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Isolation of some PUFA from edible oils by argentated silica gel chromatography

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RESUMEN

Aislamiento de ácidos grasos poliinsaturados (PUFA) de aceites comestibles mediante cromatografía líquida de gel de sílice impregnado con nitrato de plata.

Los aceites comestibles de lino, girasol, borraja y el de hígado de marrajo (*Isurus oxyrinchus*), se fraccionaron mediante cromatografía líquida en columna abierta, usando como fase estacionaria gel de sílice parcialmente impregnado con nitrato de plata, con el objetivo de purificar varios ácidos grasos poliinsaturados (PUFA). Mediante este método, se aislaron los ésteres metílicos de los ácidos linoleico (LA, 18:2n6), α-linolénico (ALA, 18:3n3), γ-linolénico (GLA, 18:3n6), estearidónico (SDA, 18:4n3), eicosapentaenoico (EPA, 20:5n3) y docosahexaenoico (DHA, 22:6n3) con altas purezas y rendimientos.

PALABRAS-CLAVE: Ácidos grasos poliinsaturados – Borraja –Cromatografía en columna de gel de sílice impregnado con nitrato de plata - Girasol – Lino – Marrajo (Isurus oxyrinchus)

SUMMARY

Isolation of some PUFA from edible oils by argentated silica gel chromatography.

Four edible oils, linseed, borage and sunflower seed oils, and the liver oil of shortfin mako (*Isurus oxyrinchus*), have been fractionated by open column liquid chromatography, using partially argentated silica gel as stationary phase, looking for purify polyunsaturated fatty acids (PUFA). By this method, linoleic (LA, 18:2n6), α-linolenic (ALA, 18:3n3), γ-linolenic (GLA, 18:3n6), stearidonic (SDA, 18:4n3), eicosapentaenoic (EPA, 20:5n3) and docosahexaenoic (DHA, 22:6n3) fatty acid esters have been isolated from these oils with high purity percentages and yields.

KEY-WORDS: Argentated silica gel column chromatography - Borage – Linseed –Polyunsaturated fatty acid - Shortfin mako (Isurus oxyrinchus) -Sunflower.

1. INTRODUCTION

Essential fatty acids (EFA) are polyunsaturated fatty acids (PUFA) whose absence in a normal diet has been described as responsible for the development of a wide variety of diseases, such as cardiovascular disorders, inflammatory processes, viral infections, certain types of cancer, and autoinmune disorders (WHO/FAO, 1977). The administration of oils rich in EFA has proved to be effective in the prevention and treatment of these abnormalities. Even though the amounts of EFA found in natural oils can be enough for most therapeutic uses, there are some applications, such as pharmaceutical products, and in vitro and in vivo studies, that require higher concentrations of these acids, demanding purity up to 90% or more.

The methods used to obtain fractions rich in PUFA from natural oils are commonly based on differences in the polarity and/or spatial configuration of the fatty acids present in the extract. These differences are mostly associated with the number of double bonds in the carbon chain and hence, PUFA can be separated according to their degree of unsaturation (Kates, 1986). Winterization (Yokochi et al, 1990) and urea inclusion methods (Ratnayake et al, 1988; Robles-Medina et al, 1995b; Guil-Guerrero et al, 2000; Guil-Guerrero and Belarbi, 2001) have been successfully employed to obtain concentrates rich in PUFA, but in most cases, there is an upper limit for the highest purity attainable of a given acid by these methods. Hence, these techniques are usually coupled with liquid chromatography in order to reach purity higher than 90%. A combined process combining concentration with urea and PUFA fractionation by HPLC has been applied in the fractionation of blackcurrant seed oil, obtaining fractions of 95% GLA (Traitler et al, 1988). A similar combined process has been carried out to purify SDA, arachidonic acid (AA, 20:4n6), EPA and DHA from cod liver oil and marine microalgae (Robles-Medina et al, 1995a; Giménez-Giménez et al., 1998). By the same procedure, high purity GLA has been obtained from, *Anchusa azurea* and *Echium fastuosum* seed oil (Guil-Guerrero and Campra-Madrid, 2002).

