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Purification of Stearidonic Acid from Modified Soybean Oil by Argentation Silica Gel Column Chromatography

Leslie Kleiner-Shuhler · Luis Vázquez · Casimir C. Akoh

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Abstract The objective of this study was to purify stearidonic acid (SDA, 18:4 ω-3) from modified soybean oil containing a mixture of over 20 fatty acids (23% SDA). Interest in obtaining purified fractions of SDA arises from reported health benefits associated with polyunsaturated fatty acids (PUFA), such as cardiovascular disease prevention. In addition, SDA may also provide improved stability characteristics since its unsaturation index is less than longer PUFA, such as eicosapentaenoic acid (EPA, 20:5 ω-3). First, a chemical ethanolysis of modified soybean oil was performed to transform the triacylglycerols into fatty acid ethyl esters (FAEE). Then, the FAEE were fractionated and SDA-EE was purified by argentation silica gel (10% $AgNO_3$) open column chromatography, which allows selectivity based on degree of unsaturation. Different FAEE sample loads and mobile phases were explored until the best purification of SDA-EE was achieved. The solvents used were hexane and hexane:acetone mixtures (99 and 95%). Under the optimal conditions, a fraction with high SDA-EE purity (96%) was obtained with 77% yield. Besides, it was possible to obtain another fraction enriched in α-linolenic acid-EE (37% purity and 68% yield) and γ-linolenic acid-EE (22% purity and 61% yield). A scaled-up process resulted in 840 mg of final product composed of 97% SDA-EE with 71% yield.

Keywords Argentation chromatography · Column chromatography · Fatty acid ethyl ester · Modified soybean oil · Stearidonic acid

L. Kleiner-Shuhler · L. Vázquez · C. C. Akoh (✉)
Department of Food Science and Technology, University of Georgia, Athens, GA 30602-2610, USA
e-mail: cakoh@uga.edu

Introduction

The dietary consumption of polyunsaturated fatty acids (PUFA) has been associated with a variety of health benefits. In particular, highly unsaturated PUFA such as α-linolenic acid (ALA, 18:3 ω-3), eicosapentaenoic acid (EPA, 20:5 ω-3), and docosahexaenoic acid (DHA, 22:6 ω-3), have been found to have effects on the reduction of cardiovascular disease [1], inflammation [2], cancer [3], and neurological disorders [4]. Accordingly, EPA and DHA ethyl esters are the main components (more than 90%) of Lovaza®, the first prescription omega-3 product approved by the FDA. Lovaza is used to reduce the hepatic production of triacylglycerols [5]. More recently, there has been increasing interest in stearidonic acid (SDA, 18:4 ω-3), a PUFA that is a metabolic intermediate in the conversion of ALA to EPA [6]. SDA has less degree of unsaturation than EPA and DHA and is therefore, less susceptible to lipid oxidation, the formation of undesirable free radicals, aldehydes, and off-flavors [7]. Because of its physical characteristics and its role on the metabolism of other PUFA, SDA became of particular interest to the food industry.

SDA is found naturally in low quantities in fish and some other marine life. EPA and DHA constitute 15–20% of the total fatty acid profile of fish, while SDA typically constitutes 0.5–2% of the fatty acids [8]. The increased interest in incorporating PUFA into the diet and their low bioavailability have led to overfishing and a severe decline in some fish populations [9]. For these reasons there is a current interest in the development of alternative methods to obtain PUFA. Thus, current genetic engineering technologies have been used to produce vegetable oils containing SDA.

PUFA have been traditionally purified by discriminating the fatty acids present based on their polarity and/or degree

of unsaturation. Some methods that use these principles are winterization (low temperature crystallization) [10], urea complexation methods [11], and argentation (Ag) silica gel column chromatography [12]. In order to obtain individual PUFA with purities higher than 90%, winterization and urea complexation methods need to be coupled to HPLC [13]. This extra step further increases the cost associated with the purification techniques, as well as the time needed to obtain a purified fraction.

Ag-silica gel chromatography is performed under mild conditions (no extreme temperatures or pressures are needed), and it has been shown to provide high purity fractions (>90%) of fatty acid methyl esters (FAME), without further purification by HPLC [12, 13]. Different compounds can be fractionated by their specific interactions between the stationary phase (usually silica gel) and the mobile phase (elution solvents). For Ag-silica gel chromatography, the silica gel is coupled to silver ions (Ag^+) that form reversible polar complexes with unsaturated locations in organic molecules [14]. Due to that reversible complexation, it is possible to use this technique to separate PUFA based on the number, position, and geometric configuration of double bonds. The extent and the strength of complexation control the mobility of a solute, as does the polarity of the mobile phase. The concentration of silver nitrate loaded per unit mass of silica gel influences the surface density of Ag^+ ions on the solid matrix, hence, affecting the retention time of the solute [15]. This principle has also been applied to thin layer chromatography (TLC) [16] and HPLC [17].

Ag-silica gel chromatography has been used to purify ALA, γ- linolenic acid (GLA, 18:3 ω-6), EPA, DHA, and SDA from a variety of free fatty acids (FFA) [18] and FAME [12, 13, 15] mixtures. For example, Ryu et al. [18] obtained 95% ALA from a hydrolysate of perilla oil. Sajilata et al. [15] and Guerrero et al. [12] used this technology to purify GLA methyl ester from *Spirulina platensis* and several plant sources, respectively. In a different study, Guil-Guerrero et al. [13] obtained ALA methyl ester from linseed oil, GLA methyl ester from Borage oil, and EPA, DHA, and SDA methyl esters from Shortfin Mako (*Isurus oxyrinchus*) liver oil. The elution solvents employed were solutions of hexane:acetone, and the maximum sample load on the silver ion silica gel did not exceed 4% (w/w) (weight of sample/weight of stationary phase) [12, 13, 15, 18].

