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ORIGINAL PAPER

Concentration of Stearidonic Acid in Free Fatty Acid and Fatty Acid Ethyl Ester Forms from Modified Soybean Oil by Winterization

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Abstract The concentration of stearidonic acid (SDA, 18:4 ω-3) in free fatty acid (FFA) and fatty acid ethyl ester (FAEE) forms by low temperature crystallization (winterization) was studied. For this purpose, modified soybean oil (initial SDA content, ~23%) was transformed into its corresponding FFA and FAEE by chemical hydrolysis and ethanolysis, respectively. In the first study, the FFA and FAEE were used as starting material for winterization and variables such as winterization time, type of solvent, and the oil:solvent ratio were evaluated until optimization of the process was achieved. In the second study, changes in the winterization procedure were introduced to obtain a remarkable improvement on the SDA purity of the final products. Since winterization of FAEE was not efficient due to its low melting points, the second study focused on FFA. The best relationship between SDA purity (59.8%) and SDA yield (82.3%) was attained by performing winterization of FFA with hexane at 10% oil:solvent ratio for 24 h. Scaled-up processes were also performed to obtain 59 g of FFA (purity 59.6%; yield 82.6%) enriched in SDA. The products obtained can be used as starting materials for the production of functional lipids and for clinical trials.

Keywords Fatty acid ethyl ester · Free fatty acid · Low temperature crystallization · Modified soybean oil · Stearidonic acid · Winterization

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Introduction

Polyunsaturated fatty acids (PUFA) have been the subject of much attention because of their special physiological effects in humans that include reductions in cardiovascular disease [1], inflammation [2], cancer [3], and neurological disorders [4]. It has been suggested that the typical western diet, which is high in ω-6 and low in ω-3, may not supply the appropriate balance of PUFA for proper biological function of the body. These health benefits have been attributed to long chain ω-3 PUFA, mainly to eicosapentaenoic acid (EPA, 20:5 ω-3) and docosahexaenoic acid (DHA, 22:6 ω-3) [5].

The traditional source of EPA and DHA is fish oil. However, it has been reported that fish oil has negative aspects such as its typical fishy smell, unpleasant taste, and poor oxidative stability [6]. Also, because of problems associated with over-fishing there is an increased interest in the development of alternative sources for PUFA. Currently, alpha-linolenic acid (ALA, 18:3 ω-3) is the main ω-3 fatty acid available in vegetable oils. However, there is poor conversion of ingested ALA to the longer-chain ω-3 fatty acids, EPA and DHA [7]. For these reasons, there has been growing interest in stearidonic acid (SDA, 18:4 ω-3), a PUFA that is a metabolic intermediate in the conversion of ALA to EPA [8]. SDA has less degree of unsaturation than EPA and DHA and is therefore, less susceptible to lipid oxidation, formation of undesirable free radicals, aldehydes, and off-flavors [9]. Because of its physical characteristics and its role in the metabolism of other PUFA, SDA became of particular interest to the food industry.

SDA is naturally found as a minor fatty acid in seafood, contributing 0.5–2% of the total fatty acids (EPA and DHA contribute 15–20%), and some species of seaweed.

Although it is rarely found in terrestrial plant sources, species from the Boraginaceae, Grossulariaceae, Caryophyllaceae, and Primulaceae families may have high SDA content. Hence, echium oil is the richest commercially available plant source of SDA (3.5–9.0%).

A new review [8] reported the bioactive properties of SDA, which favorably compared its physiological effects to that of other ω-3 PUFA, such as EPA, DHA or ALA in a limited number of studies. Similar effects were observed between SDA and EPA on the inhibition of tumorigenesis in a rodent model of colorectal cancer, in ex vivo platelet aggregation studies, on changes in tissue arachidonic acid content and eicosanoid formation, and similarities on biomarkers of inflammation and modification of plasma lipid profiles. These studies suggest the physiological importance of ω-3 PUFA with 4 or more double bonds. SDA, as a metabolic surrogate for EPA, has the potential to augment health promotion and mimic disease reduction observed with ω-3 PUFA. In addition, SDA appeared to be as safe as other ω-3 PUFA [8].

Some common procedures used to obtain PUFA concentrates are enzymatic purification [10], urea complexation [11, 12], low temperature crystallization (winterization) [13], and argentation silica gel chromatography [14]. However, only few are suitable for large-scale production [6]. The methods used to concentrate PUFA are commonly based on differences in the polarity and/or spatial configuration of the fatty acids present in the extract. According to these characteristics, the degree of unsaturation can play an essential role in the separation [15]. Although urea complexation methods have some advantages such as low cost, simple equipment and mild conditions, these methods may lead to the formation of ethyl carbamate (also known as urethane), which has been found to be a carcinogen in animals [16]. For that reason, urea complexation methods may not be desirable in the food or pharmaceutical industry.

The process of winterization involves a partial crystallization followed by separation of the solids from the liquid portion. The term "winterization" was originally applied decades ago when seed oil was subjected to winter temperatures to accomplish the process of the removal of solids by controlled crystallization and filtration [15]. The melting point of fatty acids changes considerably with the type and degree of unsaturation and thus, separation of mixtures of saturated and unsaturated fatty acids may become possible. At low temperatures, long chain saturated fatty acids, which have higher melting points, crystallize out and PUFA remain in the liquid form [5]. It has been reported that use of different organic solvents and temperatures affect the concentration of PUFA and thus, proper choice of solvent and temperature is necessary to achieve optimal concentration yield of the ω-3 PUFA [5, 17].

Chen and Ju [18] concentrated GLA by means of a two-step solvent crystallization process of *Borago officinalis* free fatty acids. Nevertheless, this procedure was attempted using acetonitrile as solvent, which should be avoided in any bioprocess to obtain dietary products, because it is widely reported to be harmful to humans. In the low temperature crystallization of the FFA from seal blubber oil, hexane gave higher values of purity and yield of ω-3 PUFA than acetone [5]. Likewise, for concentrating GLA by crystallization of FFA from *Borago officinalis* and *Echium fastuosum*, Lopez-Martinez et al. [15] reported that hexane at an oil:solvent ratio of 10% (w/w) gave higher GLA purity and yield than acetone. These results were significantly better than those obtained at ratios of 20 and 40% [15]. In these studies the best results were achieved at the lowest temperature (−70 °C) tested [5, 15]. Other studies reported the separation by winterization of sodium salts of fatty acids produced by saponification, according to their solubility differences in ethanol [13, 19]. However, concentration of SDA has not been studied so far, since this fatty acid was absent or present at very low amounts in the starting materials employed. The melting point of SDA is −57 °C.

