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Concentration and stabilization of $C_{20–22}$ *n*-3 polyunsaturated fatty acid esters from the oil of *Sardinella longiceps*

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ABSTRACT

Methyl esters of $C_{20–22}$ *n*-3 polyunsaturated fatty acids derived from sardine oil triglycerides were concentrated to 86% purity with greater than 30% recovery by argentated chromatography. The synergistic effect of ethyl acetate fractions of seaweeds *Kappaphycus alvarezii, Hypnea musciformis* and *Jania rubens* used in 0.1:0.2:0.2 (%, w/w) ratio in arresting oxidative degradation of the *n*-3 PUFA methyl ester concentrate was demonstrated during accelerated storage. The induction time (6.8 h) and antioxidant activity indices (>24) were greater for *n*-3 PUFA concentrates supplemented with seaweed extracts than antioxidants BHT and α-tocopherol (<5 h and <17, respectively). Nuclear Magnetic Resonance spectroscopy was employed to study the oxidative changes of fatty acid signals of PUFA concentrate during accelerated storage. Potential of seaweeds to improve the storage stability of $C_{20–22}$ *n*-3 fatty acid methyl esters was studied. This study has applications in development of food and pharmaceutical products.

1. Introduction

The marine fish oils rich in $C_{20–22}$ *n*-3 polyunsaturated fatty acids (PUFA) are conditionally essential nutrients for adequate growth, development and function in humans (Gil, Serra-Majem, Calder, & Uauy, 2012). Although fish oil is a natural source of *n*-3 PUFAs in the diet, EPA and DHA contents varies from 5 to 26 percent by weight of the total fatty acids (Alkio, Gonzalez, Jäntti, & Aaltonen, 2000). The $C_{20–22}$ *n*-3 PUFAs are currently in demand in pure form in nutraceutical formulations, and are being studied to understand their potential roles in human health. The technologies available for purifying individual PUFAs and PUFA concentrates are based on the differences in physicochemical properties associated with the number of double bonds in the molecule or the chain length (López-Martínez, Campra-Madrid, & Guil-Guerrero, 2004). Common procedures used to obtain PUFA concentrates include molecular distillation, liquid chromatography, supercritical fluid extraction (Alkio et al., 2000), enzymatic purification (Shimada, Sugihara, & Tominaga, 2001), low-temperature crystallization (Crexi, Monte, Monte, & Pinto, 2012), urea complexation and argentation silica gel chromatography (Chakraborty & Paulraj, 2009).

The concentrated long chain *n*-3 PUFAs are greatly susceptible toward chemical, physical, and biochemical deterioration leading to the formation of low molecular weight secondary oxidation and intramolecular rearranged products along with other intermediates, which result in reduced nutritional value and negative consumer acceptance due to the formation of non-desirable off-flavors and odors. The formation of these undesirable secondary metabolites can be arrested by using antioxidative compounds such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tocopherols, propyl gallate, gallic acid, lactoferrines, etc., which have been widely studied to prevent lipid oxidation in bulk fish oil and PUFAs (Kamal-Eldin & Yanishlieva, 2002). Recently, the use of natural antioxidants as an alternative to synthetic compounds to stabilize the bulk fish oil and fish oil-in-water emulsions has acquired greater significance due to safety and greater consumer preference.

Antioxidative compounds derived from the marine sources may work synergistically with fish oils to produce stable product(s) (Airanthi, Hosokawa, & Miyashita, 2011). Seaweeds are predominantly available marine flora, which have protective antioxidative defense systems with potential resource of natural antioxidant

Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; NMR, Nuclear Magnetic Resonance; FTIR, Fourier transform infra red; PV, peroxide value; p-AnV, *para*-anisidine value; TOTOX, total oxidation; TBARS, thiobarbituric acid reactive species; IT, induction time; AAI, antioxidant activity index; % SYN, percent synergism; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ANOVA, analysis of variance; FAME, fatty acid methyl esters; MDAEQ, malondialdehyde equivalence; CSO, crude sardine oil; LCFA, low-temperature crystallized fatty acids; FAC, fatty acid concentrate.