The main goal of the current work was to isolate SDA ethyl ester (SDA-EE) with high purity and yield by Ag-silica gel open column chromatography from high SDA modified soybean oil. Different variables were investigated, such as the maximum sample load, the optimal polarity of mobile phases and volumes of the elution solvents. In particular, this work focused on the FAEE load optimization to enhance the amount of final product obtained in a single process.

Materials and Methods

Materials

High SDA modified soybean oil was provided by the Monsanto Company (St. Louis, MO, USA). Modified soybean oil is not yet commercially available. SDA-enriched soybean oil was produced through the overexpression of lipid biosynthetic genes in normal soybean oil (Monsanto Co., St. Louis, MO, USA) resulting in the presence and increased levels of SDA in soybean oil. This modified soybean oil can provide a sustainable and inexpensive plant source of omega-3 fatty acids. Palmitic acid ethyl ester (purity, >99%) and stearic acid ethyl ester (purity, 99%) were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). Sodium ethoxide, 21% (w/w) in ethanol, was obtained from Alfa Aesar, a Johnson Matthey Company (Ward Hill, MA, USA). Stearidonic acid methyl ester was purchased from the Cayman Chemical Company (Ann Arbor, MI, USA). Silica gel (63–200 μm particle size, Catalog No: 196724) was purchased from Selecto Scientific (Suwanee, GA, USA). Silver nitrate (ACS grade crystals) was obtained from EMD Biochemicals (Darmstadt, Germany). Pasteur pipettes (5¾ inch length), crystalline sodium chloride, anhydrous sodium sulfate, acetone, hexane and ethanol (96%, v/v) were purchased from Fisher Scientific (Pittsburgh, PA, USA). All the solvents and compounds were ACS grade.

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 [19]. The reaction was performed in a 1-L cylindrical vessel. 200 mL of the oil was mixed with sodium ethoxide (2.625%, w/v) in absolute ethanol at a ratio of 4:2 (v/v) (2.25 fold molar excess of ethanol). The mixture was heated at 60 °C with mechanical shaking for 40 min, under a 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%, with yield over 95%. This product was used as the starting material for subsequent fractionation of FAEE.

Argentation of Silica Gel (10% $AgNO_3$)

The argentation of the silica gel was based on the method described by Ryu et al. [18] and Guil-Guerrero et al. [13] as follows: 100 g of silica gel (63–200 μm particle size) were placed in a beaker containing 200 mL ethanol (96%, v/v). This slurry was agitated with a mechanical stirrer for 10 min. Then, 10 g $AgNO_3$ were dissolved in 35 mL of ethanol:water (70:30,v/v), and the solution obtained was added to the slurry. The mixture was agitated for another 10 min and the solvent evaporated in a rotary evaporator at 60 °C. $AgNO_3$ silica gel was dried at 100 °C for 2 h, cooled at room temperature and kept in the dark under dry atmosphere until further use.

Fractionation of FAEE by Ag-silica Gel Open Column Chromatography (analytical scale)

The chromatography columns employed for all analytical scale experiments were Pasteur pipettes (146 mm length and approximately 5.7 mm diameter), with the ends plugged with glass wool. Prior to packing the column, the Ag-silica gel was activated for 2 h at 100 °C. Approximately 1 g of the Ag-silica gel was dry packed into the column as the stationary phase. For all experiments the packed height was 70 mm measured from the top of the glass wool. The column was tapped while packing in order to ensure compact packing of the gel. Conditioning of the column was by gravity after adding 4 mL hexane as elution solvent. At this time, the volumetric flow rate was measured to be 0.286 mL/min (1 mL/3.5 min). Sample of FAEE was then loaded in the column. For the optimization of FAEE sample loads, samples of 5, 10, 15, 20, 25, 30 and 50% w/w (weight of sample/weight of stationary phase) were explored. The samples were prepared by dissolving, respectively, 50, 100, 150, 200, 250, 300, and 500 mg of FAEE in 1 mL of the solvent of the first fraction (hexane).

The solvents used in this work were: hexane; hexane:acetone (99:1, v/v); hexane:acetone (95:5, v/v) and hexane:acetone (90:10, v/v). In order to simplify the nomenclature, these solvents were designated as 100, 99, 95 and 90% hexane, respectively. These solvents were introduced into the column in order of increasing polarity and were allowed to elute by gravity. The number of fractions and the volumes of the solvents were investigated and discussed in "Results."

After the elution, each fraction was then washed with NaCl solution (1%, w/v). The volume of the NaCl solution employed was half of the volume of each fraction. The organic layer was collected, dried with anhydrous sodium sulfate, and the solvent evaporated under nitrogen. It was crucial to wash the anhydrous sodium sulfate with another 2 mL of hexane to effectively recover all the FAEE of each fraction. The residue obtained was then analyzed by GC. Experiments under optimal conditions were performed in triplicate. The mean values of purity, yield, total weights and their corresponding standard deviations are shown in tables.

Scale-up

A glass column of 140 mm length and 27.8 mm diameter was employed for the scale-up process. Based on the diameter of the column, the following parameters were calculated: volumetric flow rate, sample load, and volume of the elution solvents. The packed height of the column was maintained at 70 mm, as measured from the top of the glass wool plugging it.