In the present study, the winterization of FAEE and FFA obtained from modified soybean oil with high initial content of SDA (~23%) was investigated. The main aim of this work was to optimize the SDA purity and yield using winterization process for the concentration or enrichment of SDA in FAEE and FFA forms. The products obtained can be used to produce structured lipids, nutraceuticals, and functional foods.

Materials and Methods

Materials

Modified soybean oil with high SDA ω-3 content (~23%) was donated by Monsanto Company (St. Louis, MO). Sodium ethoxide, 21% (w/w) in ethanol, and 2,2,4-trimethylpentane (isooctane) were purchased from Alfa Aesar, a Johnson Matthey Company (Ward Hill, MA). Absolute ethanol was purchased from Decon Labs Inc. (King of Prussia, PA). NaCl, KOH (pellets), HCl 35–38% (w/w) and acetone were obtained from J.T. Baker (Phillipsburg, NJ). Hexane (HPLC grade) was purchased from EMD Biochemicals (Darmstadt, Germany). Boron trifluoride (BF_3, 14% in methanol), palmitic acid ethyl ester and stearic acid ethyl ester were obtained from Sigma Chemical Co. (St. Louis, MO). Stearidonic acid methyl ester was purchased from Cayman Chemical Company (Ann Arbor, MI). Methanol and anhydrous sodium sulfate were purchased from Fisher Scientific (Pittsburgh, PA).

Ethanolysis of High SDA ω-3 Oil

A transesterification reaction (ethanolysis) of the high SDA ω-3 oil (modified soybean oil) was carried out in order to transform all the TAG into their corresponding FAEE. The methodology employed was based on the method described by Vázquez and Akoh [20]. The reaction was performed in a 1 L cylindrical vessel. 200 mL of the oil were mixed with sodium ethoxide (2.625%, w/v) in absolute ethanol at a ratio of 4:2 (vol/vol) (2.25-fold molar excess of ethanol). The mixture was heated at 60 °C with mechanical shaking for 40 min, under nitrogen atmosphere. The product was washed twice in order to completely remove the remaining ethanol, glycerin or any other polar compounds. First washing was done with a saturated NaCl solution, and the second with distilled water. Separation of two phases was done using a separatory funnel (2-L capacity) and centrifugation was not necessary. The volume used in each washing was half of the volume of oil utilized. Finally, the product of the ethanolysis reaction was dried over anhydrous sodium sulfate and vacuum filtered. Using this methodology, the FAEE content in the final product was ~99%. This product was used as the starting material for subsequent crystallizations of FAEE and its composition reported in Table 1.

Hydrolysis of High SDA ω-3 Soybean Oil

Saponification was done to hydrolyze the TAG of high SDA ω-3 oil, following a procedure based on the methods described by Torres et al. [21] and Senanayeke and Shahidi [22], with some modifications. According to this methodology, 150 g of the high SDA ω-3 oil were mixed with 480 mL of a solution of KOH 3.7 N in ethanol:water (50:50, v/v) with 0.15% w/v of EDTA, in a 1-L cylindrical vessel. The mixture was heated at 60 °C for 1 h with mechanical stirring. Then, the reaction was stopped by adding 120 mL of distilled water. The hydroalcoholic mixture, containing the soaps, was acidified by adding 4 M HCl and adjusted to pH 2 to release the FFA. Both phases were separated in a separatory funnel. Then, the liberated fatty acids were washed with 100 mL of distilled water to ensure a neutral pH. The FFA remaining in the hydroalcoholic phase were extracted twice with 200 mL of hexane. Hexane was separated from the fatty acids by evaporation in a rotary evaporator at 40 °C and all the FFA obtained were pooled. This pooled fraction was dried over anhydrous sodium sulfate and filtered under vacuum. This product was used as the raw material for subsequent crystallizations involving FFA and its composition reported in Table 2.

Winterization Procedure

The products obtained from chemical ethanolysis (FAEE) and chemical hydrolysis (FFA) were fractionated by low temperature crystallization at different conditions to yield a liquid fraction and a solid or crystallized fraction. Only organic solvents permitted for the food sector were studied. Particular preference was given to hexane, ethanol, acetone, and mixtures of these solvents.

Study 1-Preliminary Study

In this study, four different blends of solvents were used to prepare the FAEE solutions. These were pure acetone; hexane:acetone (10:90, v/v); hexane:acetone (20:80, v/v); and hexane:ethanol (30:70, v/v). Later on, winterization of FAEE was performed by using methanol and ethanol as solvents, according to the patent by Luthria [23]. On the other hand, four different proportions of acetone in hexane were used as solvents for FFA winterization: 0, 10, 20 and 30% (v/v). In the winterization of FFA, one further experiment was carried out by using 2,2,4-trimethylpentane (isooctane). These mixtures of solvents were selected based on the enhanced levels of PUFA achieved in previous works [5, 15, 24]. Same solvents as for TAG were selected to perform the winterization of FAEE, based on similar polarity of FAEE and TAG provided by the ester bonds in the molecules. Thus, mixtures composed mainly of acetone were selected for FAEE, whereas mixtures composed mainly of hexane were selected for FFA.

Two different oil:solvent ratios: 0.75:30 (w/v) and 1:10 (w/v) were investigated for all samples (FAEE and FFA) in Study 1. In order to simplify the nomenclature, these ratios were designated as 2.5 and 10%, respectively. Thus, 0.75 g of sample were dissolved in 30 mL of solvent at 2.5%, whereas 1 g of sample was dissolved in 10 mL of solvent at 10% ratio. Lopez-Martinez et al. [15] reported the best concentration of GLA by winterization at the lowest oil:solvent ratio tested (10%). Based on that, we selected a lower range of oil:solvent ratio (2.5 and 10%) for investigation.

Each solution was stirred by vortexing until the entire sample was dissolved. All solutions were then stored and cooled from room temperature to −80 °C. The winterization temperature was set at −80 °C, according to the best results reported in previous works [5, 15, 17, 24]. In these studies, PUFA enrichment was more effective when winterization was carried out at the lowest temperatures. Still lower temperatures can also be used, but these are usually uneconomical because of the increased energy costs. Two winterization times were explored (4 and 24 h) only for FAEE.