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molecules (Chakraborty, Joseph, & Praveen, 2014). However, very few studies have focused on using these marine derived extracts as antioxidative additives to the fish oil and PUFA concentrates (Senevirathne et al., 2006).

In the present work, the concentrated *n*-3 PUFA esters were prepared from the fatty acid concentrate of a marine finfish species Oil Sardine (*Sardinella longiceps*) using argentated chromatography. This study further aimed to examine the antioxidative effect of a combination of ethyl acetate fractions from the red seaweeds, *Kappaphycus alvarezii, Hypnea musciformis* and *Jania rubens* on oxidative stability of the concentrated *n*-3 PUFA esters. The oxidative changes in the concentrated PUFA methyl esters were assessed by analyzing the primary and secondary oxidation products of various lipid peroxidation assays, and free radical scavenging activities during accelerated storage at 65 °C. The compositional differences of the C_{20-22} *n*-3 fatty acid methyl esters before and after the accelerated experimental storage period were determined to understand the potential of the seaweeds to improve their storage stability. The synergistic effect of seaweed extracts on the oxidative stability of the PUFA concentrate were compared with synthetic antioxidants. The secondary oxidation products and lipid molecular species derived from the PUFAs in various treatments were mapped in a non-destructive way by ^{1}H NMR spectroscopy during accelerated experimental storage.

2. Materials and methods

2.1. Materials

S. longiceps (Oil Sardine) was collected from the fishing harbor at the Southwest coast of India (9.97°N 76.28°E). Three seaweeds, *K. alvarezii* (Doty), *H. musciformis* (Wulfen) J.V. Lamouroux and *J. rubens* (Linnaeus) J.V. Lamouroux were harvested from the Gulf of Mannar in Mandapam region (8°48′ N, 78°9′ E and 9°14′ N, 79°14′E) on the Southeast coast of India. All other chemicals used in the study were analytical grade and purchased from E-Merck (E-Merck, Germany).

2.2. Preparation of concentrated C_{20-22} n-3 fatty acid methyl esters by argentated column chromatography

Crude sardine oil (CSO) obtained from *S. longiceps* was refined by a step-wise process of bleaching and deodorization as described earlier (Chakraborty & Joseph, 2015). The refined oil was then subjected to purification by sequential process of saponification, low temperature crystallization (to obtain low-temperature crystallized fatty acids, LCFA), urea fractionation (to obtain fatty acid concentrate, FAC), and *trans*-esterification (to obtain fatty acid methyl esters) as described in the previous studies (Crexi et al., 2012; Chakraborty & Paulraj, 2009). The long chain C_{20-22} *n*-3 fatty acid methyl esters were then concentrated by normal pressure liquid column chromatography with $AgNO_3$ impregnated neutral alumina and silica gel as the stationary phases.

2.2.1. Preparation of silver impregnated alumina and silica

Silver nitrate powder ($AgNO_3$, 10 g) was added to ethanol (80% v/v, 60 ml) and dissolved by stirring for 10 min. About 50 g of neutral alumina (70–230 mesh) or silica gel (0.06–0.2 mm, 70–230 mesh ASTM; mean pore diameter of 6 nm, specific surface area of 500 m^2/g), was slurried in ethanol (95% v/v, 100 ml) and added to the $AgNO_3$ solution under stirring for 2 h. Ethanol was evaporated under vacuum at 60 °C, and the silver impregnated alumina/silica was activated by heating overnight (110 ± 2 °C) in the hot air oven to prepare Ag-alumina/Ag-silica powder. This material was cooled and kept in the dark in a desiccator until further use.

