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ORIGINAL PAPER

Purification of GLA-Triglycerides from Evening Primrose Oil by Gravimetric Column Chromatography

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Abstract Gravimetric normal-phase silver ion–silica gel column chromatography has been used for the novel application of purification of GLA-containing triglycerides (GLA-TGs) from evening primrose seed oil (EPO). Gradient elution with increasing polarity enabled separation of valuable TG species containing γ-linolenic acid (GLA, 18:3n-6). Enzymatic hydrolysis revealed the distribution of fatty acids (FAs) in the isolated TG species, with GLA in the *sn*-2 position in different percentages, depending on the degree of unsaturation. A novelty of this work was the successful use of the procedure to improve the purification of raw GLA species from EPO up to preparative scale, thus enabling use of this methodology for industrial purposes.

Keywords Gamma-linolenic acid · Evening primrose seed oil · *Oenothera biennis* · Pancreatic lipase · Positional analysis · Gas chromatography · HPLC

Introduction

Gamma-linolenic acid (GLA, 18:3n-6) is a polyunsaturated FA (PUFA) belonging to the n-6 PUFA family. It is found naturally in the FA and TG fractions of some seed oils. The richest sources of GLA include EPO, borage seed oil, blackcurrant seed oil, hemp seed oil, and a number of fungal sources, for example oil of javanicus produced from *Mucor javanicus* [1]. GLA is also found in some microbial sources, for example *Spirulina* species [2], and a minimal amount is produced in the human body as a downstream metabolite of Δ6-desaturase enzyme-induced conversion from the essential FA (EFA) linoleic acid (LA, 18:2n-6). Under some conditions, for example reduced enzymatic activity of Δ6-desaturase, GLA may become conditionally essential [2, 3]. GLA formation is dependent of the activity of the Δ6-desaturase, which is hindered by several factors, including aging, nutrient deficiency, smoking, and excessive alcohol consumption [3, 4].

GLA has anti-inflammatory, antithrombotic, and lipid-lowering potential. It also enhances smooth muscle relaxation and vasodilatation. In addition, EFAs including GLA are important constituents of membrane phospholipids, including the mitochondrial membrane, where they enhance the integrity and fluidity of the membrane [2].

EFAs bioavailability is sometimes low. In these circumstances, when TGs are processed by human metabolism, the FAs present in positions *sn*-1 and *sn*-3 of the glycerol backbone are released by pancreatic lipase whereas the FAs esterified in position *sn*-2 remains unreacted, being adsorbed by intestinal mucosa as *sn*-2 monoglycerides (*sn*-2 MGs). So, PUFAs that are esterified in *sn*-2 position of TGs are more easily adsorbed than those which are in the *sn*-1 and *sn*-3 positions, and are, therefore, more bioavailable for metabolic needs [5]. Triglycerides with GLA esterified in the *sn*-2 position could thus be used as a valuable product by the pharmaceutical and/or alimentary industries with no need to apply the whole oil.

Although the synthesis of structured GLA-TGs has been achieved successfully by enzymatic procedures, the possibility of obtaining *sn*-2 GLA-TGs from natural sources on a large scale has remained untested until now. Thus, it

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would be of interest to develop a suitable technique enabling simplification or avoidance of enzymatic synthesis, taking into account that any *sn*-2 EFA-TG would be used as a raw reagent to obtain structured TGs.

In this work, EPO has been used to attempt purification of GLA-TGs. The choice of EPO was based on several considerations:

(a) EPO is a commercial product which is inexpensive and readily available from chemists' shops and health food stores.
(b) It is a relatively rich source of GLA (8–14%) [1].
(c) Although a higher level of GLA is present in seeds oil of blackcurrant (*R. nigrum*) and borage (*B. officinalis*), EPO seems to have the most biologically active form of GLA [6, 7].
(d) EPO has a relatively simple TG profile, which possibly makes separation of the target TGs easier [8].