The samples were immediately centrifuged at −20 °C and 12,000 rpm (17,210 g) for 2 min in a Sorvall RC 6

Table 1 Purity and yield of main fatty acids in the liquid fraction using different solvents and oil:solvent ratios in winterization of FAEE in Study 1

Solvent[a]	Acetone		A90		A80		H:ET		Methanol		Ethanol		Starting FAEE[c]
Oil:solvent ratio (%, w/v)	10	2.5	10	2.5	10	2.5	10	2.5	10	2.5	10	2.5	
Fatty acid (purity, %)[b]													
Palmitic C16:0	6.5	4.3	8.0	6.2	8.9	8.8	6.5	6.0	0.3	1.2	0.5	1.5	12.1
Stearic C18:0	2.0	1.3	2.5	1.8	2.8	2.7	2.0	1.8	0.1	0.4	0.1	0.3	4.1
Oleic C18:1 (ω-9)	16.9	17.1	16.7	17.1	16.5	16.6	17.4	17.3	16.5	18.3	18.5	18.4	15.5
Linoleic C18:2 (ω-6)	27.1	28.1	26.4	27.2	26.0	26.1	27.0	27.2	28.6	29.0	29.3	29.1	24.5
γ-Linolenic C18:3 (ω-6)	7.6	7.8	7.4	7.6	7.3	7.2	7.6	7.6	8.6	8.2	8.2	8.1	7.2
α-Linolenic C18:3 (ω-3)	11.8	12.1	11.5	11.7	11.3	11.3	11.8	11.8	13.2	12.8	12.9	12.7	10.7
Stearidonic C18:4 (ω-3)	26.1	27.1	25.5	26.2	25.1	24.9	26.1	26.2	30.7	27.9	28.1	27.7	23.8
Fatty acid (yield, %)													
Palmitic C16:0	37.7	28.0	52.5	42.1	61.3	66.0	39.0	40.7	0.4	6.0	1.0	9.0	–
Stearic C18:0	34.0	24.3	48.0	36.5	56.9	60.5	35.3	35.7	0.3	5.4	0.7	6.2	–
Oleic C18:1 (ω-9)	76.8	85.8	85.4	90.4	87.9	96.3	80.7	91.5	17.9	70.4	27.5	86.5	–
Linoleic C18:2 (ω-6)	77.9	89.1	85.5	91.1	87.8	95.5	79.3	91.3	19.7	70.7	27.6	86.6	–
γ-Linolenic C18:3 (ω-6)	74.5	84.2	82.1	86.0	84.3	90.2	76.2	86.3	20.0	67.8	26.4	82.5	–
α-Linolenic C18:3 (ω-3)	77.4	87.8	85.2	89.7	87.4	94.8	78.8	90.2	20.8	71.0	27.7	86.5	–
Stearidonic C18:4 (ω-3)	77.3	88.3	84.9	90.2	87.2	93.9	78.8	90.3	21.7	69.9	27.3	84.9	–

Each value reports the average of at least two different experiments. Standard deviation was less than 5%

[a] Solvents: (A90) hexane:acetone (10:90, v/v); (A80) hexane:acetone (20:80, v/v); (H:ET) hexane:ethanol (30/70, v/v)

[b] Minor fatty acids contributing to 100% of total fatty acids were: C14:0, C16:1, C17:0, C18:1 ω-7, C18:2 (trans), C18:4 (trans), C20:0, C20:1 ω-9 and C22:0

[c] Raw FAEE: Product obtained from chemical ethanolysis of high SDA ω-3 modified soybean oil

Plus superspeed centrifuge (Thermo Fischer Scientific Inc., Waltham, MA). Centrifugations were performed at 8,000 rpm (11,270 g) in the scaled-up processes. The acceleration and deceleration rate was set at the maximum provided by the equipment during the centrifugations. The amount of time used in this separation should be minimized to avoid exposing the sample to warmer temperatures, which may impact crystallization. After centrifugation, the samples were stored again at −80 °C for 5 min, and then the crystallized fraction was separated from the liquid fraction by decantation. After separation of phases, the organic solvent was removed from the liquid fraction by using vacuum rotary evaporator at 50 °C. Likewise, the organic solvent remaining in the solid fraction was removed under nitrogen. The fatty acid content of both fractions was determined by GC. The solid fraction consists mainly of higher melting point fatty acids, while the liquid fraction contained low melting point fatty acids, with a higher concentration of PUFA, specifically SDA.

Study 2-Optimized Methodology

In a further study, the methodology of centrifugation was improved by keeping the rotor of the centrifuge at −80 °C for at least 24 h prior to the centrifugation process (performed at −20 °C). This procedure was not applied in the previous study to prevent possible damage of the rotor. The objective of this study was to investigate the possible effect of rotor cooling on SDA purity and yield by minimizing the impact of warmer temperatures on low melting components during this step. For that reason, this methodology was used mainly for the experiments that provided best results in the previous study. Two winterization times (4 and 24 h) were studied and oil:solvent ratio of 1:5 (w/v) was also investigated. This ratio was designated as 20% and it was obtained by dissolving 2 g of sample in 10 mL of solvent.

Derivatization of Samples for GC

Samples obtained from crystallizations of FFA (from the hydrolysis), were converted to fatty acid methyl esters (FAME) for determining their fatty acid profiles by GC analyses. These derivatizations followed AOAC Official Method 996.01, Section E [25], with minor modifications. Briefly, 70 mg of FFA were weighed into a Teflon-lined test tube. Then, 2 mL of 0.5 N NaOH in methanol was added followed by incubation for 5 min at 100 °C to

Table 2 Purity and yield of main fatty acids under different conditions in winterization of FFA in Study 1

Oil:solvent ratio (%, w/v)	10								Starting FFA[c]
Solvent[a]	Hexane		H90		H80		H70		
	LF	SF	LF	SF	LF	SF	LF	SF	
Fatty acid (purity, %)[b]									
Palmitic C16:0	1.2	24.5	1.3	23.5	1.9	25.9	7.3	27.5	12.2
Stearic C18:0	0.4	8.4	0.4	8.0	0.6	8.9	2.5	9.4	4.1
Oleic C18:1 (ω-9)	11.9	20.3	15.2	16.6	17.2	14.4	16.9	13.8	15.9
Linoleic C18:2 (ω-6)	22.6	26.7	27.4	21.7	29.2	18.5	27.0	17.0	24.6
γ-Linolenic C18:3 (ω-6)	11.6	2.3	9.8	4.5	8.6	5.3	7.7	5.5	7.2
α-Linolenic C18:3 (ω-3)	10.5	10.9	12.4	8.9	12.7	8.0	11.6	7.6	10.7
Stearidonic C18:4 (ω-3)	39.8	5.0	31.7	14.9	28.1	17.1	25.3	17.2	23.5
Fatty acid (yield, %)									
Palmitic C16:0	4.7	99.4	5.3	97.1	8.4	95.3	10.0	99.0	–
Stearic C18:0	4.3	100.8	4.9	98.4	7.9	96.6	9.5	100.6	–
Oleic C18:1 (ω-9)	37.2	63.2	45.8	52.6	57.8	40.5	61.5	38.3	–
Linoleic C18:2 (ω-6)	45.7	53.9	53.4	44.4	63.5	33.8	67.5	30.6	–
γ-Linolenic C18:3 (ω-6)	80.2	15.9	65.2	31.6	64.3	32.9	64.7	33.6	–
α-Linolenic C18:3 (ω-3)	48.8	50.4	55.7	41.8	63.4	33.7	66.5	31.3	–
Stearidonic C18:4 (ω-3)	84.4	10.7	64.8	31.9	64.0	32.9	65.7	32.3	–