However, these combined processes involve the use of too many processing operations that reduce overall PUFA recovery and magnify costs. Besides, HPLC is an expensive technique and it is not easily scalable to obtain tonnage quantities of purified PUFA.

The isolation of PUFA by argentated silica gel open column chromatography has only been reported in a few works. It is a simple, time-saving and easily scaleable liquid chromatography technique that uses columns packed with partially argentated silica gel as stationary phase. The

differential elution of fatty acid methyl esters (FAME) is carried out through the elution of a series of increasing polarity solvents. The isolation of a desired FAME is achieved by the adjustment of the volume and polarity of these solvents. This method has been employed to purify ALA from perilla oil (Ryu et al., 1997). Highly purified GLA has also been obtained by this technique, by fractionation of urea concentrates from seed oils of several wild species (Guil-Guerrero et al. 2000).

Alternatively, a fractionation process of microalgal (FAME) to obtain EPA concentrates that avoids the previous urea concentration step, employing argentated silica gel open column liquid chromatography, has been described (Belarbi et al., 2000).

This work reports the fractionation of FAME from linseed, sunflower, and borage seed oils, and from shortfin mako liver oil by argentated silica gel chromatography to obtain different fractions rich in LA, ALA, GLA, SDA, EPA and DHA methyl esters.

2. MATERIALS AND METHODS

2.1. Shortfin mako liver fatty acids extraction

Previous to fatty acid extraction, the liver was lyophilised. Saponification was carried out in a 2.5 L controlled temperature reactor. Ethanol (96%) (v/v) was the extraction solvent. In a typical experiment, 5 g of lyophilised liver was treated with 350 mL of freshly prepared solvent containing 8 g of KOH for lipid extraction and simultaneous saponification. Extractions/saponification was carried out at 60 ^{o}C for 1 h, with constant agitation in an argon atmosphere. The mixture obtained was filtered through sintered glass filters (100-160 %m pore size) to remove the biomass residue. The residue was washed with 100 mL of ethanol (96%), and the resulting filtrate was added to the combined filtrate, and unsaponificables were separated by extraction with hexane (5x200 mL). The hydroalcoholic phase, containing the soaps, was acidified to pH 1 with HCl:H_2O (1:1, v/v) and the free fatty acids were recovered by extraction with hexane (8x200 mL). The extract was washed with water to neutral pH and the total volume was made up to 500 mL.

2.2. Simultaneous fatty acids extraction and transesterification

Seed oil samples or Shortfin mako liver fatty acids (20 mg) were mixed with 20 mL of a solution of methanol and acetyl chloride (20:1, v/v) and 20 mL hexane, as previously described (Lepage and Roy, 1984).

The mixture was heated at 100 ^{o}C for 30 min under stirring. After cooling to room temperature, 20 mL of water were added and the FAME extracted in the hexanic layer. Three more extractions with hexane were made to ensure complete removal of methyl esters.

2.3. Fractionation of fatty esters by column chromatography

Argentated silica gel column chromatography was used for fractionation of FAME. Ag-silica gel was prepared as follows (Ryu et al., 1997): 100 g of silica gel for column chromatography (0.06–0.2 mm, 70–230 mesh ASTM; mean pore diameter of 60 nm, specific surface area of 500 m^2/g) for column chromatography (Scharlau, Barcelona, Spain) were slurried in ethanol (200 mL, 10 min). A solution of silver nitrate (10 g) in 70% (v/v) ethanol (~35 mL) was added. Agitation was continued for a further 10 min. Ethanol was evaporated in a rotary evaporator under vacuum at ~60 ^{o}C. The silver impregnated silica gel was activated by overnight heating at 120 ^{o}C. This material was cooled and kept in the dark under dry atmosphere until needed.

