing 15 g of NaOH. The contents were refluxed for 1 h with stirring. The hydrolysis reaction was monitored by TLC, and the reaction was complete within 1 h. The FFAs were recovered by lowering the pH to 2 using 12 N HCl. Recovered FFAs were washed with water to neutralize the acid and then dried over anhydrous sodium sulfate.

The obtained FFA mixture was enriched in PUFA content by the urea inclusion method as described by Hayes et al.[17] Briefly, 50 g of FFAs, 150 g of urea, and 550 mL of 96% ethanol were heated at 65 °C until a homogeneous solution was obtained. The contents were rapidly cooled under running tap water for 10 min to allow crystallization. The crystallized and noncrystallized fractions were separated by filtration. The method for preparation of fatty acid methyl esters (FAME) and the program for FAME analysis by GC were as described by Mbatia et al.[11]

2.3. Lipase-Catalyzed Synthesis of Lipophilic Esters. *2.3.1. Enzymatic Synthesis of Rutin Fatty Acid Esters.* This was performed following a method reported by Lue et al.[13] Briefly, rutin (1 g) and PUFA concentrate (1.9 g) 1:4 rutin/PUFA molar ratio were solubilized in dried acetone (300 mL). Immobilized lipase (6 g) and molecular sieves (15 g) were added, and the reaction was agitated at 200 rpm and 50 °C for 96 h. To terminate the reaction, enzyme and molecular sieves were filtered off and acetone was evaporated. The residue was transferred into four centrifuge tubes. Heptane/water 30 mL (4:1 v/v) was used to extract unreacted PUFA. The heptane phase was discarded. Rutin and rutin−PUFA esters were separated using 35 mL of ethyl acetate/hot water (60 °C, 1:6 v/v). Esters were extracted into the ethyl acetate phase. The ethyl acetate phases were pooled, dried over anhydrous sodium sulfate, and evaporated using a rotary evaporator to recover the rutin esters of PUFA as a solid dark-yellow product (0.43 g, 30%).

2.3.2. Enzymatic Synthesis of Vanillyl Fatty Acid Esters. This was performed as described by Kobata et al.[14] with slight modifications. To a mixture of fish oil PUFA (6 g) and vanillyl alcohol (4.5 g) (1:1.5 PUFA/vanillin molar ratio) in acetone (25 mL) were added lipase (2 g) and 2.5 g of molecular sieves, and the mixture was agitated at 200 rpm for 48 h at 50 °C. To stop the reaction, enzyme and molecular sieves were separated by filtration and the filtrate was concentrated in vacuo. The crude product was purified by silica gel column chromatography using hexane/ethyl acetate (96:4 v/v) to obtain the vanillyl esters of PUFA as an oily colorless liquid (5.16 g, 60%).

2.4. Analytical Methods. The reaction products of both rutin and vanillyl esters were monitored by TLC and HPLC and confirmed by LC-MS of purified samples. For rutin esters, mixtures of chloroform/methanol (80:20 v/v) and for vanillyl esters pure chloroform were used as mobile phase for TLC analysis. TLC plates were visualized under UV light (254 nm).

HPLC analysis was carried out using a Dionex Ultimate HPLC system equipped with a Varian 385-LC evaporative light scattering detector (ELSD) and a Luna RP-C18 column (250 × 3.0 mm, 5 μm particle size). This system was also equipped with an autosampler, online degasser, and column heater. The injection volume was 20 μL. Acetonitrile and water containing 0.05% acetic acid were used as mobile phases A and B, respectively, in the following gradient elution: 78−100% A over 50 min, 100% A for 5 min, followed by 10 min re-equilibration time between samples. The column temperature was 25 °C, the flow rate was 0.43 mL/min, and detection was carried out in ELSD using evaporator and nebulizer temperatures of 25 and 40 °C, respectively, and a gas flow of 1.7 standard liters per minute (SLPM).

LC-MS analysis was carried out on a hybrid QSTAR Pulsar quadrupole time-of-flight mass spectrometer (PE Sciex Instruments, Toronto, Canada), equipped with an electrospray ionization (ESI) source. The software used was Analyst QS 1.1, also from PE Sciex. LC-MS was used to characterize the purified vanillyl and rutin PUFA esters. The elution program was similar to that used during RP-HPLC analysis. The scan range was m/z 200−1500. Negative ESI mode was used for PUFA and rutin−PUFA esters, whereas positive ionization mode was used for vanillyl−PUFA esters.

