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ORIGINAL PAPER

Triarachidonoyl Glycerol Purification Process

E. Venegas-Venegas · M. A. Rincón-Cervera · J. L. Guil-Guerrero

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Abstract Purification of arachidonic acid (AA, 20:4n-6) from different sources has been previously reported, but in most cases AA is obtained after triacylglycerol (TAG) hydrolysis. In this work, gravimetric normal-phase chromatography with gradient elution has been used to purify an AA-enriched fraction of TAG from the commercial single cell oil named ARASCO (38–44% AA content). A TAG fraction with more than 90% AA content was obtained, employing appropriate solvents for alimentary processing. The process was scaled up with satisfactory results. Due to the use of food-safe solvents in the whole process, it could be applied with alimentary or pharmaceutical purposes.

Keywords Arachidonic acid · Triacylglycerol · Chromatographic purification · HPLC · ARASCO

Introduction

Arachidonic acid (AA) is a long-chain polyunsaturated fatty acid (LCPUFA) belonging to the n-6 family. The novo synthesis of AA does not occur in the human metabolism [1], thus AA must be obtained from the diet or synthesized by desaturation, elongation and peroxisomal partial β-oxidation reactions from the parent linoleic acid (LA, 18:2n-6), which is an essential FA (EFA). Production of AA through the metabolic pathway is rate limited by the poor conversion of LA to gamma-linolenic acid (GLA, 18:3n-6), catalyzed by the Δ6-desaturase enzyme [2]. AA acts as a precursor for the biosynthetic production of prostaglandin PGE_2, leukotrienes, thromboxanes and related metabolites [3, 4], that influence several metabolic activities such as platelet aggregation, inflammation and the immune function. AA is found in human breast milk and can directly affect infant growth by affecting the expression of growth-related early genes [5]. So, AA should be considered as a supplement in infant formulas, especially if they content also docosahexaenoic acid (DHA, 22:6n-3) [1], in order to support an adequate n-6/n-3 balance for the correct development of formula fed infants. Because of that, there is a growing demand of AA from pharmaceutical and infant alimentary industries. In fact, a number of companies are already marketing term and preterm infant formula that have been enriched in AA and DHA [6].

Purification of AA from different sources has been reported, but no suitable sources both from vegetal or animal origin are still available for the commercial production of this PUFA [7]. AA is mainly obtained from micro-organisms, such as the microalga *Porphyridium cruentum* [3, 8], *Parietochloris incisa* [9] and *Pythium insidiosum* [10]; the fungus *Mortierella* sect.*schmuckeri* [11] and especially from the fungus *Mortierella alpina* [12, 13]. Phototrophic and heterotrophic algae exhibited low percentages of AA in biomass and low biomass densities, but *M. alpina* can produce more than 10 g/l of AA [6]. Therefore, this species is the most employed micro-organism for commercial production of AA [14], and also for the production of ARASCO® [15], the oil used for the purification of AA in this work.

Different methods for AA obtainment have been employed achieving from 57.1 to 97.0% purity, with recoveries ranging from 20.6 to 81.9% (Table 1). As a

E. Venegas-Venegas · M. A. Rincón-Cervera ·
J. L. Guil-Guerrero (✉)
Food Technology Division, University of Almería,
04120 Almería, Spain
e-mail: jlguil@ual.es

Table 1 Methods for arachidonic acid purification

Reference	Method	Purity (%)	Recovery (%)
Zhu et al. [16]	Urea inclusion method.	57.1	81.9
Shimada et al. [17]	Enzymatic hydrolysis, enzymatic esterification, urea inclusion method, second enzymatic esterification.	75.0	71.0
Vali et al. [4]	Saponification, low-temperature crystallization, esterification, solvent extraction.	95.3	71
Giménez-Giménez et al. [8]	Saponification, urea inclusion method, HPLC purification.	81.4	20.6
Guil-Guerrero et al. [3]	Saponification, urea inclusion method, argentated column chromatography.	97.0	39.5
Zhang et al. [18]	Saponification, urea inclusion method, $CuSO_4$ column chromatography.	90.8	39.9

result of all these methods, AA is purified as free FA (FFA) form, or as methyl/ethyl ester. However, AA presents a high degree of unsaturation, and the FFA form is more prone to autoxidation processes than in the unaltered TAG form. Furthermore, it has been reported that TAG have about 50% more absorption and metabolic activity than any FA ester [19]. So TAG seem to be the best form to supply EFA. Although triarachidonoyl glycerol could be obtained by enzymatic synthesis [19], natural sources have been not obtained until now.

In this work, gravimetric normal-phase column chromatography with gradient elution has been employed to purify an AA-enriched fraction of TAG from ARASCO® (38–44% AA). This method has been used previously to purify PUFA-enriched TAG from different natural sources by our research group [20]. In this work, tri-AA-TAG was obtained, employing appropriate solvents for alimentary processing.