Oil:solvent ratio (%, w/v)	2.5									
Solvent	Hexane		H90		H80		H70		Isooctane	
	LF	SF	LF	SF	LF	SF	LF	SF	LF	SF
Fatty acid (purity, %)										
Palmitic C16:0	1.3	23.6	1.6	19.0	2.0	34.2	2.8	37.6	1.8	24.9
Stearic C18:0	0.4	8.1	0.5	6.5	0.6	11.9	0.8	13.2	0.5	8.7
Oleic C18:1 (ω-9)	9.4	22.4	14.7	17.2	18.7	10.1	18.7	8.5	9.6	21.6
Linoleic C18:2 (ω-6)	20.8	28.2	29.3	20.4	30.4	12.2	29.2	12.1	19.0	26.8
γ-Linolenic C18:3 (ω-6)	12.6	1.7	10.0	5.2	8.0	5.4	8.0	5.1	13.1	1.8
α-Linolenic C18:3 (ω-3)	10.0	11.3	12.8	9.1	12.7	6.5	12.4	6.1	8.8	10.9
Stearidonic C18:4 (ω-3)	43.6	3.0	29.3	21.1	26.0	17.6	26.5	15.1	45.3	3.4
Fatty acid (yield, %)										
Palmitic C16:0	5.0	100.9	4.6	99.6	10.3	103.6	16.0	98.2	6.9	97.5
Stearic C18:0	4.3	102.7	4.3	101.1	8.9	107.1	14.1	101.9	5.5	100.7
Oleic C18:1 (ω-9)	28.3	73.4	33.1	69.1	74.0	23.4	79.8	17.1	27.5	64.8
Linoleic C18:2 (ω-6)	40.4	59.8	42.7	53.1	77.8	18.4	80.6	15.8	35.1	51.9
γ-Linolenic C18:3 (ω-6)	83.7	12.4	50.2	46.2	70.3	27.6	75.4	22.7	82.6	12.1
α-Linolenic C18:3 (ω-3)	44.5	55.2	43.0	54.2	74.4	22.3	78.8	18.1	37.4	48.5
Stearidonic C18:4 (ω-3)	88.7	6.7	44.8	57.6	69.9	27.7	76.7	20.7	87.7	6.9

Each value reports the average of at least two different experiments. Standard deviation was less than 5%

LF Liquid fraction; *SF* Solid fraction

[a] Solvents: (H90) hexane:acetone (90:10, v/v); (H80) hexane:acetone (80:20, v/v); (H70) hexane:acetone (70:30, v/v)

[b] Minor fatty acids contributing to 100% of total fatty acids were: C14:0, C16:1, C17:0, C18:1 ω-7, C18:2 (trans), C18:4 (*trans*), C20:0, C20:1 ω-9 and C22:0

[c] Starting FFA: Product obtained from chemical hydrolysis of high SDA ω-3 modified soybean oil

saponify the lipid. After incubation, 2 mL of 14% boron trifluoride (BF_3) in methanol were added. The sample was vortexed for 1 min and incubated again for 5 min at 100 °C to allow methylation. To stop the reaction and extract the FAME, 2 mL of hexane and 2 mL of saturated NaCl solution were added to the sample, vortexed for exactly 2 min at room temperature, and centrifuged for 5 min at 1,000 rpm (76 g) to separate the organic and aqueous phases. The upper organic layer was filtered through an anhydrous sodium sulfate column and recovered into a GC vial and analyzed. Samples obtained from crystallizations of FAEE were analyzed directly by GC.

GC Analysis

FAME and FAEE were analyzed using an Agilent Technology (Santa Clara, CA) 6890 N gas chromatograph equipped with a flame ionization detector. Separation was achieved with an SP-2560 column, 100 m 0.25 mm i.d., and 0.20 μm film (Supelco Inc., Bellefonte, PA). Injection (1 μL) was performed at a split ratio of 20:1. Helium was the carrier gas at a constant flow rate of 1.1 mL/min. The injector temperature was 250 °C, and the FID was 300 °C. The temperature was held at 140 °C for 5 min, and then increased up to 240 °C with ramping at 4 °C/min and held isothermally for 15 min. FAEE relative content was calculated by integration using a GC Chemstation software. Identification of the various fatty acid methyl and ethyl esters was based on the retention times and relative area percentages of a Supelco 37 Component FAME mix (Supelco Inc., Bellefonte, PA). Quantification was via an external standard of palmitic acid ethyl ester, stearic acid ethyl ester, and stearidonic methyl ester. These standards were selected to calculate the response factors of different fatty acids according to their chain length. FAEE samples were dissolved in hexane for GC analyses at 20–25 mg/mL.

Statistical Analyses

Software package ORIGIN 8.0 (Northampton, MA) was used to calculate averages, standard deviations and to perform the analysis of variance (ANOVA). The significance level was $p < 0.05$. All experiments were performed at least in duplicate, and the mean values are shown in the tables and figure.

Results and Discussion

Low temperature crystallization was performed to obtain fractions with high purity and yield of SDA. As mentioned in Methods section, FFA from hydrolysis and FAEE from ethanolysis of modified soybean oil were used as starting materials.

Two main responses were evaluated

a. Purity or composition (%)

$$= \frac{\text{weight of a fatty acid in a fraction(mg)}}{\text{weight of the entire fraction(mg)}} \times 100$$

b. Yield(%)

$$= \frac{\text{weight of a fatty acid in a fraction(mg)}}{\text{weight of this fatty acid present in the starting material(mg)}} \times 100$$

Study 1-Preliminary Study

FAEE Low Temperature Crystallization

Winterization of the FAEE produced by chemical ethanolysis of the original oil was performed in Study 1. In the first approach, acetone; hexane:acetone (10:90, v/v); hexane:acetone (20:80, v/v); and hexane:ethanol (30:70, v/v) were used as solvents.