The scale up was performed by using Eqs. (1) and (2) in order to calculate the new sample load and volumetric flow rate, respectively. In these equations, "s-u" refers to scale-up process, and "an" to analytical or small scale process.

$$\text{Sample load}_{s-u} = (\text{Radius}_{s-u}/\text{Radius}_{an})^2 \times \text{Sample load}_{an} \quad [20] \tag{1}$$

$$\text{Volumetric flow rate}_{s-u} = (\text{Sample}_{s-u}/\text{Sample}_{an}) \times \text{Volumetric flow rate}_{an} \quad [20] \tag{2}$$

Once the sample load and volumetric flow rate for the scaled-up process were determined, Eq. (3) was used to calculate the volume of elution solvent for each fraction.

$$\text{Volume}_{s-u} = (\text{Volume}_{an}/\text{Volumetric flow rate}_{an}) \times \text{Volumetric flow rate}_{s-u} \tag{3}$$

Equation (3) was deduced from the relationship:

$$\text{Volume}_{s-u}/\text{Volumetric flow rate}_{s-u} = \text{Volume}_{an}/\text{Volumetric flow rate}_{an}$$

The values of the different variables are reported in "Results" (scale-up). As in the analytical scale processes, prior to the packing of the column, the Ag-silica gel was activated for 2 h at 100 °C and allowed to cool down to room temperature. In the scaled-up processes, a similar procedure to that of analytical scale was performed, with slight variations. In this case, a wet packing method was performed. Thus, after plugging the exit of the column with glass wool, hexane was added to the column until approximately 50 mm height. Then, 21 g of Ag-silica gel was sequentially and homogeneously loaded. After that, more hexane was added and the stopcock was opened. The Ag-silica gel was packed while hexane flowed and if necessary more was added to reach the 70 mm height in the column. The final amount of Ag-silica gel was 21.4 g. The FAEE sample was loaded after dissolving it in 24 mL of the solvent of the first fraction (hexane).

Four fractions were sequentially collected in a separatory funnel for their subsequent washings with NaCl solution (1%, w/v) and the separation of phases. The organic layer was collected, dried with anhydrous sodium sulfate, and the solvent removed in a rotary evaporator at 50 °C. The residue obtained was then analyzed by GC. Scale-up processes were performed in duplicate. The mean values of purity, yield, total weights and their corresponding standard deviations are shown in tables.

GC Analysis

FAEE were analyzed using an Agilent Technology (Santa Clara, CA, USA) 6890N 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, USA). 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 set point 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 FAEE was based on the retention times and relative area percentages of a Supelco 37 Component FAME mix (Supelco Inc., Bellefonte, PA, USA). 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 Analysis

Software package ORIGIN 8.0 (OriginLab, Northampton, MA, USA) was used to calculate averages, standard deviations and to perform the analysis of variance (ANOVA). The significance level was $p < 0.05$.

Results and Discussion

Two main responses were evaluated:

a. $$\text{Purity or compostion}\,(\%) = \frac{\text{weight of a fatty acid in a fraction}\,(\text{mg})}{\text{weight of a entire fraction}\,(\text{mg})} \times 100$$

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

The behavior of different FAEE was investigated. Nevertheless, the study was mainly focused on the isolation of SDA ethyl ester (SDA-EE).

Method Development-Preliminary Experiments

Two preliminary experiments were performed to determine the optimal solvents to fractionate FAEE by Ag-silica gel chromatography and obtaining high purity and yield of SDA-EE. According to previous works [12, 13, 15, 18], pure hexane, and hexane:acetone solutions were used as elution solvents. It had been previously shown that by increasing the solvent polarity, the composition of the eluents followed this order: saturated, monounsaturated, and PUFA [13].

In the current study the FAEE load was higher than that reported in the literature (4%, w/w) [12, 13, 18], with the aim of processing a larger amount of FAEE in a single step. At the end of the separation, solutions with remarkably increased acetone content were added to force the elution of all polyunsaturated FAEE from the column. Hence, all FAEE loaded in the column were eluted with approximately 100% recovery of FAEE.

Table 1 shows the conditions investigated and SDA-EE purity and yield obtained in the preliminary studies (small scale). Purification of SDA-EE was not achieved under conditions of the first experiment, since SDA-EE was distributed in several fractions, and the highest SDA-EE yield (53.3%) was low in purity (48.6%).

In the second experiment, fractionation of FAEE was improved and thus, most of the SDA-EE was recovered between 99% hexane and 90% hexane. However, the SDA-EE yield was distributed in several fractions and thus, SDA-EE isolation was also inadequate. The reason could be that in these experiments, an overload of the chromatographic column may occur with 300 mg of sample. Because of that, optimization of the FAEE load was carried out.

Optimization of FAEE Sample Load

Different FAEE sample loads were investigated: 5% (w/w), 10% (w/w), 15% (w/w), 20% (w/w), 25% (w/w), 30% (w/w), and 50% (w/w). In this optimization, the volume of 100% hexane was higher than that of preliminary experiments. The aim was to avoid fast changes in the polarity by

Table 1 SDA-EE purity and yield in the preliminary experiments of Ag-silica gel column chromatography of FAEE

Fraction (%)[a]	Volume (mL)	SDA-EE purity (%)	SDA-EE yield (%)	Weight (mg)
Experiment 1				
100 F1	10	6.2	15.4	180.9
99 F1	18	48.6	53.3	79.4
98 F1	6	94.2	4.3	3.3
98 F2	4	98.4	2.3	1.7
70 F1	10	66.8	16.0	17.4
70 F2	10	14.7	0.5	2.6
70 F3	10	3.1	0.1	1.2
Experiment 2				
100 F1	5	1.4	2.7	139.7
100 F2	5	20.4	10.0	36.0
100 F3	5	27.9	3.7	9.8
100 F4	5	30.3	2.6	6.2
99 F1	5	31.8	2.0	4.6
99 F2	5	32.7	7.0	15.7
99 F3	5	59.0	29.2	36.1
99 F4	5	88.2	5.5	4.6
90 F1	10	88.3	24.5	18.4
90 F2	10	73.3	5.5	5.5
80 F1	10	0.1	0.5	5.9