The technique used in this work to separate the TGs of interest was gravimetric normal-phase column chromatography with silica gel and silver nitrate as stationary phase. The resolving power of silica gel–silver nitrate is attributed to reversible charge-transfer complexation of Ag^+ with carbon–carbon double bonds [9]. The extent and strength of complexation between the stationary phase and the TGs, and the polarity of the mobile phase affects the mobility of solutes—the most polar solutes are more retained within the stationary phase and it is necessary to use more polar eluents to elute them. This procedure has several important advantages in comparison with other purification techniques, for example HPLC: much lower cost, no need of exhaustive training of staff, capability of adaptation to industrial scale, and use the same stationary phase several times. Although this technique has been used successfully to purify valuable PUFAs, it has never been used to purify TGs. Although this procedure has some drawbacks, for example lack of reproducibility and loss of silver ion, results obtained in this work show they may not be highly relevant.

Experimental Procedures

Column Purification of TGs

The stationary phase was prepared by weighing 8 g silica gel (130–270 mesh, 60 Å; Sigma–Aldrich, Germany) and adding 50 mL absolute ethanol. Silver nitrate (0.8 g) was dissolved in absolute ethanol in an opaque flask, with magnetic stirring, and the solution was then added to the silica gel in absolute ethanol and stirred for 10 min. The solvent was evaporated in a rotary evaporator under vacuum (50 °C) and the silver-impregnated silica gel was kept in an oven at 105 °C for 2 h [9]. The chromatographic column was prepared as is previously described for purification of FA esters [9].

To optimize the chromatographic process, it has been reported that the optimum amount of FA methyl esters (FAMEs) that can be loaded into the column is 4% (*w*/*w*) of the stationary phase [10]. Nevertheless, in this work a slightly larger amount (5%) was used. This choice was effected by considering that recoveries for TGs fractions 1 and 2 (Fig. 1) were 97 ± 3% (100% purity) and 61 ± 2% (100% purity), respectively, for oil loaded on to the column at 5% (*w*/*w*) of the stationary phase, which was determined

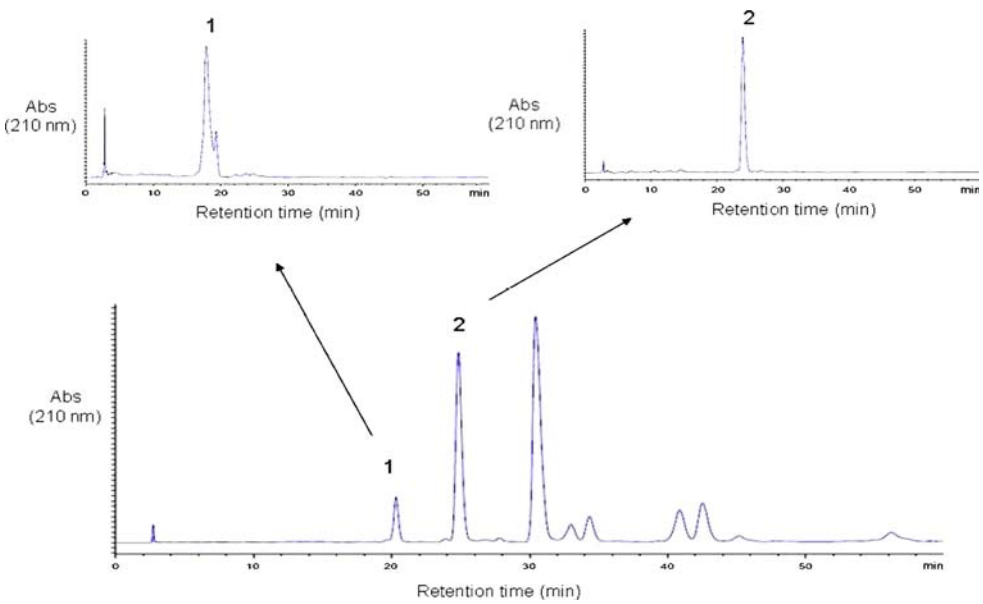

Fig. 1 Reversed-phase HPLC profile of the TGs of EPO (for chromatographic variables, see "Experimental Procedure" section) and purified peaks 1 and 2 after chromatographic separation

