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[Received October 3, 1984]

The Isolation of Omega-3 Polyunsaturated Fatty Acids and Methyl Esters of Fish Oils by Silver Resin Chromatography

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ABSTRACT

Multigram quantities of the highly unsaturated ω3 component from samples of fish oil fatty acids and esters were isolated by silver resin chromatography. An XN1010 resin column saturated with silver ions was utilized. Polyunsaturated fatty acid (PUFA) esters from fish oil concentrate (FOC) were fractionated based on the number of double bonds by using solvent programming (acetonitrile in methanol). Larger samples (4-9 g) of FOC acids and esters and menhaden acids and esters were enriched in ω3 polyunsaturates to 82-99% (95-99% total PUFA) by use of a larger 100% silver resin column and isocratic elution with 30, 35 or 45% acetonitrile in acetone.

INTRODUCTION

During the last decade, interest in the dietary effects of PUFAs and specifically of ω3 polyunsaturated fatty acids has increased. A number of studies have correlated the effects of PUFAs on tumors (1), atherosclerotic diseases, membrane function and blood lipid composition (2-5). Samples enriched in these fatty acids are needed to further investigate their nutritional, health and biochemical effects and to serve as secondary analytical standards. For these reasons, we have investigated the fractionation and isolation of PUFAs from relatively large samples of fish oil by silver resin chromatography.

Separation of analytical quantities of PUFA methyl esters and other FA derivatives by reverse phase chromatography (6-10) and silver impregnated silica (11) have been reported previously. While PUFA-enriched oils can be produced by low temperature crystallization (12), molecular distillation (13) and supercritical fluid extraction (Krokonis, V.J., unpublished results, Phasex Corp., Nashua, New Hampshire, 1984), these techniques resulted in enrichments to only 70 to 85% of total FA. We found that silver resin chromatography could be used to provide more highly enriched PUFA fractions and also to isolate specific fatty acid methyl esters based on the number of double bonds.

EXPERIMENTAL

Materials

XN1010 sulfonic acid resin (16/50 mesh) was obtained from Rohm and Haas. A 50% ω3 fish oil concentrate (FOC) (Jahres Frabriker, Sandefjord, Norway) was esterified by sulfuric acid/methanol. Menhaden oil (Zapata Haynie Corp., Reedville, Virginia) was transesterified (sodium metal in methanol) to obtain the fatty acid methyl esters (FAMEs) and saponified (alcoholic potassium hydroxide) to prepare the fatty acids.

Methods

The preparation (grinding, sieving, sodium and silver ion incorporation) and nomenclature of the XN1010 resin have been described previously (14-17). Two columns were prepared. Column A (2.7 × 60 cm) was slurry packed with ca. 180 ml (65 g) of 200/270 mesh, 100% Ag^+/Na^+ resin and was used for sample sizes up to 500 mg of fatty acids or methyl esters. 100% Ag^+/Na^+ means that the sulfonic acid protons of the resin were first replaced by Na^+ ions and then 100% of these Na^+ ions were replaced by Ag^+ ions (17). Solvent programming was accomplished with two 6000A HPLC pumps and a Model 660 Solvent Programmer (all Waters Associates). The eluants were monitored by an ultraviolet detector (ISCO, Model 1840) at 210 nm. Column B, a slurry packed 4.7 × 45 cm Michel-Miller column (Ace Glass, Vineland, New Jersey), contained ca. 750 ml (250 g) of 100/200 mesh, 100% Ag^+/Na^+ XN1010 resin and had a fatty acid capacity of ca. 10 g. Compounds applied to column B were eluted isocratically. The mixed solvents were pumped by a metering pump (Metering Pumps Ltd., London) and products were monitored by a refractive index detector (Waters Associates).