The experiments were performed under these conditions: FAEE load 300 mg (30%, w/w), column length 70 mm, flow rate 0.286 mL/min, argentation silica gel 1 g

[a] 100% pure hexane, 99% hexane:acetone (99:1, v/v), 98% hexane:acetone (98:2, v/v), 90% hexane:acetone (90:10, v/v), 80% hexane:acetone (80:20, v/v), 70% hexane:acetone (70:30, v/v)

delaying the introduction of more polar solvents and to slow down the separation of FAEE inside the column. Besides, the number of fractions collected was higher than in previous works [12, 13, 15, 18] in order to control and improve the fractionation of FAEE. Thus, part of 100% hexane and all 99% hexane were collected in several fractions of a reduced volume (3 mL). Table 2 shows the conditions investigated and SDA-EE purity and yield obtained in these experiments.

Poor separation of SDA-EE was achieved with 5% load. In this case, no FAEE were obtained in the intermediate fractions (100% F2 to 99% F3), and most of the FAEE co-eluted with SDA-EE in the 90% hexane first fraction. Because of that, although this fraction gave the highest SDA-EE yield (70%), it was very low in SDA-EE purity (24.4%). The low amount of sample loaded could increase the retention of FAEE, and most of them could elute together when the most polar solvent (90% hexane) was added to the column. It should be noted that good SDA-EE separation was achieved at 10% load. Under these conditions, a single fraction with high SDA-EE purity (83.0%) and yield (74.3%) was obtained in the first fraction eluted with 90% hexane.

Although no single fraction was isolated with high SDA-EE purity and yield with increasing load, under these conditions some of the fractions can be pooled to obtain a good balance between purity and yield. This possibility may be valuable on an industrial scale, since a higher load significantly enhances the amount of final product obtained in a single process with a reduced amount of solvents. Thus, a cost/benefit analysis would be needed to evaluate the best option.

Equations (4) and (5) define total purity and yield of pooled fractions.

$$\begin{aligned}\text{Purity}_{(\text{F1}+\text{F2}+\cdots+\text{Fn})} = \{&[(\text{Purity}_{\text{F1}} \times \text{Weight}_{\text{F1}})/100] \\ &+ [(\text{Purity}_{\text{F2}} \times \text{Weight}_{\text{F2}})/100] + \cdots \\ &+ [(\text{Purity}_{\text{Fn}} \times \text{Weight}_{\text{Fn}})/100] \\ &/(\text{Weight}_{\text{F1}} + \text{Weight}_{\text{F2}} + \cdots + \text{Weight}_{\text{Fn}})\} \times 100\end{aligned} \quad (4)$$

$$\text{Yield}_{(\text{F1}+\text{F2}+\cdots+\text{Fn})} = \text{Yield}_{\text{F1}} + \text{Yield}_{\text{F2}} + \cdots + \text{Yield}_{\text{Fn}} \quad (5)$$

where F1, F2,…, Fn = pooled fractions.

For example, by pooling 99% F4 and 90% F1 fractions at 20% load, SDA-EE purity and yield were 86.2 and 42.8%, respectively (Table 3). Similarly, by pooling 99% F3, 99% F4 and 90% F1 fractions at 20% load, 72.5% SDA-EE purity with 71.5% yield were achieved. Table 3 summarizes the results of SDA-EE purity and yield obtained of selected pooled fractions. As observed in this table, at 30% load, by pooling the fractions 99% F4 and 90% F1, 73.7% SDA-EE purity with 55.9% yield were attained, whereas by pooling the fractions 99% F3, 99% F4 and 90% F1, SDA-EE purity and yield were 64.5 and 64.6%, respectively. Loads of 15, 20, 25 and 30% may lead to acceptable separation of SDA-EE by pooling fractions. However, SDA-EE purity and yield obtained under these conditions were lower than those obtained with 10% load.

Table 2 SDA-EE purity and yield in the optimization of FAEE load

Fraction (%)[a]	V^b (mL)	50 mg (5%, w/w)			100 mg (10%, w/w)			150 mg (15%, w/w)			200 mg (20%, w/w)		
		P (%)	*Y* (%)	*W* (mg)	*P* (%)	*Y* (%)	*W* (mg)	*P* (%)	*Y* (%)	*W* (mg)	*P* (%)	*Y* (%)	*W* (mg)
100 F1	20	0.4[c]	0.1[c]	1.8[c]	0.2	0.1	10.4	0.0	0.1	58.4	0.2	0.5	120.9
100 F2	3				0.1	0.0	1.8	0.1	0.0	3.2	3.0	0.5	7.6
100 F3	3				0.3	0.0	0.7	0.1	0.0	2.6	4.6	0.2	2.6
100 F4	3				0.2	0.0	1.4	0.1	0.0	2.2	6.6	0.4	2.6
99 F1	3				0.2	0.0	1.3	0.2	0.0	2.0	8.9	0.3	1.8
99 F2	3				0.2	0.0	1.7	0.1	0.0	4.9	39.8	17.6	21.6
99 F3	3				0.1	0.1	20.3	19.0	20.7	39.1	58.6	28.6	23.9
99 F4	3	0.1	0.1	10.9	6.3	8.6	32.9	76.8	22.5	10.5	78.9	9.2	5.7
90 F1	10	25.4	70.0	33.0	83.0	74.3	21.4	85.9	40.5	16.9	88.4	33.6	18.6
90 F2	10	79.8	14.0	2.1	61.9	10.4	4.0	78.8	3.1	1.4	63.3	2.2	2.3
90 F3	10	67.8	5.1	0.9	14.3	0.5	0.9	14.7	0.8	2.0	14.9	0.7	1.7