as the optimum amount. Larger amounts of oil loaded on to the column reduced peak 2 recovery to less than 55% (maintaining 100% purity). So, 400 mg EPO (purchased in a local shop) was weighed and dissolved in 5 mL *n*-hexane. This solution was applied to the top of the column and was eluted with mixtures of *n*-hexane and acetone in different proportions, the eluates being collected as 10-mL fractions. A number was assigned to each fraction according to its elution (Table 1).

To study the capacity of the stationary phase to purify TGs, and the loss of silver ion in the fractions, the process was repeated three more times consecutively after finishing the first separation. Thus, the same stationary phase was used to process a total of 1600 mg EPO.

After separate collection of the fractions for each replicate, an adequate volume (0.5 mL) of each was put in vials and analyzed by HPLC (30 μL) to check the TG composition. HPLC analysis were carried out on an HP-1100 chromatograph (Hewlett–Packard, Waldbronn, Germany) with a reversed-phase column (Phenomenex Luna ODS-C18(2); 250 × 4.6 mm i.d., 5 μm particle size) and isocratic elution with acetonitrile–*i*-propanol 65:35 (*v*/*v*). The column temperature was 30 °C and the flow rate 1 mL/min. Detection was by UV absorption at 210 nm [8].

Two fractions of TGs were purified (Fig. 1). All 0.5 mL-volumes previously collected from each of these fractions were combined, being the solvent evaporated under an inert atmosphere (N_2) and then the residue redissolved in *n*-hexane (1 mL). Solutions were methylated to obtain the FA profile (see method in "Hydrolysis Enzymatic Reaction") by gas–liquid chromatography (GLC) (see method in "GLC Analysis"). Results are listed in Table 2.

Table 1 Sequence of solvents used for elution of the chromatographic column

Solvents[a]	Numbers assigned to the fractions
8 × 10 mL H:A 98:2	1–8
8 × 10 mL H:A 95:5	9–16
8 × 10 mL H:A 90:10	17–24
8 × 10 mL H:A 70:30	25–32
8 × 10 mL H:A 50:50	33–40

[a] Proportions (*v*/*v*) of *n*-hexane (H) and acetone (A)

Table 2 FA composition (area percentage on total FAs) of the TG fractions of EPO purified by gravimetric column chromatography with silica gel and silver nitrate

FAs	Total TGs (original oil)	Chromatographic eluates	
		13–24	26–34
16:0 (PA)	6.3 ± 0.2	2.1 ± 0.2	3.4 ± 0.2
18:0 (EA)	1.9 ± 0.1	0.9 ± 0.1	3.0 ± 0.2
18:1n-9 (OA)	6.4 ± 0.3	2.3 ± 0.1	2.6 ± 0.1
18:2n-6 (LA)	75.3 ± 1.1	66.9 ± 1.8	38.4 ± 1.3
18:3n-6 (GLA)	10.1 ± 0.4	27.8 ± 1.0	52.6 ± 1.7

Mean and SD values for four consecutive separations

Determination of Silver Ion Impurities

All obtained eluates containing purified peaks 1 and 2 were combined, separately. Silver ion was quantified in both fractions by adding excess 0.2 M NaCl, shaking, and centrifuging at 3,500 rpm for 5 min. The organic phase was removed for further analysis and the aqueous phase was discarded. The solid (AgCl) was dried at 105 °C overnight and weighed to obtain the amount of silver ion.