2.5. Determination of Antioxidant Activity. *2.5.1. DPPH Radical Scavenging Activity.* The antioxidant activity was determined by the radical scavenging ability using the stable DPPḢ radical as described by Akowuah et al.[18] Briefly, 200 μL of methanolic solution of the synthesized phenolic lipids (1 or 2 mM) was added to 2 mL of methanolic solution of DPPḢ radical (0.1 mM), and the total volume was made up to 3 mL with methanol. After 60 min of incubation at 30 °C in the dark, the absorbance of the mixture was measured at 517 nm against methanol as blank in an Ultrospec 1000 UV spectrophotometer.

BHT and α-tocopherol were used as positive controls and their concentrations were kept equal to that of synthesized phenolic lipids. The free radical scavenging activity (FRSA in %) of the tested samples was evaluated by comparison with a control (2 mL of DPPH radical solution and 1 mL of methanol). Each sample was measured in triplicate, and an average value was calculated. Antioxidant activity was expressed as a percentage of DPPḢ radical scavenging activity compared to control. The FRSA was calculated using the formula FRSA = $[(A_c - A_s)/A_c] \times 100$, where A_c is the absorbance of the control and A_s is the absorbance of the tested sample after 60 min.

2.5.2. Stabilization of Fish Oil against Oxidation Employing Synthesized Antioxidants. The potential of the synthesized antioxidants to protect fish oil acylglycerol concentrate prepared in our laboratory was evaluated. The acylglycerol concentrate was spiked with vanillyl−PUFA, rutin−PUFA, α-tocopherol, or BHT to a final concentration of 5 or 25 mM. A control sample with no antioxidant added was included. The samples were heated for 6 h in a water bath set at 70 °C with agitation at 170 rpm. The extent of oxidation was determined by the thiobarbituric acid assay (TBARS) as described under section 2.5.4.

2.5.3. Stabilization of Fish Oil Emulsion against Oxidation Using Synthesized Antioxidants. The potential of synthesized antioxidants to protect an emulsion against oxidation was tested. An oil emulsion was prepared from the acylglycerol concentrate as described by Huber et al.[19] Acylglycerol concentrate (10 mg/mL) was dissolved in buffer (pH 7.0) containing 50 mM Tris-HCl, 150 mM KCl, and 1% Tween 20. The contents were sonicated for 20 s (ultrasonic cleaner Branson 200). The emulsion was maintained by agitating the tubes with the emulsion at 400 rpm. The emulsion sample (2 mL) was mixed with vanillyl−PUFA, rutin−PUFA, α-tocopherol, or BHT to a final concentration of 5 or 25 mM. A control without antioxidant was included. The samples were heated at 70 °C for 6 h, and the extent of oxidation was determined using TBARS assay as described under section 2.5.3. The experiment was performed in triplicate.

2.5.4. Thiobarbituric Acid Assay. This assay was performed on the basis of the method described by Huber et al.,[19] with slight modifications.

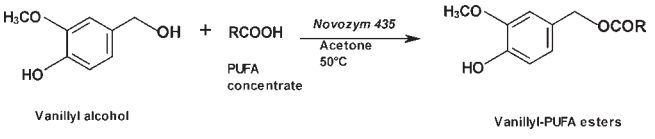
Briefly, 100 μL of lipid sample or 400 μL for emulsion sample was mixed with 2 mL of TBARS reagent (0.375% (w/v) TBA in 250 mM HCl) by agitating the contents at 200 rpm for 5 min. The contents were centrifuged at 2150*g* for 5 min. The lower phase (approximately 1 mL) was carefully transferred into Eppendorf tubes and heated at 80 °C for 20 min. The tubes were then allowed to cool to room temperature and again centrifuged at 2150*g* for 5 min. The absorbance of the lower phase was recorded at 535 nm. Distilled water was used as a blank. The degree of oxidation inhibition was calculated using the formula % inhibition = $(1 - (A_s/A_c)) \times 100$, where A_s is the absorbance of sample and A_c is the absorbance of control.