2.2.2. Argentation chromatography using Ag-alumina and Ag-silica

The slurry of Ag-alumina/Ag-silica (each 5 g) in *n*-hexane (5 ml) was poured into a water-jacketed column (45 cm × 50 mm i.d.) previously half-filled with *n*-hexane. The sardine methyl esters obtained after trans-esterification (5 g) was dissolved in *n*-hexane (5 ml) and applied on the chromatography column. The Ag-alumina column was eluted with a sequence of organic solvents and seven different fractions of concentrated methyl esters ($CMEA_{1-7}$) were collected, 100% *n*-hexane ($CMEA_1$), 1% acetone:*n*-hexane ($CMEA_2$), 3% acetone:*n*-hexane ($CMEA_3$), 5% acetone:*n*-hexane ($CMEA_4$), 8% acetone:*n*-hexane ($CMEA_5$), 10% acetone:*n*-hexane ($CMEA_6$) and 100% acetone ($CMEA_7$). Similarly, Ag-silica was also eluted with a sequence of organic solvents and seven different fractions of concentrated methyl esters ($CMES_{1-7}$) were collected, 100% *n*-hexane ($CMES_1$), 1% acetone:*n*-hexane ($CMES_2$), 3% acetone:*n*-hexane ($CMES_3$), 5% acetone:*n*-hexane ($CMES_4$), 8% acetone:*n*-hexane ($CMES_5$), 10% acetone:*n*-hexane ($CMES_6$) and 100% acetone ($CMES_7$). The recovered fatty acid methyl esters from column chromatography were resolved by silver-ion thin-layer chromatography ($AgNO_3$/TLC, 5 cm × 20 cm) using *n*-hexane/diethylether/glacial AcOH (80:20:0.5, v/v/v) as described earlier (Chakraborty & Paulraj, 2009). The purity of the concentrated methyl esters eluted with the most efficient method has been further validated using FTIR and ^{1}H NMR analyses.

2.3. Preparation and fractionation of red seaweed extracts

The dried and pulverized powder (1 kg) derived from seaweeds were extracted with methanol (3500 ml × 3) at 50–60 °C for a period of 3 h. The contents were thereafter filtered to afford the filtrate (10 l), which was concentrated (50 °C) to about one fifth of the initial volume. The resultant mixture was partitioned with ethyl acetate (1500 ml × 3), and concentrated to obtain the ethyl acetate fractions of *K. alvarezii* (KAF 1.0 g), *H. musciformis* (HMF 10.7 g) and *J. rubens* (JRF 11.7 g).

2.4. Selection of potent antioxidant combination imparting stability to the concentrated fatty acid methyl esters

To select the potential combination of seaweed extracts, the ethyl acetate fractions of *K. alvarezii* (KAF), *H. musciformis* (HMF) and *J. rubens* (JRF) was added to the concentrated methyl esters $CMES_6$ (at 0.5%, w/w) individually and in nine different factorial treatments. The treatments were KAF:HMF:JRF in the ratios of 0.5:0:0 (KAF), 0:0.5:0 (HMF), 0:0:0.5 (JRF), 0.2:0.2:0.1 ($CMES_{6a}$), 0.2:0.1:0.2 ($CMES_{6b}$), 0.1:0.2:0.2 ($CMES_{6c}$), 0.3:0.1:0.1 ($CMES_{6d}$), 0.1:0.3:0.1 ($CMES_{6e}$), 0.1:0.1:0.3 ($CMES_{6f}$), 0.4:0.05:0.05 ($CMES_{6g}$), 0.05:0.4:0.05 ($CMES_{6h}$) and 0.05:0.05:0.4 ($CMES_{6i}$). The induction time was evaluated using rancimat analysis. The potential antioxidant combinations derived from the seaweeds were selected based on the degree of synergism (% SYN). The samples were subjected to accelerated oxidative conditions using rancimat analysis (Metrohm 743, Herisau, Switzerland) by keeping $CMES_6$ as control at 80 °C with an air flow of 20 l/h. The intersection point of the two extrapolated linear portion of the conductivity curve was taken as the induction time (oxidative stability index, OSI). The degree of synergism (% SYN) was calculated on the basis of the induction times using the formula (% SYN) = $[IT_{MIX} - IT_0] - [(IT_1 - IT_0) + (IT_2 - IT_0) + (IT_3 - IT_0)] * 100/[(IT_1 - IT_0) + (IT_2 - IT_0) + (IT_3 - IT_0)]$, where IT_{MIX}, IT_0, IT_{1-3} were the induction periods of the samples containing the mixture of additives, control, and samples containing the additives, respectively (Guzman, Tang, Salley, & Simon, 2009). A positive value for % SYN defines a synergistic effect between each additive, while a negative value corresponds to an antagonistic effect.