Chromatography columns were packed as follow: Either a slurry of Ag-silica gel (2 g) in hexane (4 mL) was poured into a column that had been half filled with hexane, or dry support material was sprinkled into a similarly filled column. A slight flow of hexane was allowed to occur during packing. The packed height of glass chromatography columns was from 7 to 8 cm in a 0.5 cm diameter column. Hexane level was lowered until it was 1 cm above that of the stationary phase. The exit of the chromatography column was plugged either with glass wool or a sintered glass disc to retain solids.

About 30 mg of the crude methylated extract obtained in the transesterification step were dissolved in hexane (2 mL). Portions of this solution were applied to chromatography columns at specified loadings of extract-to-stationary phase. A maximum acceptable fatty ester loading on the silver-silica gel stationary phase of 4% (w/w) has been determined (Belarbi et al., 2000). In a typical experiment, 5 mg of sample were loaded. The column was then eluted with a sequence of increasing polarity eluents that was established after several essays for each extract. Eluents polarity was adjusted by increasing proportions of acetone in hexane, as previously described (Ryu et al., 1997). Unless specified, a fixed volume of 5 mL of every eluent was consecutively passed through the column by gravity, at a flow rate of about 1-2 mL/min. The eluates were then collected as corresponding fractions.

2.4. Fatty acids analysis

An HP 5890 series II gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) equipped with an

autoinjector (model HP 6890) and FID detector was used. The separation was carried out in a Supelco Omega wax 250 (30 m x 0.25 mm) fused silica capillary column (0.25 %m). The oven temperature profile was: 205°C (10 min), 6ºC/min to 240 °C (9 min), giving a total heating time of 25 min. Heptadecanoic acid (17:0) methyl ester was used as internal standard for quantitative analyses.

All essays were performed al least in triplicate, and the values of a typical experience are shown in the tables.

3. RESULTS AND DISCUSSION

The purification of PUFA esters were outlined by means of a simple process, which involves single stage oil methylation to obtain FAME methyl esters, followed by FAME chromatography fractionation. For Shortfin mako liver PUFA, a previous step involving simultaneous fatty acid saponification/extraction was accomplished.

Silver-silica gel fractionation of FAME is based upon differences in the polarity of the esters eluted. Saturated esters elute first, followed by the unsaturated and polyunsaturated ones. The number and geometric configuration of double bonds determine the order of elution of esters. When the mixture is eluted through the column, FAME form polar complexes with silver ion Ag+ and can be selectively eluted in order of their polarities, from low to high polarity by changing the polarities of the eluents. These polarities can be regulated increasing the percent of acetone in the eluents (Ryu et al., 1997).

The process for LA methyl ester purification is shown in Table I. Sunflower fatty acids profile is suitable for the isolation of this fatty acid ester, because it accounts for more than 56% of total seed oil fatty acids, followed by oleic acid (OA, 18:1n9) (29.3%). The two saturated fatty acid esters, palmitic (PA, 16:0) and stearic (SA, 18:0), eluted in the first two pure hexanic fractions (100 A and B). These fractions partially eluted also the OA ester (2.3 and 26.6% yield, respectively). The following fraction 99A concluded quantitatively the elution of the monounsaturated ester, and began the elution of methyl LA. The following fractions 99B, 98A and 98B eluted only pure methyl LA, for 34.8, 27.3 and 5.7% partial recoveries, respectively. The late-eluting fraction (98C) was composed by the LA and ALA esters, concluding the chromatographic sequence. The recovery of pure methyl-LA was 65.8%, considering only the appropriate fractions (99B, 98A and 98B).