Table 1. Fatty Acid Composition of Salmon Oil and PUFA Concentrate Obtained by Urea Complexation[a]

	mol %	
fatty acid	before urea enrichment	after urea enrichment
C 14:0	5.4 ± 0.06	0.7 ± 0.06
C 15:0	0.4 ± 0.00	0.2 ± 0.01
C 16:0	15.8 ± 0.06	0.1 ± 0.02
C 16:1	5.6 ± 0.01	6.2 ± 0.39
C 18:0	3.5 ± 0.01	0.4 ± 0.41
C 18:1 n-9	28.0 ± 0.11	8.5 ± 1.46
C 18:1 n-7	3.6 ± 0.04	1.6 ± 0.25
C 18:2	7.9 ± 0.01	16.9 ± 0.01
C 18:3 (ALA)	2.8 ± 0.00	6.7 ± 0.06
C 18:4	1.2 ± 0.00	3.9 ± 0.11
C 20:0	5.1 ± 0.03	0.4 ± 0.09
C 20:4	0.5 ± 0.01	1.5 ± 0.05
C 20:5 (EPA)	5.5 ± 0.01	17.8 ± 0.58
C 22:1	4.3 ± 0.03	0.2 ± 0.05
C 22:4	0.2 ± 0.01	0.5 ± 0.02
C 22:5 (DPA)	2.5 ± 0.00	7.4 ± 0.32
C 22:6 (DHA)	7.6 ± 0.02	26.9 ± 1.26

[a] The PUFA concentrate was enzymatically esterified to vanillyl alcohol or rutin to yield PUFA−phenolic derivatives.

Scheme 1. Enzymatic Synthesis of Vanillyl−PUFA Esters



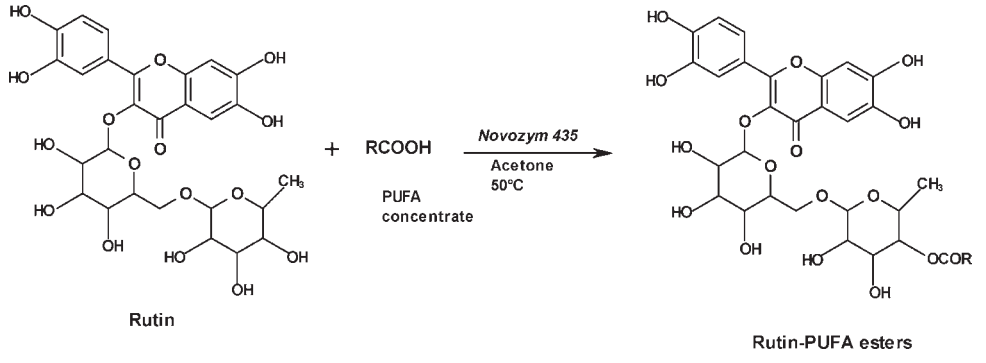
3. RESULTS AND DISCUSSION

In the present study, byproducts from fish processing were used as a source of PUFAs. Annually, an estimated amount of 63.6 million metric tonnes (MMT) of fish waste is generated globally from an annual total fish production of 141.4 MMT.[20] The oil content of byproduct from fish ranges between 1.4 and 40.1% depending on the species and tissue.[21] Such waste represents a rich source of lipids that can be used for omega-3 PUFA recovery. Because fish oils contain a mixture of saturated, monounsaturated, and polyunsaturated fatty acids, the PUFA content was enriched by urea crystallization. The fatty acid compositions before and after urea enrichment are shown in Table 1. The concentrate yield was 19% of the starting FFAs. The content of omega-3 fatty acids, α-linolenic acid (ALA, C18:3 n-3), eicosapentaenoic acid (EPA, C20:5 n-3), and docosahexaenoic acid (DHA, C22:6 n-3), accounted for 67.8 mol % of all fatty acids in the concentrate, and the mean molecular weight of the PUFA concentrate was determined to be 312 g/mol.

For clinical applications, concentrated forms of n-3 PUFAs devoid of saturated and monounsaturated fatty acids are preferred.[22] However, due to the presence of multiple double bonds in PUFAs, they are highly susceptible to oxidation, and the oxidation products can have adverse health effects to the consumer due to their cytotoxic and genotoxic effects.[23,24] The high rate of oxidation of PUFA can be controlled by the addition of synthetic antioxidants such as BHT, BHA, TBHQ, and synthetic or natural α-tocopherol. Furthermore, lipophilic derivatives of natural polyphenolic compounds such as lipophilic rutin esters have been reported to inhibit oxidation of lipids.[9]