Experimental Procedures

Oil Characterization

The TAG profile of the fungal oil ARASCO® (Martek Biosciences Corporation, Columbia, MD, USA) was obtained by RP-HPLC in a Finnigan Surveyor chromatograph (Thermo Electron, Cambridge, UK) with reverse-phase column (C_{18} Hypersil Gold, 250 × 4.6 mm i.d., 5 µm particle size; Thermo Electron, Cambridge, UK) and isocratic elution with acetonitrile/i-propanol (80:20, v/v). The flow-rate of the eluent was 1 ml/min. Column temperature was 30 °C. Detection was made by UV absorption at 210 nm [20].

Peaks appearing in the TAG profile (Fig. 1) have been clustered into eight fractions named from F1 to F8. The HPLC eluates from these fractions were collected separately, then the solvent was evaporated under vacuum, and the residue was redissolved in 1 ml of *n*-hexane for FA analyses by GLC.

FA Analyses

For FA analyses, oil samples were transmethylated by means of the method of Lepage and Roy [21]: 1 ml of freshly prepared transesterification reagents (methanol/acetyl chloride, 20:1, v/v) was added to samples in glass tubes as well as 100 µl of a solution of internal standard (heptadecanoic acid 17:0, 10 mg/ml). The tubes were shaken and then placed in a hot block (100 °C, 30 min). After that, the mixture was cooled to room temperature, and 1 ml of distilled water was added to each tube. Samples were shaken again and centrifuged (3,000 rpm, 3 min). The upper hexane phase was collected for GLC analysis.

The resulting FA methyl esters (FAME) were analyzed in a Focus GLC (Thermo Electron, Cambridge, UK) equipped with flame injection detector (FID) and a Omegawax 250 capillary column (30 m × 0.25 mm i. d. × 0.25 µm film thickness; Supelco, Bellefonte, PA, USA). The temperature program was: 1 min at 90 °C, heating until 200 °C at a rate of 10 °C/min, constant temperature at 200 °C (3 min), heating until 260 °C at a rate of 6 °C/min and constant temperature at 260 °C (5 min). The injector temperature was 250 °C with split ratio 50:1. Injection volume was 4 µl. Detector temperature was 260 °C. Nitrogen was used as the carrier gas (1 ml/min).

Chromatographic Column for TAG Purification

The stationary phase (silica gel impregnated with silver nitrate) was prepared as previously described to purify TAG from *Oenothera biennis* oil [20] and the chromatographic column was filled as explained for FAME purification [22].

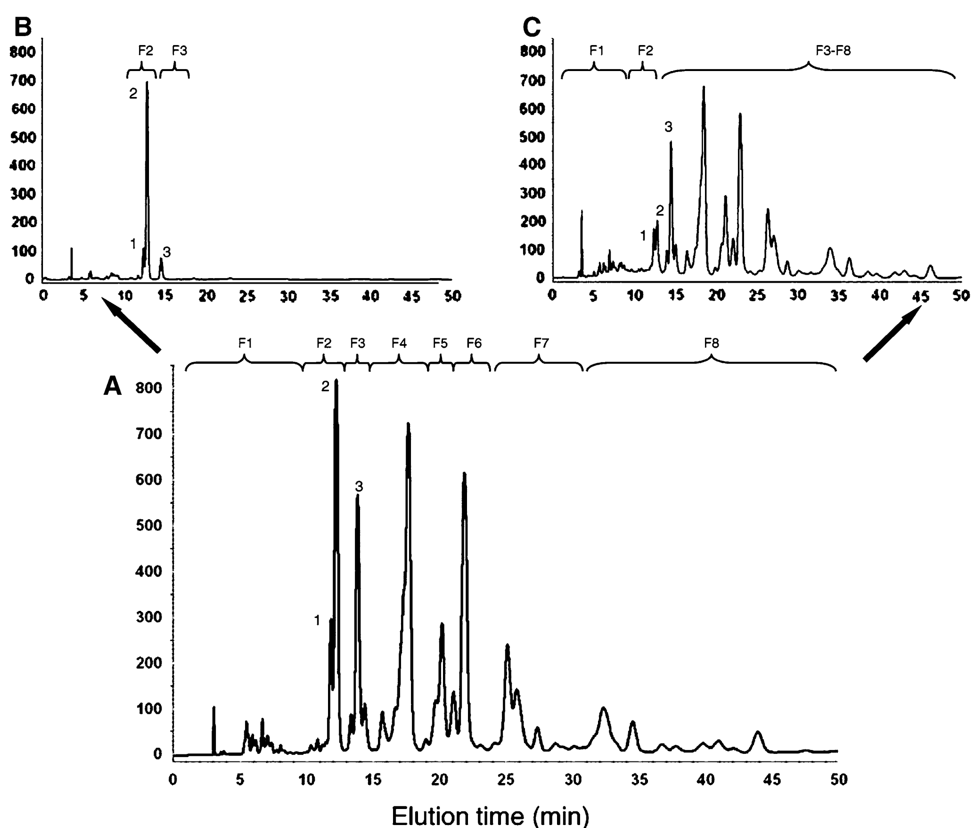

Fig. 1 Reverse-phase HPLC TAG profile of ARASCO oil (**a**), and for selected Fractions (F1–F8) after elution on the silver ion chromatographic column: enriched fraction (**b**) and the remaining oil (**c**)

The stationary phase was packed in a glass column (8 cm height × 0.5 cm diameter).