Fraction	*V* (mL)	250 mg (25%, w/w)			300 mg (30%, w/w)			500 mg (50%, w/w)		
		P (%)	*Y* (%)	*W* (mg)	*P* (%)	*Y* (%)	*W* (mg)	*P* (%)	*Y* (%)	*W* (mg)
100 F1	20	2.8	7.6	164.0	7.0	18.9	194.5	15.2	48.1	376.5
100 F2	3	27.8	1.1	2.4	31.9	1.2	2.7	33.3	0.3	0.9
100 F3	3	34.0	1.3	2.3	32.5	0.9	1.9	34.0	0.7	2.3
100 F4	3	31.5	0.8	1.6	33.2	1.1	2.4	34.6	0.5	1.7
99 F1	3	32.8	0.8	1.5	33.6	1.4	3.0	31.5	0.6	2.1
99 F2	3	33.9	0.9	1.6	34.8	1.3	2.6	33.1	0.4	1.6
99 F3	3	48.9	28.5	34.8	35.7	8.7	17.5	49.7	16.0	38.3
99 F4	3	82.6	19.9	14.4	64.5	27.9	31.1	80.5	12.0	17.8
90 F1	10	84.2	29.6	21.0	86.0	28.0	23.4	87.6	13.2	18.0
90 F2	10	26.2	0.7	1.6	13.1	0.7	3.7	55.7	1.6	3.4
90 F3	10	0.1	0.0	1.7	5.6	0.1	3.7	0.1	0.0	2.2

All the experiments were performed under these conditions: column length 70 mm, flow rate 0.286 mL/min, Ag-silica gel 1 g

[a] 100% pure hexane, 99% hexane:acetone (99:1, v/v), 90% hexane:acetone (90:10, v/v)

[b] *V* volume (mL), *P* SDA-EE purity (%), *Y* SDA-EE yield (%), *W* weight (mg)

[c] Fractions from 100% F1 to 99% F3 were pooled before analysis due to the very low weight recovered in each fraction

Furthermore, as reported in Table 2, with loads higher than 20%, an initial decrease in SDA-EE yield was found with the 100% hexane fractions following this order: 25% (7.6%) <30% (18.9%) <50% (48.1%). This suggests an overload of the column, since under these conditions, the Ag-silica gel was saturated with FAEE, and there is an amount of SDA-EE that elutes without retention inside the column. Figure 1 shows the increase in SDA-EE yield in 100% F1 at different FAEE loads. At 50% load, there was a loss of 48.14% in SDA-EE yield in the first 100% hexane fraction collected, indicating very poor separation and column overload.

Table 2 proved that the 90% F1 was the fraction with the highest SDA-EE purity and yield in all experiments. Figure 1 shows how these responses varied in 90% F1, based on FAEE sample load. It was observed that for all sample loads, with the exception of 5%, similar SDA-EE purity was obtained. However, SDA-EE yield significantly decreased with increasing FAEE load. As observed in Fig. 1 and Table 2, 10% load was optimal to collect the highest purity and yield of SDA EE in one single fraction. It should be remarked that this load is 2.5 times higher than the maximum acceptable sample load (4%, w/w) previously reported in the literature [12, 13, 15, 18].

Optimization of Fractions, Solvents and Volumes-Study of Reproducibility

In this part, some fractions obtained with the same solvents were pooled to obtain one single fraction. Therefore, the first two fractions were composed of 29 mL hexane and 12 mL 99% hexane, respectively. In addition, some

Table 3 SDA-EE purity and yield obtained by pooling fractions in the study of optimization of FAEE load

	Pooled fractions[a]			
	99% F3 + 99% F4 + 90% F1		99% F4 + 90% F1	
FAEE load (%) (w/w)	Purity (%)	Yield (%)	Purity (%)	Yield (%)
15	–	–	82.4	62.9
20	72.5	71.5	86.2	42.8
25	66.3	77.9	83.5	49.5
30	64.5	64.6	73.7	55.9
50	66.3	41.2	84.1	25.2

All the experiments were performed at these conditions: FAEE load: 100 mg (10%, w/w); column length: 70 mm; flow rate: 0.286 mL/min; argentation silica gel: 1 g

[a] 99%: hexane:acetone (99:1, v/v); 90%: hexane:acetone (90:10, v/v)

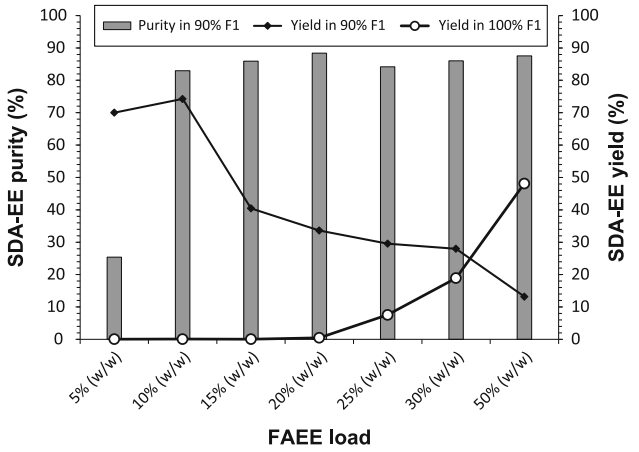

Fig. 1 Values of SDA-EE purity in 90% F1, SDA-EE yield in 90% F1 and SDA-EE yield in 100% F1, after optimization of FAEE load. F1, First fraction; 100%, pure hexane; 90% hexane:acetone (90:10, v/v)

variations were introduced in the fractionation method with the objective of improving the isolation of SDA-EE.