3.1. Enzymatic Synthesis of Rutin and Vanillyl Esters of PUFA. The synthesis of lipophilic derivatives of rutin and vanillyl alcohol (Schemes 1 and 2) was performed employing reported methods with slight modifications.[13,14] The yields obtained for isolated rutin and vanillyl PUFA esters after purification were 30 and 60%, respectively. Previously, lipophilization of phenolic derivatives was mainly reported with pure fatty acids in the range of C4−C18.[6,9,13,25] In this study, a PUFA concentrate with fatty acids mainly in the range of C18−C22 was used. All of the fatty acids were incorporated into the phenolic derivatives despite the differences in chain length and degree of unsaturation (Figure 1b,c). The shift in the retention times in reversed phase HPLC of the PUFA concentrate components after reaction with either vanillyl alcohol or rutin (Figure 1) was an indication of ester formation. This was further ascertained by LC-MS. The observed and expected molecular weights of selected fatty acid esters are given in Table 2.

Scheme 2. Enzymatic Synthesis of Rutin−PUFA Esters



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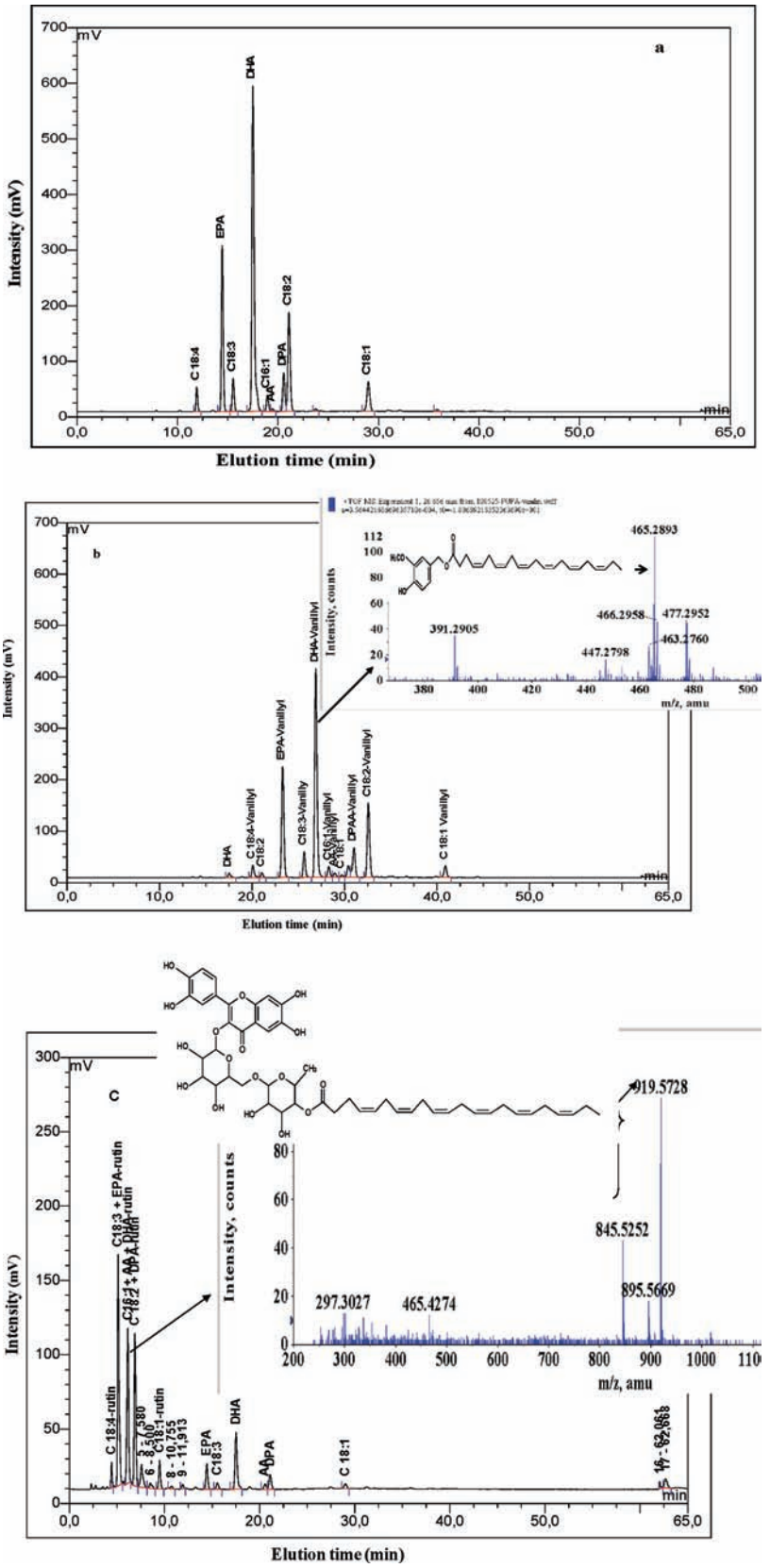