No statistical differences were detected between the two winterization times (4 and 24 h) under all conditions investigated. Therefore, the variable, winterization time, had no effects on purity and yield of SDA. Results of purity and yield of FAEE obtained in the liquid fraction, are reported in Table 1. In this table, results obtained at different winterization times were combined, since it was shown that this variable had no effect in the experimental results.

It was found that the amount of material recovered in the solid fraction was, overall, very low. Hence, the percentage of total weight of FAEE crystallized based on the starting amount of FAEE, was in the range 7–36%, depending on the solvent and the oil:solvent ratio used. Regarding the behavior of different solvents at both ratios, the total weight of crystallized FAEE significantly increased following this order: hexane:acetone (20:80, v/v) < hexane:acetone (10:90, v/v) < acetone. This pattern indicates a direct relationship with the polarity of the solvents involved, which decreases with increase in hexane content. SDA purity averages followed the same pattern and thus, SDA purity was higher when greater amounts of total FAEE crystallized. Due to the low polarity of FAEE, solvents with increased polarity were required to decrease the solubility of certain FAEE in the liquid fraction and thus, enhance the selectivity of crystallization. On the contrary, SDA yield averages followed an inverse relationship with amount of FAEE crystallized: acetone < hexane:acetone (10:90, v/v) < hexane:acetone (20:80, v/v). In other words, it was observed that as long as a higher amount of FAEE was crystallized, the SDA yield was lower. In all the responses evaluated hexane:ethanol (30:70, v/v) provided intermediate values compared to hexane:acetone (10:90, v/v) and acetone. Few statistical differences were obtained in the study of FAEE due to the high homogeneity of the results.

A significantly higher amount of total FAEE was crystallized with 10% oil:solvent ratio than with 2.5% ratio for all solvents. Overall, FAEE are compounds with low polarity and relatively low melting points. For that reason, almost all FAEE, regardless of chain length or number of double bonds, tend to remain in the liquid fraction at the temperature investigated (−80 °C), especially at low

oil:solvent ratios and with less polar solvents, due to the increased solubility of FAEE at these conditions. Because of this, very low improvements in the purity of SDA and high SDA yields were attained by performing crystallization of FAEE at all conditions investigated.

A further study of winterization of FAEE was performed to concentrate SDA by employing methanol and ethanol as solvents, according to the patent by Luthria [23]. Results of SDA purity and yield are included in Table 1. It should be noted that at 10% ratio, SDA purity was significantly improved by using methanol and ethanol compared to the rest of the solvents. In addition, at 10% ratio, SDA purity obtained with methanol (30.7%) was significantly higher than that obtained with ethanol (28.1%). The higher polarity of methanol compared to ethanol may explain the increase in SDA purity. However, no significant differences were found at 2.5% ratio, between SDA purity obtained with methanol, ethanol and acetone. In addition, the improvement in the crystallization extent by using methanol and ethanol led to significantly lower SDA yields compared to the rest of the solvents, at both ratios. As observed in Table 1, SDA yield was remarkably higher at 2.5% than at 10% ratio, for both methanol and ethanol.

Therefore, although some concentration of SDA was achieved by using the most polar solvents (acetone, methanol and ethanol), winterization of FAEE was not efficient, due to the low melting points of FAEE. Temperatures lower than −80 °C might provide an increased purity of SDA.

FFA Low Temperature Crystallization

Winterization of the FFA produced by chemical hydrolysis of the original oil was performed in Study 1. As described in the Methods section, four solvents were selected: pure hexane; hexane:acetone (90:10, v/v); hexane:acetone (80:20 v/v) and hexane:acetone (70:30, v/v), with oil:solvent ratios of 2.5 and 10%. The lowest temperature possible (−80 °C) was selected and the winterization time was 4 h.

Table 2 shows the purity and yield of the main fatty acids obtained in the liquid fraction and the solid fraction after winterization. Regarding the different solvents tested, SDA purity and yield were significantly higher with hexane than with the rest of hexane:acetone mixtures at both oil:solvent ratios. Moreover, no statistical differences in SDA purity and yield were found between hexane:acetone (80:20, v/v) and hexane:acetone (70:30, v/v). Hexane:acetone (90:10, v/v) gave intermediate values of SDA purity between hexane and the rest of the solvents, at both ratios. In terms of yield, at 10% ratio, hexane:acetone (90:10, v/v) gave the same result as hexane:acetone (80:20, v/v) and hexane:acetone (70:30, v/v), and significantly the lowest yield at 2.5% ratio.

For the oil:solvent ratios tested, SDA purity and yield were significantly higher at 2.5% (43.6% purity, 88.7% yield) than at 10% ratio (39.8% purity, 84.4% yield) with hexane only. SDA yield followed the opposite pattern with hexane:acetone (90:10, v/v) and no significant differences in SDA purity and yield were detected between both ratios in other solvents tested.

Therefore, the most remarkable enhancements in SDA purity (43.6%) and yield (88.7%) were achieved by using hexane at 2.5% oil:solvent ratio. Other authors also reported hexane as the best solvent for concentration of PUFA by winterization [5, 15]. This result can be explained by the increased polarity of FFA against other compounds, such as TAG or FAEE. Differences in polarity of solvents and compounds can play an important role in the winterization process. Hence, polar solvents were better for concentration of SDA in winterization of TAG (low polarity) [5, 15, 24] or FAEE (very low polarity), whereas a different behavior was observed with FFA. Due to the higher polarity of FFA, a solvent with very low polarity (hexane) was required to decrease the solubility of certain FFA in the liquid fraction and thus, enhance the selectivity of crystallization.

For reasons stated above, further experiment with isooctane, which provided lower polarity than hexane, was performed. This experiment was carried out at 2.5% oil:solvent ratio and at the same conditions of time (4 h) and temperature (−80 °C) as in previous experiments. Although a slight increase in SDA purity (45.3%) was achieved, no significant differences were detected in SDA purity and yield between isooctane and hexane. These results are also included in Table 2.

The differences observed between the winterization extent of FFA and FAEE could derive from the lower melting point of FAEE compared with that of FFA. The higher melting FFA permits the crystallization of a larger amount of raw material, enhancing, in this case, the efficiency and selectivity of the winterization process.