ARASCO© (40 mg) was diluted in *n*-hexane (0.5 ml) and the solution was applied on the top of the chromatographic column. Different mixtures of solvents with increasing polarity (Table 2) were eluted through the stationary phase and the eluates were collected in order of elution (numbered from 1 to 40) for further HPLC analysis.

Once the eluates were analyzed by HPLC according to the method described in "oil characterization", they were joined in two different fractions: one that included the eluates containing the TAG of interest (38–40, from now "enriched fraction") and the other one comprising the remaining oil (1–37, from now "remaining fraction").

Table 2 Sequence of eluates applied to the chromatographic column

Solvents[a] (ml)	Numbers assigned to fractions
10 × 1 H:A (93:7)	1–10
10 × 1 H:A (90:10)	11–20
5 × 1 H:A (85:15)	21–25
5 × 1 H:A (50:50)	26–30
5 × 1 A	31–35
5 × 1 A:Et (50:50)	36–40

[a] *H* hexane, *A* acetone, *Et* ethanol

Because of the use of solvents of high polarity in the mobile phase, the presence of silver ions has been detected in the collected fractions. To remove silver impurities, a saturated aqueous solution of sodium chloride was added. After vigorous shaking, the resulting two phases were allowed to separate, the upper one was collected and the solvent was evaporated under vacuum in a rotatory evaporator.

The enriched fraction was analyzed again by HPLC (see method in "oil characterization") in order to verify whether the purification process has been properly developed (Fig. 1). An aliquot of the enriched fraction was analyzed by GLC (see method in "GLC analysis").

Purification Process Characterization

In order to characterize the purification process for an oil by column chromatography, some parameters are needed. The percentage of TAG recovered after the purification process in column (TAG yield) is calculated according to the following expression:

$$Y_{\mathrm{TAG}} = \frac{X_{\mathrm{EI}}}{X_{\mathrm{A}}} \times 100$$

where Y_{TAG} refers to the TAG yield, X_{A} is the TAG amount of the oil applied in the chromatography column, and X_{EI} is the TAG amount recovered in the combined eluates after the purification process.

Table 3 FA profiles of *Mortierella alpina* oil, ARASCO, F2 fraction and enriched fraction (mean ± SD)

Fatty acid[a]	*Mortierella alpina* [23]	ARASCO©	F2 fraction (purified by HPLC)	Enriched fraction (purified by silver ion chromatography)
14:0	–	0.8 ± 0.0	2.4 ± 0.2	–
16:0	13.6 ± 1.3	11.5 ± 0.2	2.2 ± 0.4	0.6 ± 0.1
18:0	17.2 ± 1.8	7.4 ± 0.8	1.3 ± 0.2	–
18:1n-9 (n-7)	14.8 ± 1.5	20.8 ± 0.5	2.2 ± 0.4	–
18:2n-6	9.0 ± 0.7	6.9 ± 0.0	1.2 ± 0.3	–
18:3n-6	4.2 ± 0.5	3.1 ± 0.1	6.3 ± 0.5	2.5 ± 0.1
20:4n-6 (AA)	30.3 ± 2.6	43.9 ± 0.1	84.4 ± 5.1	95.3 ± 0.1
20:5n-3	0.1 ± 0.0	–	–	–
22:6n-3	–	1.3 ± 0.0	–	–
Others undetermined FA	10.9 ± 0.8	4.3 ± 0.5	–	1.8 ± 0.0

[a] % FA area on total FA area detected by GLC

The aim of this work was to obtain a TAG target, which corresponds to a peak signal (identified by HPLC) included in an enriched fraction (obtained by chromatography column), so the following expression describe the yield of a TAG after oil processing:

$$Y_{\mathrm{Peak}} = \frac{H_{\mathrm{En}}}{H_{\mathrm{A}}} \times 100$$

where Y_{Peak} is the peak yield, H_{En} is the amount of peak in the enriched fraction (calculated as % HPLC peak area in the enriched fraction × mg enriched fraction) while H_{A} is the amount of peak in the starting oil (calculated as % HPLC peak area in the starting oil × mg oil before processing).