Enzymatic Hydrolysis Reaction

To determine the proportion of GLA in the *sn*-2 position in the purified TGs for each assay, enzymatic hydrolysis with porcine pancreatic lipase (E.C. 3.1.1.3) was carried out according to the method of Lopez-Lopez et al. [11]. The different hydrolysis products were separated by TLC. The solutions were applied to an activated (105 °C, 60 min) plate of silica gel (20 × 20 cm) divided into two zones (one for each solution) which was then placed in a well-saturated developing tank. The plate was developed with *n*-hexane–diethyl ether–acetic acid 70:30:1 (*v*/*v*/*v*). Lipid standards containing mono, di, and triglyceride mixtures (Sigma–Aldrich, St Louis, MO, USA) were used to identify the TLC bands corresponding to the hydrolysis products. After development the plate was dried at room temperature and the spots were revealed with iodine vapor.

Bands corresponding to *sn*-2 monoglycerides (*sn*-2 MGs), diglycerides (DGs), FAs, and TGs were scraped from the plate and placed in test tubes capped with PTFE-lined caps. Samples were methylated as follows. Methylating mixture (methanol–acetyl chloride 20:1 *v*/*v*; 2 mL) and a standard solution of pentadecanoic acid (15:0) (10 mg/mL, 100 μL) were added to each tube. Tubes were closed and placed at 100 °C for 30 min. They were then cooled to room temperature and 1 mL *n*-hexane was added. After shaking and centrifuging (2500 rpm, 3 min), the hexane phase was removed for GLC analysis. Results from GLC analysis are shown in Table 3.

GLC Analysis

FAMEs were analyzed by use of a Focus GC (Thermo Electron, Cambridge, UK) equipped a with flame-ionisation detector (FID) and a Omegawax 250 capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Supelco,

Table 3 FA composition (area percentage of total FAs) of the hydrolysis products from the two fractions purified by gravimetric column chromatography with silica gel and silver nitrate

FAs	Products from hydrolysis of EPO TGs					
	sn-2 MGs		FFAs		TGs	
	Eluates		Eluates		Eluates	
	13–24	26–34	13–24	26–34	13–24	26–34
16:0 (PA)	1.9 ± 0.3	–	2.8 ± 0.2	5.7 ± 0.5	–	–
18:0 (EA)	–	–	1.0 ± 0.1	2.2 ± 0.2	–	–
18:1n-9 (OA)	3.0 ± 0.4	–	3.1 ± 0.3	5.6 ± 0.7	–	–
18:2n-6 (LA)	54.1 ± 2.6	29.2 ± 0.8	73.9 ± 0.5	42.0 ± 1.3	67.1 ± 1.4	40.0 ± 1.0
18:3n-6 (GLA)	41.0 ± 2.0	70.8 ± 1.2	19.2 ± 0.5	44.6 ± 0.4	31.5 ± 0.1	59.7 ± 0.8

Mean and SD values for four consecutive separations

Bellefonte, PA, USA). The temperature programme was: 1 min at 90 °C, heating until 220 °C at a rate of 10 °C/min, constant temperature at 220 °C (2 min), heating until 250 °C at a rate of 10 °C/min and constant temperature at 250 °C (1 min). The injector temperature was 250 °C and the split ratio 50:1. Injection volume was 4 μL. The detector temperature was 260 °C. Nitrogen was used as carrier gas (1 mL/min). Oil Reference Standard AOCS No. 2 (Sigma–Aldrich, St. Louis, MO, USA) was used to identify FAMEs. Pentadecanoic acid (15:0) was used as internal standard.

Results and Discussion

An HPLC chromatogram obtained from EPO and from the collected eluates is shown in Fig. 1. Notice that two of the peaks (labeled 1 and 2) from the EPO HPLC chromatogram (used as reference) were purified by means of the silica–silver nitrate column; these were the two peaks with the largest amounts of GLA [8]. Peak 1 was purified within the seven last fractions of *n*-hexane–acetone 70:30 and the two-first fractions of *n*-hexane–acetone 50:50 (26–34), whereas peak 2 was purified within the four last fractions of *n*-hexane–acetone 95:5 and the eight fractions of *n*-hexane–acetone 90:10 (13–24) (Table 1). Fraction 25 is a mixture of both.