Study 2-Optimized Methodology

As described in Methods section, the methodology of centrifugation was improved by chilling the rotor of the centrifuge at −80 °C for at least 24 h prior to the centrifugation process. This procedure was applied mainly to the solvents that provided best results in Study 1. The winterization time was investigated again to check for any differences under the new conditions. The optimized procedure significantly minimized the sample exposure to warmer temperatures during centrifugation, and this may be critical in the crystallization process, especially for components with the lowest melting points. It was found

Table 3 Purity and yield of main fatty acids with hexane as solvent under different conditions in winterization of FFA in Study 2

Winterization time (h)	4					
Oil:solvent ratio (%, w/v)	2.5		10		20	
	LF	SF	LF	SF	LF	SF
Fatty acid (purity, %)[a]						
Palmitic C16:0	1.4	22.2	0.7	18.3	1.5	16.4
Stearic C18:0	0.4	7.7	0.1	6.3	0.5	5.6
Oleic C18:1 (ω-9)	9.2	21.5	2.6	21.0	4.6	19.8
Linoleic C18:2 (ω-6)	19.7	29.0	15.9	29.4	10.8	30.1
γ-Linolenic C18:3 (ω-6)	12.7	2.0	14.7	3.1	16.3	3.5
α-Linolenic C18:3 (ω-3)	9.8	11.6	9.0	11.8	6.5	12.6
Stearidonic C18:4 (ω-3)	44.2	4.1	51.2	8.3	57.0	9.9
Fatty acid (yield, %)						
Palmitic C16:0	5.6	93.6	2.2	98.5	3.4	97.4
Stearic C18:0	4.9	94.6	1.1	98.8	3.2	97.9
Oleic C18:1 (ω-9)	27.7	70.8	6.2	88.7	8.1	91.6
Linoleic C18:2 (ω-6)	37.8	60.6	23.3	78.5	12.0	88.5
γ-Linolenic C18:3 (ω-6)	82.7	14.2	72.9	28.6	61.0	34.9
α-Linolenic C18:3 (ω-3)	42.8	55.2	29.9	71.8	16.3	83.8
Stearidonic C18:4 (ω-3)	87.6	8.8	77.8	23.4	65.2	29.9
Winterization time (h)	24					
Oil:solvent ratio (%, w/v)	2.5		10		20	
	LF	SF	LF	SF	LF	SF
Fatty acid (purity, %)						
Palmitic C16:0	2.6	18.3	0.6	17.5	1.2	16.0
Stearic C18:0	0.8	6.3	0.2	6.0	0.4	5.5
Oleic C18:1 (ω-9)	4.3	22.7	3.4	21.0	3.7	19.5
Linoleic C18:2 (ω-6)	12.3	32.6	9.5	31.6	8.4	30.1
γ-Linolenic C18:3 (ω-6)	15.4	1.8	17.1	2.6	17.5	3.6
α-Linolenic C18:3 (ω-3)	7.4	13.1	6.3	12.9	5.3	12.8
Stearidonic C18:4 (ω-3)	53.8	3.6	59.8	6.4	61.4	10.3
Fatty acid (yield, %)						
Palmitic C16:0	8.3	89.5	1.7	99.0	2.5	94.5
Stearic C18:0	8.0	90.1	1.6	99.3	2.4	94.8
Oleic C18:1 (ω-9)	10.9	86.6	7.1	92.7	6.2	90.4
Linoleic C18:2 (ω-6)	19.7	78.8	12.7	88.5	8.9	88.4
γ-Linolenic C18:3 (ω-6)	83.1	14.6	76.7	24.5	63.0	35.9
α-Linolenic C18:3 (ω-3)	26.6	71.9	18.9	82.4	12.7	85.2
Stearidonic C18:4 (ω-3)	88.5	8.9	82.3	18.7	67.6	31.2

Each value reports the average of at least two different experiments. Standard deviation was less than 5%

LF Liquid fraction; *SF* Solid fraction

[a] Minor fatty acids contributing to 100% of total fatty acids were: C14:0, C16:1, C17:0, C18:1 ω-7, C18:2 (trans), C18:4 (trans), C20:0, C20:1 ω-9 and C22:0

that keeping the rotor at −80 °C for only 4 h was not enough time to improve the winterization.

Study 2 focused on FFA since Study 1 showed low efficiency of winterization of FAEE at −80 °C due to its low melting points. Hexane was used as solvent for winterization of FFA, since this solvent provided the best results of SDA purity and yield in Study 1. Two winterization times (4 and 24 h) were investigated at 2.5, 10 and 20% oil:solvent ratios.

Table 3 reports the purity and yield of the main fatty acids obtained in the liquid and solid fraction after winterization of FFA in Study 2. Winterization time had a significant effect on SDA purity at all oil:solvent ratios investigated and thus, SDA purity was significantly higher at 24 h than at 4 h in all cases. On the contrary, no statistical differences were found in SDA yield between both times under all conditions. Regarding the differences between different oil:solvent ratios, at both 4 and 24 h, SDA purity was significantly higher with 10 or 20% as ratios than with 2.5%, whereas no statistical differences were found between 10 and 20%. Moreover, at both winterization times, SDA yield followed this pattern: $20 < 10 < 2.5\%$. Only these results were significantly different from each other at 24 h. The reason why SDA yields were not statistically different at 4 h, could be that the crystallization was not complete at this winterization time.

The results of Study 1 and Study 2 performed at the same conditions (hexane as solvent and 4 h) were compared. At a 2.5% oil:solvent ratio no statistical differences were found between Study 1 and Study 2 in SDA purity (43.6 and 44.2%, respectively) and yield (88.7 and 87.6%, respectively). However, at 10% oil solvent ratio, SDA purity was significantly improved in Study 2 (51.2%) than in Study 1 (39.8%), with a slight decrease in SDA yield (77.8% in Study 2 and 84.4% in Study 1). It is remarkable that simple optimization in the methodology carried out in Study 2 of cooling the rotor at −80 °C for 24 h prior to centrifugation, could play a decisive role in the winterization process and remarkably improved the SDA purity in the final products. Nevertheless, the feasibility and costs for performing or maintaining this optimization must be evaluated on an industrial scale.