In the previous experiments, SDA-EE was the last FAEE to be eluted and hence, solvents with high polarity were added in the last fractions to ensure complete elution of FAEE through the column. In these experiments, it was found that, SDA-EE purity was lower than 90% in all cases. However, in fractions with 90% hexane (highest polarity), almost only SDA-EE were detected as FAEE in the GC analyses. In other words, in these fractions approximately 5–15% of the mass fraction was composed of compounds not detected by GC. This suggests that other relatively polar compounds co-eluted with SDA-EE, and this led to a little drop in purity. Likewise, when the acetone content was higher than 10% in the preliminary experiments, we observed that some fractions contained a white residue after solvent evaporation. A possible explanation is that part of the stationary phase may have been dissolved by the high polar solvents. The main objective of the next optimization was to determine the most adequate polarity of the final solvent that will ensure total recovery of FAEE and not dissolve part of the stationary phase. This polarity was modulated by the acetone content. Guil-Guerrero et al. [13] reported the highest SDA methyl ester purity and yield with an intermediate fraction composed of hexane:acetone (95:5, v/v). For that reason, in our work, 90% hexane was replaced with 95% hexane as solvent for the last fraction.

Furthermore, the volume of this fraction was optimized and hence, 95% hexane was added to several fractions until the FAEE obtained was less than 1 mg. Although 20 mL gave similar results, 24 mL was established as optimal, since after the elution of this volume no FAEE remained in the column. Therefore, the last fraction was composed of 24 mL 95% hexane. Under these conditions, the fraction was free of unknown compounds and led to remarkable SDA-EE purity improvements (Table 4) with a recovery of FAEE over 95%.

In a further optimization, another 2 mL of 99% hexane were added in order to keep eluting FAEE different from SDA-EE and thus, improving the SDA-EE purity in the final fraction. In other words, the goal of this optimization was to obtain a purer SDA-EE, free of other FAEE, in the final fraction. For this purpose, after the first 12 mL, four fractions of 1 mL 99% hexane were collected. Table 4 reports the conditions and results of this optimization. As observed in this table, after addition of 12 mL + 1 mL + 1 mL fractions of 99% hexane, SDA-EE purity were significantly increased to 83.3 and 90.0% in the last 1 mL fractions. For that reason, a final volume of 14 mL was established for the 99% hexane fraction.

After these last optimizations, the process under optimal conditions was repeated three times to study the reproducibility of the separation. Table 5 reports the mean values of purity and yield of main fatty acids, the weights of fractions and their corresponding standard deviations. It should be noted that the fraction obtained with 95% hexane was remarkably improved in SDA-EE purity (96.3%), with 77.0% yield. The table also showed the high reproducibility of this process.

A reduction from 29 to 20 mL in the volume of the 100% hexane fraction was also attempted (data not shown in tables). However, although SDA-EE purity in 95% fraction was high (92.5%), SDA-EE yield decreased to 62.1 and 18.0% of total SDA-EE was obtained in the 99% (12 mL) fraction. For that reason the volume of 100% hexane was maintained at 29 mL, since it was proved that this volume played an essential role in the FAEE fractionation.

Table 4 SDA-EE purity and yield in the optimization of fractions, solvents and volumes

Separated fractions						Pooled fractions				
Fraction (%)[a]	V^b (mL)	P (%)	Y (%)	W (mg)		Fraction	V^b (mL)	P (%)	Y (%)	W (mg)
100 F1	29	0.1	0.0	18.4	→	100% F1	29	0.1	0.0	18.4
99 F1	12	3.7	8.1	51.5	→	99% F1	12	3.7	8.1	51.5
99 F2	1	23.9	3.4	3.4	→	99% F2	2	32.0	5.8	4.3
99 F3	1	62.5	2.4	0.9						
99 F4	1	83.3	3.5	1.0	→	95% F1	22	94.2	75.7	18.7
99 F5	1	90.0	2.1	0.7						
95 F1	12	98.4	54.8	12.7						
95 F2	4	81.4	9.2	2.7						
95 F3	4	91.3	6.1	1.6						

All the experiments were performed under these conditions: FAEE load 100 mg (10%, w/w), column length 70 mm, flow rate 0.286 mL/min, argentation silica gel 1 g

[a] 100% pure hexane, 99% hexane:acetone (99:1, v/v), 95% hexane:acetone (95:5, v/v)

[b] V volume (mL), P SDA-EE purity (%), Y SDA-EE yield (%), W weight (mg)

Table 5 Purity, yield and standard deviation of main FAEE in the study of reproducibility