The yield of a FA target (Y_{FA}) can be calculated by the expression:

$$Y_{\mathrm{FA}} = \frac{G_{\mathrm{En}}}{G_{\mathrm{A}}} \times 100$$

where G_{En} is the amount of FA in the enriched fraction (calculated as % of the FA target on total FA in the enriched fraction × mg enriched fraction) and G_{A} is the amount of the FA in the starting oil (calculated as % FA on total FA in starting oil × mg oil before processing).

Statistical Analysis

Five replicates were performed for each analysis. The average values and standard deviations are shown in the tables. Statistical analysis was done using Excel Version 8.0 software. Significance level was defined at $p < 0.05$.

Results and Discussion

ARASCO© (Martek Biosciences Corporation, Columbia, MD, USA) is a single cell oil obtained from the fungus *Mortierella alpina*, in which AA content is increased by applying a safe standardized process [23]. For oil production, the fungus is cultured in a bioreactor, being the resulting biomass harvested by centrifugation. Once the biomass is dried, the oil is continuously extracted with hexane. The FA profile of the oil produced by the fungus is shown in Table 3 [24]. After oil extraction, solids as well as solvent are removed. Then the oil is winterized to remove the more highly saturated TAG fractions, refined, bleached and deodorized using standard procedures [23]. After that, the oil is mixed with high oleic sunflower oil to standardize a product of 40% AA [15]. The FA profile obtained from ARASCO© in this work is shown in Table 3. Notice that AA purity is higher than in *M. alpina* oil due to the winterization process. Also, oleic acid content is increased, probably due to high oleic sunflower oil addition. AA percentage (43.9% on total FA) was in good agreement with previous reports [15].

Reverse-phase HPLC TAG profile of ARASCO© oil is shown in Fig. 1a FA profiles of each TAG cluster were obtained in order to identify TAG fractions with the highest AA content. The cluster named F2 presents an AA percentage higher than the rest of the oil and exceeding 84% of total FA. The main peak belonging to this cluster contributes in a 12.7% FA to the whole oil. Different mixtures of solvents (*n*-hexane, acetone and ethanol) have been tested in an analytical-size column to find an adequate sequence of elution for the separation of the main peak in the F2 cluster from the remaining oil. The optimized sequence of solvents is presented in Table 2, while the HPLC chromatograms of the whole oil (a), the enriched fraction (b) and the remaining fraction (c) are shown in Fig. 1.

The chromatographic process in the column allowed us to obtain an AA-enriched fraction of TAG (enriched

Table 4 Comparison of results between analytical and preparative-size chromatographic columns

	TAG yield (%)[a] Y_{TAG}	Peak 2 yield[b] Y_{Peak}	AA purity[c]	AA yield[d] Y_{FA}
Analytical-size column	95.1 ± 1.0	45.2 ± 0.1	95.3 ± 0.1	14.8 ± 0.3
Preparative-size column	98.4 ± 3.2	56.3 ± 2.1	90.9 ± 3.0	16.9 ± 2.4

[a] TAG percentage recovered from ARASCO after purification process

[b] Peak 2 percentage recovered from ARASCO after purification process

[c] AA percentage on total FA in the enriched fraction

[d] AA percentage recovered from ARASCO in the enriched fraction

fraction) which partially corresponded with the previously identified F2 fraction by HPLC. The enriched fraction showed the same peaks that appeared in F2 fraction, but the contribution of the main peak (Peak 2) increased after chromatographic purification in the column. This fact explains the higher AA purity in the enriched fraction when comparing with the F2 fraction (Table 3). The AA content in the enriched fraction was 95.3% on total FA (45.2% yield) in the analytical scale, by collecting the eluates labelled from 38 to 40 (Table 2).

Once the analytical scale was optimized, the process was scaled up by a factor of10. Data for FA yield, AA purity and yield about enriched fraction obtainment are shown in Table 4. Notice that although AA purity in the enriched fraction decreases to 90.9%, the recovery for Peak 2 increased to 56.3% after three consecutive extractions of the TAG fraction by using *n*-hexane, during silver ion removal. The presence of silver ions in some of the collected fractions could be argued to be a drawback with this process. However, it has been previously shown that small amounts of this ion detected in the eluates can be successfully removed [20].

Although the AA contained in the enriched fraction present a low yield with respect to AA content in ARASCO© (14.8% for the analytical-size column and 16.9% for preparative-size column), it is necessary to emphasize that the remaining and unaltered oil could be used as an AA source with several purposes, such as pure AA obtainment or to enrich other food formulations.

This purification method allows us to obtain a highly enriched AA fraction from ARASCO, with the advantage of coming from a natural and renewable source and also to be available in the TAG form. By considering that this method is also feasible to scale up [22], and due to the use of food-safe solvents in the whole process, it could be applied with alimentary or pharmaceutical purposes.

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