Therefore, with the optimized methodology, the best relationship between SDA purity (59.8%) and SDA yield (82.3%) was attained by performing winterization of FFA with hexane at 10% oil:solvent ratio for 24 h. These conditions and low temperature (−80 °C), were shown to be the most expedient for concentration of SDA by winterization of FFA, and provided the best efficiency and selectivity in the process. It should be noted that at these conditions, 99.0% total palmitic acid, 99.3% total stearic acid, 92.7% total oleic acid, 88.5% total linoleic acid, and 82.4% total α-linolenic acid were crystallized and removed, and this contributed to the high purity of SDA in the liquid fraction (59.8%). On the contrary, only 24.5% of total γ-linolenic acid was crystallized at these conditions, and thus most of the γ-linolenic acids were obtained with SDA. Therefore, highest values of γ-linolenic acid purity (17.1%) with 76.7% yield were also reached by using hexane at 10% oil:solvent ratio and 24 h at conditions of Study 2. Although α-linolenic and γ-linolenic acids have the same melting point (−11 °C), these compounds showed different

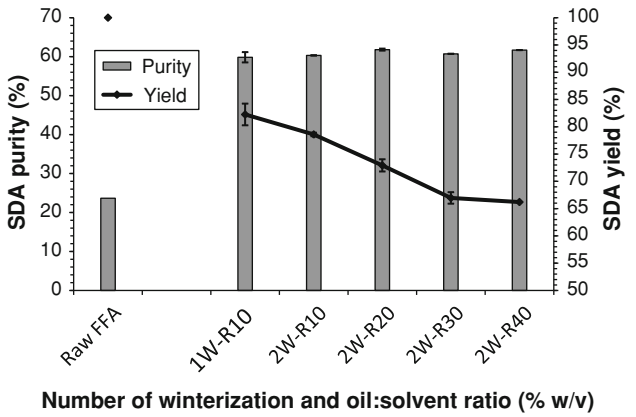

Fig. 1 SDA purity and yield of liquid fraction obtained after first and second winterization of FFA, with hexane as solvent, 24 h and conditions of Study 2 (optimized methodology). Solvents: (H) hexane; (ISO) isooctane. Processes: (1 W) First winterization; (2 W) Second winterization. Oil:solvent ratios: (R10) 1:10 (w/v); (R20) 2:10 (w/v); (R30) 3:10 (w/v); (R40) 4:10 (w/v). *Error bars* represent the standard deviation between different experiments

behavior during the winterization process. As reported by Lopez-Martinez et al. [15], the solubility of α-linolenic is higher in the more polar solvents than in the less polar hexane. Because of that, α-linolenic crystallized and decreased in the liquid fraction, while γ-linolenic acid was more concentrated in hexane solutions as well as SDA. Polarity and differences in spatial conformation between α-linolenic and γ-linolenic acid carbon chains must determine the different crystallization behavior of both isomers.

An experiment with isooctane was also performed at a 10% oil:solvent ratio and 24 h, since in Study 1 this solvent provided similar results to hexane. Likewise, in Study 2 no significant differences in SDA purity were found between both solvents (57.0% with isooctane and 59.8% with hexane), and SDA yield was significantly lower with isooctane (71.3%) than with hexane (82.3%).

Double Crystallization at Low Temperature

A double crystallization process was performed by using, as starting material, the liquid fraction obtained from the first winterization, and employing the conditions of the optimized methodology (Study 2). Following the previous step, hexane was selected as solvent for the double crystallization process. The winterization time was 24 h, and four oil:solvent ratios were investigated: 1:10, 2:10, 3:10 and 4:10 (w/v). Results of SDA purity and yield obtained in the first and subsequent second crystallizations are shown in Fig. 1. As observed in this figure, only slight increments in SDA purity were obtained after the second crystallization, whereas SDA yield remarkably decreased with increasing oil:solvent ratios. For that

Table 4 Purity and yield of main fatty acids in small scale and scale-up winterization of FFA

	Small scale[a]		Scale-up	
	LF	SF	LF	SF
Fatty acid (purity, %)[b]				
Palmitic C16:0	0.6 ± 0.2	17.5 ± 0.3	0.4 ± 0.2	17.8 ± 0.4
Stearic C18:0	0.2 ± 0.1	6.0 ± 0.1	0.1 ± 0.1	6.1 ± 0.1
Oleic C18:1 (ω-9)	3.4 ± 0.2	21.0 ± 0.2	3.4 ± 0.1	21.1 ± 0.3
Linoleic C18:2 (ω-6)	9.5 ± 0.9	31.6 ± 0.2	9.5 ± 0.6	31.7 ± 0.1
γ-Linolenic C18:3 (ω-6)	17.1 ± 0.4	2.6 ± 0.1	17.0 ± 0.4	2.5 ± 0.1
α-Linolenic C18:3 (ω-3)	6.3 ± 0.6	12.9 ± 0.1	6.6 ± 0.7	12.9 ± 0.1
Stearidonic C18:4 (ω-3)	59.8 ± 1.3	6.4 ± 0.4	59.6 ± 1.1	6.1 ± 0.5
Fatty acid (yield, %)				
Palmitic C16:0	1.7 ± 0.7	99.0 ± 4.4	1.0 ± 0.6	98.9 ± 0.3
Stearic C18:0	1.6 ± 0.7	99.3 ± 4.5	0.8 ± 0.8	99.1 ± 0.6
Oleic C18:1 (ω-9)	7.1 ± 0.3	92.7 ± 3.8	7.2 ± 0.3	91.5 ± 1.5
Linoleic C18:2 (ω-6)	12.7 ± 1.2	88.5 ± 4.6	12.7 ± 0.9	87.3 ± 2.2
γ-Linolenic C18:3 (ω-6)	76.7 ± 1.7	24.5 ± 1.7	76.9 ± 1.1	23.1 ± 2.0
α-Linolenic C18:3 (ω-3)	18.9 ± 1.7	82.4 ± 4.7	19.9 ± 2.1	80.4 ± 3.3
Stearidonic C18:4 (ω-3)	82.3 ± 2.0	18.7 ± 1.6	82.6 ± 1.0	17.3 ± 2.0
Product total weight (g)[c]	0.325 ± 0.002	0.671 ± 0.001	59.0 ± 0.4	121.0 ± 1.5

Each value reports the average of at least two different experiments. The value after the ± symbol represents the standard deviation between the different assays

LF Liquid fraction; *SF* Solid fraction

[a] Conditions. Solvents: Hexane; oil:solvent ratio (10%, w/v); winterization time (24 h)

[b] Minor fatty acids contributing to 100% of total fatty acids were: C14:0, C16:1, C17:0, C18:1 ω-7, C18:2 (trans), C18:4 (trans), C20:0, C20:1 ω-9 and C22:0

[c] The starting material was 1 g for small scale and 180 g in the scale-up processes

reason, second crystallization of FFA is not a valuable process for scale-up.