	Raw FAEE[b]	Fractions[a]			
		100% F1 (29 mL)	99% F1 (12 mL)	99% F2 (2 mL)	95% F1 (24 mL)
Fatty acid (purity, %)					
Palmitic C16:0	12.1	61.0 ± 3.7	0.6 ± 0.4	ND	0.1 ± 0.0
Stearic C18:0	4.1	20.8 ± 1.2	0.1 ± 0.1	ND	0.0 ± 0.1
Oleic C18:1 (ω-9)	15.5	12.7 ± 4.8	23.5 ± 0.5	0.3 ± 0.0	0.1 ± 0.0
Linoleic C18:2 (ω-6)	24.5	ND	44.5 ± 2.6	4.1 ± 2.6	0.1 ± 0.1
γ-Linolenic C18:3 (ω-6)	7.2	1.8 ± 0.1	10.2 ± 0.6	23.2 ± 1.5	0.6 ± 0.1
α-Linolenic C18:3 (ω-3)	10.7	1.4 ± 0.0	15.2 ± 1.1	42.5 ± 1.7	1.3 ± 0.3
Stearidonic C18:4 (ω-3)	**23.8**	**1.1 ± 0.0**	**3.5 ± 0.7**	**28.5 ± 3.2**	**96.3 ± 1.9**
Fatty acid (yield, %)					
Palmitic C16:0	–	98.2 ± 3.5	2.5 ± 1.7	ND	0.1 ± 0.1
Stearic C18:0	–	99.7 ± 3.7	1.7 ± 1.3	ND	0.2 ± 0.4
Oleic C18:1 (ω-9)	–	16.4 ± 7.9	76.3 ± 4.8	0.1 ± 0.0	0.1 ± 0.1
Linoleic C18:2 (ω-6)	–	ND	91.3 ± 1.6	0.9 ± 0.5	ND
γ-Linolenic C18:3 (ω-6)	–	4.7 ± 0.1	71.3 ± 6.3	17.5 ± 6.2	1.5 ± 0.4
α-Linolenic C18:3 (ω-3)	–	0.7 ± 0.1	71.3 ± 7.7	21.4 ± 7.0	2.3 ± 0.6
Stearidonic C18:4 (ω-3)	–	**0.1 ± 0.0**	**7.4 ± 1.7**	**6.3 ± 1.3**	**77.0 ± 1.7**
Total weight (mg)	100	19.6 ± 2.0	50.5 ± 2.0	5.4 ± 1.6	19.3 ± 0.7

All the experiments were performed in triplicate under these conditions: FAEE load 100 mg (10%, w/w), column length 70 mm, flow rate 0.286 mL/min, argentation silica gel 1 g; *ND* Not detected

[a] 100% pure hexane, 99% hexane:acetone (99:1, v/v), 95% hexane:acetone (95:5, v/v)

[b] Raw FAEE product obtained from ethanolysis of high SDA ω-3 modified soybean oil

Enrichment of ALA and GLA Ethyl Ester

A further experiment was performed to optimize the isolation of ALA ethyl ester (ALA-EE) and GLA ethyl ester (GLA-EE) in different fractions from that of SDA-EE. Since most of total GLA-EE and ALA-EE co-eluted with other FAEE in the 12 mL 99% hexane fraction, in this experiment this volume was collected in several 1 mL fractions. Table 6 reports the purity and yield of ALA-EE and GLA-EE obtained in the different fractions. The yield of these compounds significantly increased when fractions from 99% F4–F6 were pooled with 99% F7–F9 fractions.

Table 6 Purity and yield of ALA-EE and GLA-EE in the optimization of fractions, solvents and volumes

Fraction (%)[a]	V[b] (mL)	W (mg)	P (%) ALA-EE	Y (%) ALA-EE	P (%) GLA-EE	Y (%) GLA-EE
100 F1	29	22.0	0.1	0.9	0.3	4.7
99 F1	6	4.2	0.1	0.6	0.0	0.1
99 F2	1	1.9	0.0	0.1	0.0	0.0
99 F3	1	9.0	0.1	0.6	0.0	0.4
99 F4	1	12.4	0.6	5.9	0.6	8.3
99 F5	1	11.0	2.3	21.4	1.7	23.0
99 F6	1	10.7	3.7	34.6	2.4	32.8
99 F7	1	6.0	2.5	23.6	1.5	20.8
99 F8	1	2.3	0.9	8.2	0.5	6.5
99 F9	1	1.0	0.2	1.4	0.1	1.0
95 F1	24	18.7	0.1	1.3	0.1	0.7

Pooled fractions (%)	P (%) ALA-EE	Y (%) ALA-EE	P (%) GLA-EE	Y (%) GLA-EE
(99) F4 + F5 + F6 + F7 + F8 + F9	23.7	95.2	15.4	92.4
(99) F5 + F6 + F7 + F8 + F9	31.1	89.3	19.7	84.2
(99) F6 + F7 + F8 + F9	36.6	67.9	22.2	61.2
(99) F7 + F8 + F9	38.6	33.2	22.1	28.4

The experiments were performed under these conditions: FAEE load 100 mg (10%, w/w), column length 70 mm, flow rate 0.286 mL/min, argentation silica gel 1 g

[a] 100% pure hexane, 99% hexane:acetone (99:1, v/v), 95% hexane:acetone (95:5, v/v)

[b] V volume (mL), P purity (%), Y yield (%), W weight (mg)

However, a decreased ALA-EE and GLA-EE purity was also observed, mainly due to the presence of linoleic acid (18:2 ω-6) ethyl ester. Therefore, the elution volume obtained with 99% hexane was divided into two fractions, consisting of 10 and 4 mL. The last 4–5 mL of 99% hexane gave a good balance between purity (31.1–36.6% for ALA-EE and 19.7–22.2% for GLA-EE) and yield (67.9–89.3% for ALA-EE and 61.2–84.2% for GLA-EE) (Table 6). Therefore, the optimal fractions used for the scale-up were: 29 mL 100%, 10 mL 99%, 4 mL 99% (ALA-EE and GLA-EE), and 24 mL 95% (SDA-EE).

Scale-up

Fractionation of FAEE by Ag-silica gel chromatography was scaled-up to 2,380 mg of the starting FAEE in a single step. The best conditions obtained on an analytical scale were used for the scale-up processes.

