Concentration and Stabilization of n-3 Polyunsaturated Fatty Acids from Sardine Oil

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ABSTRACT: A simple and relatively inexpensive procedure to obtain 90% eicosapentaenoic acid + docosahexaenoic acid concentrates from sardine oil involved a two-step winterization of the oil (10 and 4ºC), followed by saponification and selective precipitation of saturated and less unsaturated free fatty acids by an ethanolic solution of urea. Antioxidant effects of butylated hydroxytoluene, *dl*-α-tocopherol, and two natural antioxidants, quercetin and boldine, added to the concentrate (0.5% wt/vol), were compared in the Rancimat at 60°C. *dl*-α-Tocopherol was unable to inhibit concentrate oxidation. Butylated hydroxyanisole and butylated hydroxytoluene had induction periods of 1.7–1.8 h compared to the control (1.0 h). A mixture of quercetin + boldine (2:1 w/w) significantly increased the induction period to 4.5 h.
JAOCS 75, 733–736 (1998).

KEY WORDS: Boldine, natural antioxidant, polyunsaturated fatty acid concentrates, quercetin, Rancimat test, synthetic antioxidant.

Nutritional and pharmacological effects of n-3 polyunsaturated fatty acids from marine origin have raised interest in developing processes to concentrate eicosapentaenoic acid (20:5, EPA), and docosahexaenoic acid (22:6, DHA) (1). Samples enriched in these fatty acids are needed to further investigate their nutritional, health, and biochemical effects. Procedures to obtain EPA and DHA include low-temperature crystallization (2), silver-resin chromatography (3), acidolysis catalyzed by immobilized lipases (4), and supercritical extraction (5). Although these procedures produce 75–80% EPA and DHA concentrates, they are suitable for laboratory-scale only and are expensive for industrial application. In the present work, we adapted a simple, rapid, and relatively inexpensive procedure to concentrate EPA + DHA as free fatty acids from fresh sardine oil.

Polyunsaturated fatty acids are highly susceptible to oxidative rancidity, which produces undesirable changes in the chemical and sensory (flavor) properties of the product and generates potentially toxic end-products (6). Oxidation may be avoided or delayed by addition of antioxidants, of either synthetic or natural origin. During the last two decades, synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have raised concern because some deleterious and potentially toxic effects have been observed (7–9). Antioxidants of natural origin may offer an alternative to synthetic antioxidants (10). A number of products, extracted mainly from vegetables, may have antioxidant properties (11). We demonstrated that flavonoids, such as morin, quercetin and rutin, and boldine, an aporphine extracted from the bark of the Boldo tree (*Peumus boldus* Mol.), showed potent and synergistic antioxidative effects by inhibiting metal- or temperature-induced oxidation of fish oil (12,13). In this work, we assayed the effectiveness of quercetin and boldine, compared to BHT, BHA, and *dl*-α-tocopherol, for stabilizing temperature-induced oxidation of EPA + DHA concentrate from sardine oil.

EXPERIMENTAL PROCEDURES

Materials. Recently refined sardine oil was obtained from a local fish meal and fish oil factory (CORPESCA S.A., Mejillones, Segunda Región, Chile). BHA, BHT, and quercetin were obtained from Sigma (St. Louis, MO). *dl*-α-Tocopherol, in free form, was obtained from Productos Roche (Santiago, Chile). Boldine was crystallized to chromatographic purity from the crude alkaloidal mixture extracted from Boldo bark (14). Boron trifluoride (14% ethanol) was purchased from Merck (Merck Quimica Chilena, Santiago, Chile). The solvents (analytical grade) were obtained from Romil Chemicals (Leicestershires, England), and urea (99%) was obtained from J.T. Baker (Phillipsburg, NJ).