Figure 1. HPLC-ELSD profiles of initial PUFA concentrate (a) and products of enzymatic esterification of PUFA concentrate with vanillyl alcohol (b) or rutin (c). LC-MS spectra of DHA−vanillyl and DHA−rutin esters (one of the products) are also shown along with the HPLC chromatograms.

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Table 2. Molecular Weights of Synthesized Vanillyl−PUFA and Rutin−PUFA Esters As Observed in the LC-MS Analysis (Expected Masses Are Shown in Parentheses)

fatty acid	vanillyl−PUFA[a]	rutin−PUFA[b]
C 18:3	415.2869 (415.2848)	869.5341 (869.3596)
C18:4	413.2730 (413.2692)	867.5105 (867.3439)
C 20:4	441.2950 (441.3005)	895.5675 (895.3752)
EPA	439.2622 (439.2848)	893.5427 (893.3596)
DPA	467.2669 (467.3161)	921.6045 (921.3909)
DHA	465.2893 (465.3005)	919.5728 (919.3752)

[a] M + H. [b] M − H.

Table 3. Antioxidant Activity of Commercial Antioxidants, Substrate Phenolics, and Synthesized Rutin− and Vanillyl−PUFA Esters As Determined by the DPPH Radical Method

compound	1 mM	2 mM
α-tocopherol	92.3 ± 0.69	92.6 ± 0.71
BHT	65.8 ± 3.75	84.8 ± 0.21
rutin	91.6 ± 0.57	95.6 ± 0.26
vanillyl alcohol	87 ± 0.09	90.5 ± 1.48
rutin−PUFA esters	91.1 ± 0.32	92.2 ± 0.31
vanillyl−PUFA esters	52.3 ± 0.15	66.1 ± 1.71

Table 4. Oxidation Inhibition after 6 h of Incubation of Fish Oil or Emulsion at 70 °C in the Presence of Commercial or Synthesized Antioxidants As Determined by the TBARS Method

	oil		emulsion	
compound	5 mM	25 mM	5 mM	25 mM
BHT	43 ± 1.8	85 ± 2.7	87 ± 4.0	92 ± 2.8
α-tocopherol	60 ± 6.1	80 ± 2.5	8 ± 1.0	38 ± 3.0
rutin−PUFA	42 ± 0.3	62 ± 4.9	22 ± 1.0	67 ± 4.7
vanillyl−PUFA	42 ± 3.7	77 ± 1.2	43 ± 5.1	63 ± 3.3

The better yields of vanillyl esters than those of rutin esters may be attributed to the lower reactivity of rutin with fatty acids compared to that of vanillyl alcohol. It has been reported that although Novozym 435 is a nonspecific lipase, it shows greater selectivity toward primary hydroxyl groups compared to secondary hydroxyls.[26] Vanillyl alcohol has a primary hydroxyl group, whereas in the case of rutin, acylation occurs on the 4‴-hydroxyl group of the rhamnoside moiety.[9,27] Despite there being six secondary hydroxyl groups in the rutin molecule, LC-MS data indicated that no rutin molecule was acylated with more than one fatty acid. A previous study by Lue et al.[13] in which rutin was esterified with lauric or palmitic acid reported a rutin−laurate and rutin−palmitate ester yield of 81%. The lower rutin ester yields (30%) obtained in the current study could be due to the presence of greater amounts of long-chain PUFAs in the fatty acid mixture. It was earlier reported that as the chain length increases, the conversion yields of esters gradually decrease.[9,25] When using *C. antarctica* lipase in 2-methylbutan-2-ol, esterification of rutin with fatty acids of chain lengths C4−C12 gave a conversion yield of >50%, whereas lower yields were obtained with longer fatty acids (C12−C18).[9] Not only the chain length but also the presence of double bonds influences the lipase specificity to a large extent. Several lipases have quite low specificity for EPA and/or DHA, but the *C. antarctica* lipase B used in the present study was clearly able to incorporate those fatty acids efficiently (Figure 1b,c).