The FA composition of the fractions corresponding to peaks 1 and 2 (richer in GLA than the other fractions obtained from EPO) are shown in Table 2. Notice that LA and GLA are the main constituents in both fractions, and that they are significantly enriched in GLA compared with the original oil—the percentage of GLA is almost three times higher in peak 2 and five times higher in peak 1. Recovery for fractions 1 and 2 was 100% and 61%, respectively (mean values). The composition of both peaks is similar to that reported previously for the same fractions [8]; small differences could be attributed to differences between the EPO samples.

The possibility of using the chromatographic column several times without changing the stationary phase in order to purify EPO TGs was also tested. Reproducible results were successfully obtained from four consecutive separations: in all cases peaks 1 and 2 were purified with the same solvent sequence expressed above, so reproducibility is totally satisfactory when using the same column. In addition, the reproducibility of the HPLC signal was statistically significant ($p < 0.01$) for both peaks. These results show that the same stationary phase can be used several times consecutively, reducing not only the cost but also the operating time. Furthermore, lack of reproducibility of separation of the EPO TGs because of changing columns is reduced, because one column can be used several times.

Because of the use of silver nitrate in the stationary phase, silver ion could be eluted in some fractions. To determine the extent of this problem, silver ion was quantified in the TGs-containing fractions corresponding to peaks 1 and 2 for each replicate separation. The initial amount of silver ion in the stationary phase was 508 mg, and 36, 35, 38, and 32 mg silver ion were released, respectively. These data show that the mean loss of silver ion during each replicate chromatographic separation was 6.9 ± 0.5% of the initial amount of silver ion. However, this loss does not seem to affect the purification process to a significant extent. In addition, silver ion can be removed successfully by adding NaCl solution, so harmful effects seem to be negligible. These fractions could therefore be used for alimentary or pharmaceutical purposes.

It has been reported that porcine pancreatic lipase is not the most suitable enzyme for regiospecific studies of GLA oils because the Δ6 double bond of GLA reduces the rate of the hydrolysis. However, and despite the known drawbacks of hydrolysis with pancreatic lipase, the error in this

analysis seems to be very low [10]. In addition, by using this methodology, unsafe reagents, for example Grignard compounds, are avoided and safety and biocompatibility levels are improved, in contrast with other proposed positional methods.

Results of the hydrolysis reaction showed that in fraction number 2 (13–24), 41% of the coeluting TGs have GLA in position *sn*-2, whereas in fraction number 1 (26–34), 70.8% of the TGs have GLA in *sn*-2 (Table 3). On the basis of the percentages of FAs in the different classes of hydrolyzed products, it can be concluded that in fraction 2 the major TGs could be LLG (L = LA, G = GLA) and LGL, because the proportion of LA and GLA in the TG fraction is nearly 2:1 and in the FFAs released from positions *sn*-1 and *sn*-3 is nearly 3:1 (they do not fit exactly, because of the remaining FAs contained the eluates and because of the different proportions in which these TGs are present). In Fraction 1, the major TG must be LGG, and the remaining FAs constituents of this fraction (palmitic, stearic, and oleic) seem to be esterified in the *sn*-1 and *sn*-3 positions of TGs. These results are in agreement with those previously reported [8].

Previous studies showed the TG profile of EPO [8, 12] but no procedure to purify them, except HPLC or TLC, has been reported until now. These papers described purification on the analytical scale, but have several serious disadvantages when preparative purification is required. As a result of this work, it can be concluded that gravimetric column chromatography with silica gel and silver nitrate is a useful tool to purify GLA-TGs species, using EPO as natural source. This technique has several advantages:

(a) It enables direct purification of TGs of interest with acceptable yields.
(b) It is easily adaptable to larger scales for industrial purposes.
(c) It is much less expensive than other TGs separation techniques, for example HPLC.

Further investigations are in progress with these TGs species with GLA in the *sn*-2 position isolated from EPO by means of transmethylation reactions with medium-chain FAs to obtain structured lipids. These could be utilized by the pharmacology and/or other industries to achieve a more effective way of increasing GLA intake while avoiding excessive fat ingestion.

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