Procedure. Sardine oil was deodorized under high-vacuum distillation to reduce peroxides and cholesterol content (15). Recently distilled oil (20 L), with a peroxide value below 0.5 meq/kg (16) and a cholesterol content below 20 mg% (17), was winterized in a two-step temperature-controlled process, first at 10ºC for 48 h, and then at 4ºC for 72 h. After each winterization step, the supernatant was recovered by centrifugation (2500 × *g*, 10 min). Final volume recovered after winterization was 18.2 L of oil. Two liters of winterized oil was saponified by adding 2 vol of an ethanolic (70%) KOH (7 N) EDTA (5.0 mM) solution, heating at 90ºC under nitrogen in a stirred, steam-jacketed, 15-L kettle, and keeping under reflux for 2 h. The mixture was cooled to ambient temperature by

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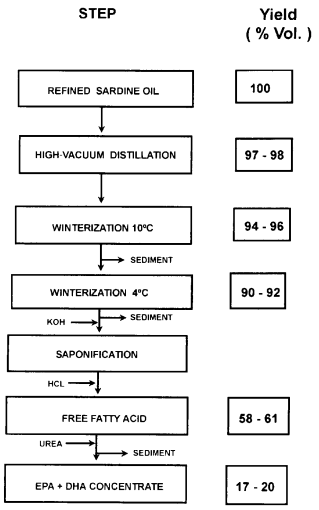

SCHEME 1

passing tap water through the kettle jacket. Then, 0.4 vol of HCl (12 N) and 1 vol of hexane were added. Finally, the mixture was vigorously mixed by mechanical agitation, the organic fraction containing the free fatty acids was separated, and hexane was evaporated at 40ºC under vacuum (335 mBar). One volume of free fatty acids was added to nine volumes of ethanol that contained 30% urea, and after stirring, the mixture was kept at 20°C for crystallization for 48 h. Crystals were separated by centrifugation at 5000 × *g* for 30 min at 20ºC. The supernatant, containing the nonurea-complexing fatty acids (largely polyunsaturated fatty acids), was rotary-evaporated at 35ºC under vacuum (289 mBar) to 1/10 of its original volume and thereafter maintained under N_2. After each concentration step, the peroxide value (16), cholesterol content (17), and iodine value (18) were assessed. The fatty acid composition of the methyl ester derivatives of the mixture was analyzed by gas–liquid chromatography as previously reported (19). Scheme 1 summarizes the concentration procedure, including the yield for the oil after each step.

Oxidative stability of EPA + DHA concentrates was analyzed in a Rancimat model 679 (Methrom, Herisau, Switzerland) at 60ºC with an air flow of 7 L/h (20). Total concentration of antioxidant assayed (single or as mixture, w/w) was 0.5% wt/vol. Stability of the samples was estimated as the time (h) necessary for induction of oxidation (induction period). Stabilization experiments were performed in quintuplicate, and results were expressed as means ± SD. The significance between means values was assessed by Student's *t*-test for unpaired results.

RESULTS AND DISCUSSION

Table 1 shows the fatty acid composition of the refined oil after distillation of the oil, after the two-step winterization and after urea precipitation. Initial concentration of EPA ±

TABLE 1
Chemical Characterization of Sardine Oil After the Different Steps of the Concentration Procedure

Fatty acid composition[a] (% methyl esters)	Refined oil	Distilled oil	Winterized (10°C)	Winterized (4°C)	Urea fractionation
$C_{12:0}$ Lauric	0.1	0.1	—	—	—
$C_{14:0}$ Myristic	8.8	8.7	8.5	6.2	—
$C_{14:1}$ Myristoleic	0.2	0.2	—	—	—
$C_{15:0}$ Decapentaenoic	0.4	0.4	0.7	0.1	—
$C_{16:0}$ Palmitic	18.1	17.9	17.1	5.2	—
$C_{16:1}$ Palmitoleic	10.9	10.9	10.7	0.1	0.2
$C_{16:2}$ Hexadecadienoic	0.4	0.3	—	—	—
$C_{18:0}$ Stearic	4.5	4.4	4.3	4.2	—
$C_{18:1n-9}$ Oleic	11.7	11.5	11.2	11.0	—
$C_{18:2n-6}$ Linoleic	1.8	1.6	1.7	1.9	0.1
$C_{18:3n-3}$ Linolenic	1.5	1.5	1.9	3.9	0.2
$C_{18:4n-3}$ Octadecatetraenoic	3.1	3.3	3.7	3.9	0.4
$C_{20:4n-6}$ Arachidonic	2.0	2.2	2.4	2.9	0.1
$C_{20:4n-3}$ Eicosatetraenoic	0.8	0.9	1.2	1.7	—
$C_{20:5n-3}$ Eicosapentaenoic	11.0	11.8	12.7	14.6	41.1
$C_{22:4n-6}$ Docosatetraenoic	1.6	1.8	1.9	2.1	3.2
$C_{22:5n-3}$ Docosapentaenoic	1.8	1.9	2.1	2.8	4.1
$C_{22:6n-3}$ Docosahexaenoic	16.5	17.7	18.2	19.9	50.2
Unidentified	4.8	2.4	1.7	1.5	0.4
EPA + DHA	27.5	29.5	30.9	34.5	91.3
Peroxide value[b]	4.2 ± 0.8	0.4 ± 0.1	0.8 ± 0.2	0.8 ± 0.4	1.8 ± 0.7
Iodine value[b]	221.5 ± 7.1	227.4 ± 5.6	236.8 ± 8.1	240.6 ± 8.6	343.5 ± 7.9
Cholesterol content (mg/100 g)[b]	480.5 ± 7.5	19.4 ± 1.8	18.6 ± 2.0	19.2 ± 0.9	<2.0