3.2. Antioxidant Activity. Esterification of PUFA concentrate with vanillyl alcohol resulted in PUFA−vanillyl esters that were more hydrophobic than the PUFA concentrate, whereas rutin−PUFA esters were less hydrophobic according to the retention times in reversed phase HPLC (Figure 1b,c). However, both products were more hydrophobic than either rutin or vanillyl alcohol with retention times of 2.26 and 2.65 min, respectively.

The well-established DPPH radical scavenging activity was used to determine the antioxidant activity for the synthesized rutin− and vanillyl−PUFA esters in two different concentrations. The results were compared with those of the reference compounds BHT and α-tocopherol as well as the parent compounds rutin and vanillyl alcohol and are given in Table 3. Both rutin− and vanillyl−PUFA esters showed radical scavenging activity in the DPPH radical assay. The antioxidant activity of the synthesized rutin−PUFA esters was higher compared to that of vanillyl−PUFA esters in both tested concentrations. The rutin−PUFA esters exhibited higher activity than the commercial antioxidant BHT and an activity similar to that of α-tocopherol. The difference in antioxidant activities between the synthesized esters may be attributed to the difference in the structure, solubility, and number of phenolic hydroxyls.[28] Unlike rutin, which has four phenolic hydroxyl groups, vanillyl alcohol has only one phenolic hydroxyl group and a methoxyl group. In addition, the solubility of the rutin derivatives in methanolic solution could be higher than that of vanillyl esters due to the presence of the carbohydrate moiety, which may be the reason for the difference in the activity in DPPH radical assay. Lipophilization of rutin did not influence the radical scavenging activity as both rutin and rutin esters showed similar radical scavenging capacities. Similar patterns have previously been reported.[9,29] Lipophilization of vanillyl alcohol, however, lowered its DPPH scavenging activity. This could be due to the greatly increased hydrophobicity, which may have resulted in decreased solubility in the tested medium.

The antioxidant activity of the prepared lipophilic phenolic derivatives was also evaluated in two types of media rich in PUFA: an acylglycerol concentrate containing 53% PUFA obtained from enzymatic treatment of fish oil and an emulsion prepared using the same acylglycerol concentrate. Both the prepared lipophilic derivatives exhibited antioxidant activity as determined by the TBARS assay. BHT and α-tocopherol were used as reference antioxidants, and the results are presented in Table 4. In the bulk oil system the antioxidant activities of rutin− and vanillyl−PUFA esters were similar at 5 mM concentration, whereas at 25 mM the activity of vanillyl−PUFA esters was higher, probably because vanillyl−PUFA esters were more hydrophobic than the rutin esters. The activities for both the synthesized derivatives were, however, lower than those of reference compounds. In the emulsion system, vanillyl−PUFA esters exhibited antioxidant activity 2 times that of the rutin−PUFA esters; however, at 25 mM, the activities were comparable (Table 4). Both of the products showed lower activity than one of the controls (BHT) but were superior to the lipophilic reference compound α-tocopherol, which exhibited the lowest antioxidant activity. BHT has previously been reported to provide a better stabilization of PUFA in emulsion against oxidation than α-tocopherol.[30]

The difference in activities in the tested methods is expected as the two media are different with respect to the solubility, distribution, and location of antioxidants.[15,31] Murata et al.[32] studied the relationship between hydrophobic nature and antioxidant activity of flavonoids and found that hydrophobicity is an important determinant for antioxidant potency. Viskupicova et al.[9] also observed that hydrophobicity may have an impact on the antioxidant capacity of a compound in lipophilic food systems, with long-chain fatty acid rutin derivatives offering better protection against oxidation of sunflower oil and β-carotene−linoleate suspension than short-chain derivatives. On the contrary, Laguerre et al.[31] observed that an increase in hydrophobicity does not necessarily improve the antioxidant activity of phenolics. A maximum antioxidant efficiency to protect emulsions against oxidation was achieved with rosmarinic acid octyl esters, with longer chain analogues showing a decreased activity. In the present study, ranges of products with various lengths of the fatty acid chain were synthesized, which can be a good strategy to ensure that at least some molecules have ideal properties for use in each system.

In conclusion, consumption of omega-3 PUFAs stimulates oxidation, which necessitates their use in the presence of an antioxidant. In this study, esterification of PUFA to natural phenolics resulted in lipophilic esters that were able to stabilize oil and emulsions against oxidation. Esterification of omega-3 PUFAs to natural phenolics that have antioxidant properties thus protects the PUFAs from oxidation, and the PUFA−phenolic derivatives carry the combined health beneficial properties of PUFAs and the phenolic molecules.