Table I

Fractionation of Sunflower Oil FAME by Silver Silica Gel Liquid Chromatography

		Applied eluent (% hexane:acetone)[a]														
		100A		100B		99A		99B		98A		98B		98C		FAME
FAME	Oil%	Purity%	Yield%	Purity %	Yield%	Purity%	Yield%	Purity%	Yield%	Purity%	Yield%	Purity%	Yield%	Purity%	Yield%	Yield(%)
16:0	10.3	71.6	57.7	27.3	39.5											97.2
18:0	2.2	19.9	76.8	3.3	22.9											99.7
18:1n9	29.3	8.2	2.3	52.3	26.6	61.4	69.8									98.7
18:2n6	56.0			17.0	4.5	38.2	22.7	100.0	34.8	100.0	27.3	100.0	5.7	38.9	0.9	96.0
18:3n3	0.8													61.1	98.1	98.1
Eluates Yield (%)			8.3		14.9		33.3		19.5		15.3		3.2		1.3	95.8[c]
Area (%)[b]	98.6	99.7		99.9		99.6		100.0		100.0		100.0		100.0		

[a] Eluent volume in each fraction was 5 mL. Each eluent code indicates hexane percentage in the eluent. Two or more eluates of equal composition are distinguished by serial letters.
[b] FAME total area (%) in each eluent detected by GLC.
[c] Eluates total yield (%).

The methyl-ALA purification process is presented in Table II. The linseed oil is highly unsaturated (90% of total fatty acids), and particularly rich in ALA (55.0%), OA (21.2%) and LA (13.8%). The saturated fatty acid esters are easily removed in the most apolar eluates (100 A and B fractions), while the monounsaturated ester methyl-OA was present in these fractions and the following eluates 99A and 99B. The LA ester is mostly eluted in fractions 99A and fraction 99B, although a low recovery was found in the 98A fraction (11.1%). The 99B solvent fraction began the elution of methyl-ALA, which also elutes in the following fractions 98A, 98B and 98C. Both two last fractions are composed only by this ester, adding a total yield of 58.4% for a 100% purity. Methyl-ALA isolation

from the fatty acid esters in the extract was possible due to the fact that it is the most unsaturated and abundant PUFA in linseed oil, and the absence of GLA is also a determinant factor in the effectiveness

Table II
Fractionation of Linseed Oil FAME by Silver Silica Gel Liquid Chromatography

		Applied eluent (% hexane:acetone)[a]														FAME
		100A		100B		99A		99B		98A		98B		98C		
FAME	Oil%	Purity%	Yield%	Purity %	Yield%	Purity %	Yield%	Purity %	Yield%	Purity%	Yield%	Purity %	Yield%	Purity %	Yield%	Yield(%)
16:0	5.3	31.0	53.8	26.4	44.8											98.6
18:0	3.4	25.9	70.1	11.3	29.9											100.0
18:1n9	21.2	42.2	18.3	60.4	25.6	62.4	54.2	2.9	2.0							100.1
18:2n6	13.8					37.0	49.3	37.1	38.4	9.6	11.1					98.8
18:3n3	55.0						0.0	58.9	15.3	90.4	26.1	100.0	31.5	100.0	26.9	99.8
Eluates Yield			9.2		9.0		17.4		14.3		15.9		17.3		14.8	97.0 [c]
Area (%)[b]	98.7	99.1		98.1		99.4		98.9		100.0		100.0		100.0		

[a] Eluent volume in each fraction was 5 mL. Each eluent code indicates hexane percentage in the eluent. Two or more eluates of equal composition are distinguished by serial letters.
[b] FAME total area (%) in each eluent detected by GLC.
[c] Eluates total yield (%).