[a]Typical profile.
[b]Results represent the mean of five assays ± SD. Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

DHA was 27.5%. The distillation and the two-step winterization procedure (10 and 4ºC) improved the EPA + DHA concentration to 29.5, 30.9, and 34.5%, respectively. Although winterization only slightly increased EPA + DHA concentration, this procedure was essential for subsequent urea precipitation (Valenzuela, A., and J. Sanhueza, unpublished results; data not shown). Urea precipitation produced a supernatant that contained 91.3% EPA + DHA. The capability of urea to form crystalline adducts with hydrocarbons, alcohols, aldehydes, acids, and esters has been described (21,22). For molecules to form urea complexes, they must have more than six carbon atoms, and the carbon atoms must be linked in a straight chain. Saturated fatty acid and, in minor proportion, mono- and diunsaturated fatty acids may form adducts with urea, which have been identified as true compounds and not solid solutions or mixed crystals (23). Highly polyunsaturated fatty acids, such as EPA and DHA without straight-chain molecular structures, do not form urea crystalline complexes. The concentration procedure has a yield (free fatty acids) of 17–20% (vol) based on the refined oil. Costs are relatively inexpensive. Considering raw material, solvent and salts (analytical grade), and energy investment (no human resources), 91.3% EPA + DHA concentrate costs around U.S. $140/L. Obtaining 8–10 L of concentrate requires 12–15 h, starting from the refined oil, but does not include the time for winterization.

Results of oxidation assays of EPA + DHA concentrate (Fig. 1) showed high susceptibility for oxidation of the untreated concentrate (control), which had a 1-h induction period. Addition of *dl*-α-tocopherol did not change the induction period of the concentrate compared to the control. BHT or BHA significantly increased the induction period to 1.7–1.8 h. Quercetin and boldine, added singly, showed good inhibitory effects on oxidation, but when these two natural products were assayed as a mixture, induction periods were significantly increased. The 2:1 w/w mixture of quercetin/boldine showed the highest efficacy of all antioxidants assayed. The antioxidant effect of quercetin and boldine, compared to *dl*-α-tocopherol BHA, and BHT, on temperature- or metal-induced oxidation of fish oil was described earlier (12,13). The individual antioxidant effects of quercetin and boldine, and the synergism exhibited by these two natural substances for protecting oxidation of the EPA + DHA concentrate, are good examples of the effectiveness of these two natural antioxidants. Both quercetin and boldine act by scavenging free radicals or excited forms of oxygen that are involved in the first steps of fatty acid oxidation (12,13). Although quercetin and boldine were nontoxic to experimental animals (11,24), further physicochemical and toxicological evaluations are required to assess their future as natural antioxidants.

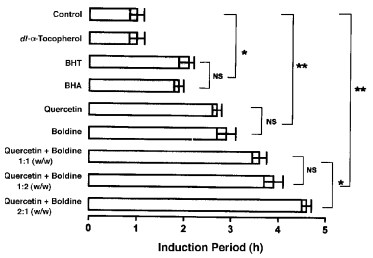

FIG. 1. Effect of synthetic and natural antioxidants (0.5% wt/vol, final concentration) on the induction period for oxidation of 91.3% EPA + DHA concentrate, assessed by the Rancimat. Results represent the mean of five assays ± SD. $*P < 0.05$, $**P > 0.01$. NS, not significant. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

ACKNOWLEDGMENTS

This work was supported by Fondo Nacional de Ciencias y Tecnología (FONDECYT). projects 1930808 (A.V.), 1950258 (H.S.), and 1940422 (S.N).

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[Received August 7, 1996; accepted November 24, 1997]