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REFERENCES

(1) Figueroa-Espinoza, M.-C.; Villeneuve, P. Phenolic acids enzymatic lipophilization. *J. Agric. Food Chem.* **2005**, *53*, 2779–2787.

(2) Iverson, F. Phenolic antioxidants: health protection branch studies on butylated hydroxyanisole. *Cancer Lett.* **1995**, *93*, 49–54.

(3) Guyot, B.; Gueule, D.; Pina, M.; Graille, J.; Farines, V.; Farines, M. Enzymatic synthesis of fatty esters in 5-caffeoyl quinic acid. *Eur. J. Lipid Sci. Technol.* **2000**, *102*, 93–96.

(4) Sabally, K.; Karboune, S.; Yeboah, F. K.; Kermasha, S. Enzymatic esterification of dihydrocaffeic acid with linoleyl alcohol in organic solvent media. *Biocatal. Biotransform.* **2005**, *23*, 37–44.

(5) Sabally, K.; Karboune, S.; St-Louis, R.; Kermasha, S. Lipase-catalyzed transesterification of dihydrocaffeic acid with flaxseed oil for the synthesis of phenolic lipids. *J. Biotechnol.* **2006**, *127*, 167–176.

(6) Kanjilal, S.; Kaki, S. S.; Kotte, S. R.; Kunduru, K. R.; Bhamidipati, V. S. K.; Rao, K. B.; Shiva, K.; Mannepalli, L. K.; Rachapudi, B. N. P. Chemo-enzymatic synthesis of lipophilic ferulates and their evaluation for antioxidant and antimicrobial activities. *Eur. J. Lipid Sci. Technol. 110*, 1175−1182

(7) Uauy, R.; Valenzuela, A. Marine oils: the health benefits of n-3 fatty acids. *Nutrition* **2000**, *16*, 680–684.

(8) Calder, P. C. Long-chain n-3 fatty acids and cardiovascular disease: further evidence and insights. *Nutr. Res. (N.Y.)* **2004**, *24*, 761–762.

(9) Viskupicova, J.; Martina, D.; Miroslav, O.; Tibor, L.; Ernest, S. Lipophilic rutin derivatives for antioxidant protection of oil-based foods. *Food Chem.* **2010**, *123*, 45–50.

(10) Shahidi, F.; Ying, Z. Novel antioxidants in food quality preservation and health promotion. *Eur. J. Lipid Sci. Technol.* **2010**, *112*, 930–940.

(11) Mbatia, B.; Adlercreutz, D.; Adlercreutz, P.; Mahadhy, A.; Mulaa, F.; Mattiasson, B. Enzymatic oil extraction and positional analysis of n-3 fatty acids in Nile perch and salmon heads. *Process Biochem.* **2010**, *45*, 815–819.

(12) Mbatia, B.; Adlercreutz, P.; Mulaa, F.; Mattiasson, B. Enzymatic enrichment of omega-3 polyunsaturated fatty acids in Nile perch (*Lates niloticus*) viscera oil. *Eur. J. Lipid Sci. Technol.* **2010**, *112*, 977–984.

(13) Lue, B.-M.; Guo, Z.; Glasius, M.; Xu, X. Scalable preparation of high purity rutin fatty acid esters. *J. Am. Oil Chem. Soc.* **2010**, *87*, 55–61.

(14) Kobata, K.; Kawaguchi, M.; Watanabe, T. Enzymatic synthesis of a capsinoid by the acylation of vanillyl alcohol with fatty acid derivatives catalyzed by lipases. *Biosci., Biotechnol., Biochem.* **2002**, *66*, 319–327.

(15) Kuduru, K. R.; Thumu, R.; Rachapudi, B. N. P.; Sanjit, K. Evaluation of the antioxidant activity of capsiate analogues in polar, nonpolar, and micellar media. *J. Agric. Food Chem.* **2011**, *59*, 564–569.

(16) Haraldsson, G. G.; Kristinsson, B. Separation of eicosapentaenoic acid and docosahexaenoic acid in fish oil by kinetic resolution using lipase. *J. Am. Oil Chem. Soc.* **1998**, *75*, 1551–1556.