Table III
Fractionation of Borago Oil FAME by Silver Silica Gel Liquid Chromatography

		Applied Eluent (% hexane:acetone)[a]														FAME
		100A		100B		99A		99B		98A		98B		98C		
FAME	Oil%	Purity%	Yield%	Purity %	Yield%	Purity %	Yield%	Purity %	Yield%	Purity %	Yield%	Purity %	Yield%	Purity %	Yield%	Yield(%)
16:0	10.9	45.3	66.1	20.3	32.2											98.3
18:0	4.1	17.4	67.8	7.3	31.0											98.8
18:1n9	19.1	24.7	20.6	67.8	61.4	22.1	18.3									100.3
18:2n6	35.8					74.2	32.7	90.3	50.7	38.1	16.2	0.3	0.1			99.7
18:3n6	20.5							9.6	9.4	60.3	44.7	94.3	40.9	96.1	4.7	99.8
18:3n3	0.5							0.1	4.0	0.4	12.2	4.2	74.8	1.9	3.8	94.7
20:1n9	4.0	8.5	33.8	6.5	28.1	9.5	37.5									99.4
22:1n9	2.6	7.4	45.3	1.8	12.0	6.8	41.3									98.6
24:1n9	1.7	5.3	49.6	1.5	15.3	3.7	34.4									99.2
Eluates Yield			15.9		17.3		15.8		20.1		15.2		8.9		1.0	94.2[c]
Area (%)[b]	99.2	92.7		96.9		100.0		100.0		98.8		98.8		98.0		

[a] Eluent volume in each fraction was 5 mL. Each eluent code indicates hexane percentage in the eluent. Two or more eluates of equal composition are distinguished by serial letters.
[b] FAME total area (%) in each eluent detected by GLC.
[c] Eluates total yield (%).

of liquid chromatography in purifying ALA, since the similar polarities of both 18:3 isomers can interfere the separation process.

Borage seed oil had a more complex fatty acids profile (Table III), having high amounts of monounsaturated fatty acids (27.4%). LA and GLA are the most abundant PUFA (35.8%, and 20.5%, respectively), with only a small amount of ALA (0.5%). As is previously described, saturated esters were eluted in the first two fractions (100A and 100B). These solvent fractions and the following 99A eluted quantitatively the monounsaturated esters, while the majority of the methyl-LA eluted in the 99A, 99B and 98A fractions. Finally, the elution of GLA and ALA esters started in the 99.B fraction, although with low yield (9.4 and 4.0%, respectively). The following solvent fractions 98A, 98B and 98C eluted the remaining polyunsaturated esters. The highest methyl-GLA purity (94.3 and 96.1%), yielding 40.9 and 4.7%, was achieved in the two last fractions. As expected, most methyl-ALA eluted along with methyl-GLA, but due to its reduced amount in the oil, this acid did not interfere significantly the GLA ester purification process.

Shortfin mako liver oil, as many other fish oils, is rich in long chain PUFA (Table IV), mainly in EPA (11.7%) and DHA (25.0%). As habitually happens in other fish oils, it is not a rich source of short chain PUFA, showing low levels of ALA (1.25%), GLA (0.29%) and SDA (2.36%). Due to the higher polarity of EPA and DHA, more polar solvents were used to elute these PUFA methyl esters, compared to the fractionation of seed oils. The first solvent fractions 100A, 100B, 98A, and 98B eluted the saturated and monounsaturated esters, and the polyunsaturated methylated LA, GLA, and ALA. The 95A solvent

Tabla IV
Fractionation of Shortfin Mako (I. oxyrinchus) Liver Oil FAME by Silver Silica Gel Chromatography