From Eqs. (1), (2), and (3), described in "Methods" (scale-up), values of FAEE sample load (2,380 mg), volume flow rate (6.7 mL/min), and volumes of elution solvents were calculated. Table 7 reports mean values of purity and yield of main fatty acids, the weights of fractions and their corresponding standard deviations, obtained in the scaled-up processes.

It should be noted that experimental conditions were successfully scaled-up to obtain 420 mg of 96.9% SDA-EE, with 71.3% yield (Table 7). The scale-up process was repeated twice, yielding 840 mg of the purified SDA-EE, as product for further applications. Regarding the isolation of SDA-EE, it should be noted that good reproducibility was achieved in both scaled-up processes (Table 7) and on an analytical scale (Table 5). ANOVA was performed for the comparison of SDA purity and yield on an analytical scale and scaled-up processes. No significant differences ($p > 0.05$) were found in SDA purity in all fractions obtained. On the contrary, significant differences ($p < 0.05$) were found in SDA yield in several fractions. Thus, SDA yield was significantly higher ($p < 0.05$) on an analytical scale (77.0% ± 1.7) than in the scaled-up processes (71.3% ± 0.2) in the 95% hexane fraction. However, these differences were not pronounced. Due to this high reproducibility, in our opinion, the process should be readily scaled-up with high SDA-EE purity and yield.

In addition, it was possible to obtain another fraction enriched in ALA-EE (31.6% purity and 47.9% yield) and GLA-EE (18.1% purity and 41.0% yield) (Table 7). It was found that, for these FAEE, the reproducibility between the analytical scale and the scaled-up process was lower than with SDA-EE. The reason could be that fractionation of ALA-EE and GLA-EE occurred between two fractions with the same composition (99% hexane), whereas SDA-EE eluted after changing the polarity of solvent (95% hexane).

Table 7 Purity, yield and standard deviation of main FAEE in the scaled-up processes

	Fractions[a]			
	100% F1 (680 mL)	99% F1 (234 mL)	99% F2 (94 mL)	95% F1 (563 mL)
Fatty acid (purity, %)				
Palmitic C16:0	40.7 ± 0.6	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
Stearic C18:0	13.8 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
Oleic C18:1 (ω-9)	34.3 ± 2.8	14.0 ± 3.0	0.8 ± 1.1	0.1 ± 0.0
Linoleic C18:2 (ω-6)	4.8 ± 2.3	57.2 ± 2.4	21.4 ± 2.8	0.3 ± 0.1
γ-Linolenic C18:3 (ω-6)	1.1 ± 0.0	4.8 ± 6.9	18.1 ± 0.9	0.6 ± 0.2
α-Linolenic C18:3 (ω-3)	0.6 ± 0.0	19.7 ± 9.1	31.6 ± 0.1	1.4 ± 0.3
Stearidonic C18:4 (ω-3)	**0.1 ± 0.0**	**2.4 ± 1.4**	**25.9 ± 4.1**	**96.9 ± 0.5**
Fatty acid (yield, %)				
Palmitic C16:0	97.7 ± 0.7	0.4 ± 0.0	0.1 ± 0.1	0.2 ± 0.2
Stearic C18:0	98.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.2	0.5 ± 0.5
Oleic C18:1 (ω-9)	64.2 ± 5.7	27.5 ± 4.0	0.8 ± 1.1	0.1 ± 0.0
Linoleic C18:2 (ω-6)	5.7 ± 2.7	71.7 ± 7.7	14.3 ± 2.9	0.2 ± 0.1
γ-Linolenic C18:3 (ω-6)	4.6 ± 0.2	44.3 ± 6.9	41.0 ± 5.1	1.5 ± 0.5
α-Linolenic C18:3 (ω-3)	1.7 ± 0.1	40.7 ± 6.4	47.9 ± 3.5	2.2 ± 0.6
Stearidonic C18:4 (ω-3)	**0.1 ± 0.0**	**3.0 ± 1.7**	**17.6 ± 1.5**	**71.3 ± 0.2**
Total weight (mg)	695 ± 7.1	735 ± 49.5	390 ± 28.3	420 ± 0.0[b]

The experiments were performed in duplicate under these conditions: FAEE load 2,380 mg (~10%, w/w), column length 70 mm, flow rate 6.7 mL/min, argentation silica gel 21.4 g

[a] 100% pure hexane, 99% hexane:acetone (99:1, v/v), 95% hexane:acetone (95:5, v/v)

[b] The scaled-up process was repeated twice to yield 840 mg SDA-EE

We attempted to reutilize the Ag-silica gel used in the scaled-up processes after its activation at 100 °C for 2 h. However, the separation was significantly worse than that achieved in the previous study. Therefore, we do not believe that reutilization of the Ag-silica gel is feasible.

Ag-silica gel open column chromatography could be used to effectively isolate SDA-EE from high SDA modified soybean oil FAEE with high purity (>96%) and yields (>70%). Due to its high purity, the SDA-EE obtained can compete with fish oil-EE (EPA-EE) and can be used for the production of structured lipids, nutraceuticals, functional lipids for clinical studies, or as fine ingredient. The process described in the paper can be scaled-up with high reproducibility, selectivity, efficiency and low cost, since no expensive equipment is needed. Besides, the optimal sample load was established at 10% (w/w), which is 2.5 times higher than the maximum acceptable sample load previously reported in the literature [12, 13, 15, 18]. In addition, in this process, no urea crystallization methods that may lead to carcinogenic carbamates were involved. For these reasons, this process and products may be useful and valuable for clinical trials and food or pharmaceutical applications.

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