(17) Hayes, D. G.; Van Alstine, J. M.; Setterwall, F. Urea-based fractionation of seed oil samples containing fatty acids and acylglycerols of polyunsaturated and hydroxy fatty acids. *J. Am. Oil Chem. Soc.* **2000**, *77*, 207–213.

(18) Akowuah, G. A.; Zhari, G. A.; Norhayati, I.; Mariam, A. HPLC and HPTLC densitometric determination of andrographolides and antioxidant potential of *Andrographis paniculata*. *J. Food Compos. Anal.* **2006**, *19*, 118–126.

(19) Huber, G. M.; Rupasinghe, V. H. P.; Shahidi, F. Inhibition of oxidation of omega-3 polyunsaturated fatty acids and fish oil by quercetin glycosides. *Food Chem.* **2009**, *117*, 290–295.

(20) Rai, A. K.; Swapna, H. C.; Bhaskar, N.; Halami, P. M.; Sachindra, N. M. Effect of fermentation ensilaging on recovery of oil from fresh water fish viscera. *Enzyme Microb. Technol.* **2010**, *46*, 9–13.

(21) Zuta, C. P.; Simpsona, B. K.; Chan, M. H.; Phillips, L. Concentrating PUFA from mackerel processing waste. *J. Am. Oil Chem. Soc.* **2003**, *80*, 933–936.

(22) Kapoor, R.; Patil, U. K. Importance and production of omega-3 fatty acids from natural sources. *Int. Food Res. J.* **2011**, *18*, 491–497.

(23) Esterbauer, H.; Schaur, R. J.; Zollner, H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biol. Med.* **1990**, *11*, 81–128.

(24) Fang, J.; Vaca, L.; Valsta, L.; Mutanen, M. Determination of DNA adducts of malonaldehyde in humans: effects of dietary fatty acid composition. *Carcinogenesis* **1996**, *17*, 1035–1040.

(25) Ardhaoui, M.; Falcimaigne, A.; Ognier, S.; Engasser, J.-M.; Moussou, P.; Pauly, G.; Ghoul, M. Effect of acyl donor chain length and substitutions pattern on the enzymatic acylation of flavonoids. *J. Biotechnol.* **2004**, *110*, 265–271.

(26) Virto, C.; Adlercreutz, P. Lysophosphatidylcholine synthesis with *Candida antarctica* lipase B (Novozym 435). *Enzyme Microb. Technol.* **2000**, *26*, 630–635.

(27) Oliveira, E. B.; De, Humeau, C.; Chebil, L.; Elaine, R. M.; Francois, D.; Maigret, B.; Ghoul, M.; Engasser, J.-M. A molecular modelling study to rationalize the regioselectivity in acylation of flavonoid glycosides catalyzed by *Candida antarctica* lipase B. *J. Mol. Catal. B—Enzym.* **2009**, *59*, 96–105.

(28) Silva, F. A. M.; Borges, F.; Guimaraes, C.; Lima, J. L. F. C.; Matos, C.; Reis, S. Phenolic acids and derivatives: studies on the relationship among structure, radical scavenging activity, and physicochemical parameters. *J. Agric. Food Chem.* **2000**, *48*, 2122–2126.

(29) Lue, B.-M.; Nielsen, N. S.; Charlotte, J.; Lars, H.; Guo, Z.; Xu, X. Antioxidant properties of modified rutin esters by DPPH, reducing power, iron chelation and human low density lipoprotein assays. *Food Chem.* **2010**, *123*, 221–230.

(30) Rupasinghe, H. P. V.; Yasmin, A. Inhibition of oxidation of aqueous emulsions of omega-3 fatty acids and fish oil by phloretin and phloridzin. *Molecules* **2010**, *15*, 251–258.

(31) Laguerre, M.; Giraldo, L. J. L.; Lecomte, J.; Figueroa-Espinoza, M.-C.; Barea, B.; Weiss, J.; Decker, E. A.; Villeneuve, P. Relationship between hydrophobicity and antioxidant ability of phenolipids in emulsion: a parabolic effect of the chain length of rosmarinate esters. *J. Agric. Food Chem.* **2010**, *58*, 2869–2876.

(32) Murata, Y.; Osaki, K.-I.; Shimoishi, Y.; Baba, N.; Tada, M. Degradation of ethyl docosahexaenoate by gamma-ray irradiation and suppression of this degradation by antioxidants. *Biosci., Biotechnol., Biochem.* **2004**, *68*, 743–745.