		Applied Eluent (% hexane:acetone)[a]																		
		100A		100B		98A		98B		95A		95B		90A		90B		90C		FAME
FAME	Oil%	Purity%	Yield%	Purity%	Yield%	Purity%	Yield%	Purity%	Yield%	Purity%	Yield%	Purity%	Yield%	Purity%	Yield%	Purity%	Yield%	Purity%	Yield%	Yield (%)
14:0	4.9	14.1	73.1	6.4	23.9	1.4	2.5													99.5
14:1	0.7	1.4	50.8	1.0	26.1	1.8	22.4													99.3
16:0	18.9	51.2	68.8	31.4	30.4															99.2
16:1n7	9.7	6.5	17.0	26.4	49.8	35.9	32.2													99.0
18:0	5.3	18.4	88.2	3.1	10.7															98.9
18:1n9	9.6	4.3	11.4	19.2	36.6	55.2	50.0													98.0
18:2n6	0.1					1.1	68.4	1.7	30.4											98.7
18:3n6	0.8							24.3	75.9	4.3	23.7									99.6
18:3n3	2.3							69.4	75.4	12.3	23.5									99.0
18:4n3	3.8									83.2	96.3	1.3	3.5							99.9
20:1n9	1.3	1.7	33.2	3.7	52.1	2.1	14.1													99.4
20:1n9	1.4	0.9	16.3	5.2	68.0	2.5	15.5													99.8
20:5n3	15.3											89.4	60.2	74.5	38.0	1.5	1.4			99.6
22:5n3	25.0											7.8	3.2	24.9	7.8	98.5	56.7	100.0	28.4	96.1
Eluates Yield			25.4		18.3		8.7		2.5		4.4		10.3		7.8		14.4		7.1	98.9[c]
Área (%)[b]	99.1	98.5		96.4		100.0		95.4		99.8		98.5		99.4		100.0		100.0		

[a] Eluent volume in each fraction was 5 mL. Each eluent code indicates hexane percentage in the eluent. Two or more eluates of equal composition are distinguished by serial letters.
[b] FAME total area (%) in each eluent detected by GLC.
[c] Eluates total yield (%).

fraction was composed mainly by the SDA ester (83.2% purity; 96.3% recovery).

EPA and DHA esters coeluted in the 95B, 90A, and 90B solvent fractions, obtaining a maximum purity for the methyl EPA of 89.4% in the in the 95B fraction, yielding 60.2% of the total methyl ester loaded. The simultaneous elution of SA and DHA esters in the methyl-EPA-fractions prevented from achieving higher purity. For DHA methyl ester, the purity reached was 100% in the 90C solvent fraction, but with a low yield (28.4%). Nevertheless, all together fractions 90B and 90C yielded almost 85.1% of total DHA methyl-ester loaded, with an overall purity of 99.0%.

The overall purity, yield and source used for the purification of the FAME isolated in this work are shown in Table V. It is concluded that the method is suitable to obtaining all PUFA, especially LA, ALA, GLA and DHA, although surely the obtainment of SDA and EPA can be improved by fine-tuning of solvent polarities and volumes, or by using a more appropriate fatty acid source.

Silver silica gel chromatography has shown to be very effective in the separation of fatty acid esters differing in their number of double bonds and/or their number of carbons. However, the use of this technique alone has shown limited effectiveness in the separation of positional isomers, such as GLA and ALA (Guil-Guerrero et al., 2000), making advisable the recourse to a previous PUFA concentration step, such as the urea inclusion method (Traitler et al., 1988; Guil-Guerrero et al., 2000), based upon differential conformation of the molecules rather than relying in polarity differences.

All purity and yield data shown in this work can be further improved by optimising volumes and polarities of mobile phases used, as well as other factors such as silver content, sample load or eluent flow rate (1-2 mL/min in this work). Time and cost can be saved as well by optimising these factors and scale up the procedure (Belarbi et al., 2000).

In conclusion, silver silica gel fractionation is a simple, one step rapid and versatile technique, easily adaptable through slight adjustments for the isolation of PUFA from oils with different fatty acids profiles. In addition, the method described here has been successfully scaled at preparative level, showing a negligibly small silver contamination in the final product (< 210ppb) (Belarbi et al., 2000).

Table V
Overall purity, yield and source used for the FAME isolated in this work

	Purity (%)	Yield (%)	Source
LA	100.0	65.8	sunflower
ALA	100.0	58.4	linseed
GLA	94.5	45.6	borage
SDA	83.2	96.3	shortfin mako
EPA	89.4	60.2	shortfin mako
DHA	99.0	85.1	shortfin mako

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