Scale-up

Winterization was scaled-up to 180 g of starting material in a single step. Best conditions obtained with the optimized methodology (Study 2) were used for the scale-up process. Hence, hexane at 10% oil:solvent ratio and 24 h were used. Six flasks containing 30 g of sample and 300 mL of solvent were used to perform the scaled-up process. Table 4 compares the purity and yield of the main fatty acids obtained in the scaled-up process with the results obtained at the same conditions in small scale. The results depict a remarkable high reproducibility attained in this work. The final product obtained after FFA scaled-up winterization was 59 g composed of 59.6% SDA, whereas the SDA yield of the process was 82.6%. In our opinion, the process should be readily scaled-up with high SDA purity and yield and excellent reproducibility.

Low temperature crystallization could be used to concentrate stearidonic acid as free fatty acids from high SDA modified soybean oil, obtaining high purity (~60%) and yield (>82%). The process described in this paper can be scaled-up to obtain 59 g of the SDA concentrate, with high reproducibility, selectivity, efficiency and low cost (only low temperature is required, −80 °C). In addition, in this process, no urea crystallization method that may lead to carcinogenic carbamates was involved. For these reasons, the products obtained can be used for production of structured lipids, nutraceuticals and functional lipids, and this process should be useful and valuable for clinical trials and food or pharmaceutical applications.

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References

1. Kris-Etherton PM, Harris WS, Appel LJ (2002) Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. Circulation 106(21):2747–2757
2. Calder PC (2006) n-3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases. Am J Clin Nutr 83(6):S1505–S1519

3. Fernandez E, Chatenoud L, La Vecchia C, Negri E, Franceschi S (1999) Fish consumption and cancer risk. Am J Clin Nutr 70(1):85–90
4. Lim GP, Calon F, Morihara T, Yang F, Teter B, Ubeda O, Salem NJ, Frautschy SA, Cole GM (2005) A diet enriched with the omega-3 fatty acid docosahexaenoic acid reduces amyloid burden in an aged Alzheimer mouse model. J Neurosci 25(12):3032–3040
5. Shahidi F, Wanasundara UN (1998) Omega-3 fatty acid concentrates: nutritional aspects and production technologies. Trends Food Sci Tech 9(6):230–240
6. Mendes A, Lopes da Silva T, Reis A (2007) DHA concentration and purification from the marine heterotrophic microalga *Crypthecodinium cohnii* CCMP 316 by winterization and urea complexation. Food Technol Biotech 45(1):38–44
7. Mantzioris E, James MJ, Gibson RA, Cleland LG (1994) Dietary substitution with an alpha-linolenic acid-rich vegetable oil increases eicosapentaenoic acid concentrations in tissues. Am J Clin Nutr 59(6):1304–1309
8. Whelan J (2009) Dietary stearidonic acid is a long chain (n-3) polyunsaturated fatty acid with potential health benefits. Am J Clin Nutr 139(1):5–10
9. Jacobsen C, Let MB, Nielsen NS, Meyer AS (2008) Antioxidant strategies for preventing oxidative flavour deterioration of foods enriched with n-3 polyunsaturated lipids: a comparative evaluation. Trends Food Sci Tech 19(2):76–93
10. Shimada Y, Sugihara A, Tominaga Y (2001) Enzymatic purification of polyunsaturated fatty acids. J Biosci Bioeng 91(6):529–538
11. Ratnayake WMN, Olsson B, Matthews D, Ackman RG (1988) Preparation of omega-3 PUFA concentrates from fish oils via urea complexation. Eur J Lipid Sci Tech 90(10):381–386
12. Senanayake SPJN, Shahidi F (2000) Concentration of docosahexaenoic acid (DHA) from algal oil via urea complexation. J Food Lipids 7(1):51–61
13. Guil-Guerrero J, López-Martínez J, Rincón-Cervera M, Campra-Madrid P (2007) One-step extraction and concentration of polyunsaturated fatty acids from fish liver. J Am Oil Chem Soc 84(4):357–361
14. Guil-Guerrero JL, Campra-Madrid P, Belarbi E-H (2000) γ-Linolenic acid purification from seed oil sources by argentated silica gel chromatography column. Process Biochem 36(4):341–354
15. López-Martínez JC, Campra-Madrid P, Guil-Guerrero JL (2004) γ-Linolenic acid enrichment from *Borago officinalis* and *Echium fastuosum* seed oils and fatty acids by low temperature crystallization. J Biosci Bioeng 97(5):294–298
16. Canas B, Yurawecz M (1999) Ethyl carbamate formation during urea complexation for fractionation of fatty acids. J Am Oil Chem Soc 76(4):537
17. Yokochi T, Usita M, Kamisaka Y, Nakahara T, Suzuki O (1990) Increase in the γ-linolenic acid content by solvent winterization of fungal oil extracted from *Mortierella* genus. J Am Oil Chem Soc 67(11):846–851
18. Chen T-C, Ju Y-H (2001) An improved fractional crystallization method for the enrichment of gamma-linolenic acid in borage oil fatty acid. Ind Eng Chem Res 40(17):3781–3784
19. Han D-S, Ahn B-H, Shin H-K (1987) Separation of EPA and DHA from fish oil by solubility differences of fatty acid salts in ethanol soaps. Korean J Food Sci Technol 19(5):430–434
20. Vázquez L, Akoh CC (2010) Fractionation of short and medium chain fatty acid ethyl esters from a blend of oils via ethanolysis and short-path distillation. J Am Oil Chem Soc 87(8):917–928
21. Torres CF, Vazquez L, Senorans FJ, Reglero G (2007) An efficient methodology for the preparation of alkoxyglycerols rich in conjugated linoleic acid and eicosapentaenoic acid. J Am Oil Chem Soc 84(5):443–448
22. Namal Senanayake S, Shahidi F (1999) Enzymatic incorporation of docosahexaenoic acid into borage oil. J Am Oil Chemists' Soc 76(9):1009–1015
23. Luthria DL (2002) Process for making an enriched mixture of polyunsaturated fatty acid esters. Assigned to OmegaTech Inc. (Boulder, CO). US 6,395,778 B1 Patent (09/758,973)
24. Fabritius D (2009) Mixtures of triglycerides of natural polyunsaturated fatty acids with high polyunsaturated fatty acid content, method for producing same and use thereof. Assigned to Nutrinova Nutrition Specialities & Food Ingredients GmbH (Frankfurt,DE). US 7,588,791 B2 Patent (10/493,116)
25. AOAC International (1998) Official Methods of Analysis of AOAC International, 17th edn. Gaithersburg, MD, Method 996.01