Solvents were removed from the eluted fractions by rotary evaporation. Fatty acids were methylated with diazomethane. The FAMEs were analyzed in a Packard Model 428 gas chromatograph equipped with a 100 m × 0.25 mm (0.2 μm coating) SP 2560 fused silica capillary column (Supelco Inc., Bellefonte, Pennsylvania). Helium carrier gas and a flame ionization detector were utilized. The oven temperature was programmed from 190 to 215 C at 10 C/min with an initial hold of 30 min. Both FAME standards and gas chromatography/mass spectroscopy (GC/MS) were used to identify the various FAMEs. GC/MS analyses were made with a Finnigan gas chromatograph/EI-CI MS system equipped with a 30 m × .319 mm DB-1 capillary column. The GC was programmed from 176 to 221 C at 2.3 C/min and then 221 to 250 C at 8 C/min with helium as carrier gas.

RESULTS

The columns employed, solvents, sample sizes and other parameters are summarized in Table I. The compositions of the starting materials are tabulated in Table II and of the

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TABLE I

LC Conditions

Sample	Sample size (g)	Column[a]	Solvent	Flow rate (ml/min)
FOC methyl esters (See Fig. 1)	0.100	A	0-30% ACN[b] in methanol Run time: 1.0 hr	7.5
FOC methyl esters (See Fig. 2)	4.15	B	30% ACN in acetone	8.5
FOC acids	5.23	B	45% ACN in acetone	8.5
Menhaden methyl esters	9.3	B	35% ACN in acetone	8.0
Menhaden acids (See Fig. 3)	8.1	B	45% ACN in acetone	8.0

[a]See "Methods" Section.
[b]Acetonitrile.

TABLE II

Initial Composition of Oils

Fatty acid components[a]	FOC methyl esters	Menhaden methyl esters
12:0	0.1	–
14:0	1.6	7.3
14:1	0.3	0.1
15:0	0.6	0.6
16:0	1.4	18.7
16:0 Br	0.4	–
16:1	6.8	9.1
17:0	0.5	0.3
17:1	0.1	–
16:2 ω4	0.6	1.1
18:0	0.1	3.2
18:1	5.6	11.2
16:3 ω4	0.8	2.9
16:4 ω4	0.4	0.3
18:2 ω6	1.7	1.6
16:4 ω1	2.0	1.7
20:0	0.3	0.2
18:3 ω6	0.5	0.2
20:1	1.7	1.5
18:3 ω3	1.0	1.5
20:2 ω6	3.5	0.5
18:4 ω3	12.0	2.4
18:5 ω3	0.3	–
20:3 ω6	–	0.2
20:4 ω6	0.6	0.8
22:1	2.0	0.2
20:3 ω3	0.2	0.2
20:4 ω3	1.0	1.6
20:5 ω3	29.1	12.5
24:0	0.1	0.1
24:1	0.1	0.4
22:4 ω3	1.1	0.6
22:5 ω3	1.0	2.3
22:6 ω3	20.5	11.1
24:6 ω3	0.8	–
Σ ω 3	67.0	32.3
Σ ω 6	6.3	3.3

[a]In order of elution from SP 2560 capillary column; some peaks not defined, so total may not equal 100%.

various fractions in Table III. Solvent programming was used for the separation of a 100 mg sample of FOC FAMEs based on the number of double bonds (Fig. 1). Larger samples (4-9 g) of both fish oil FAs and FAMEs were partially fractionated utilizing isocratic runs of mixed solvents (Figs. 2 and 3). The isocratic technique is useful if only PUFA enrichment is required. Menhaden oil methyl esters and acids were enriched by the isocratic technique from 32.2% and 93.2% ω3 (39.3% to 98.9% PUFA) when chromatographed in the acid form and from 32.2% to 81.7% ω3 (39.3% to 94.8% PUFA) as the methyl esters. A commercially available sample of ω3 FOC was enriched from 67.0% to 88.4% ω3 (76.5% to 99.8% PUFA) when chromatographed in the acid form and from 67.0 to 99.7% ω3 (76.5% to 99.9% PUFA) as the methyl esters. A methyl ester fraction containing >90% 22:6 was also isolated (Fig. 2).

Silver resin columns normally can be used almost indefinitely without loss of resolution or sample capacity, which is a distinct advantage over silica-gel/silver nitrate systems. However, on one occasion when column A was subjected to rapid changes of solvent composition, resolution was lost. The column was regenerated by the following procedure: the column, containing 65 g resin (ca. 230 meq of SO_3H groups), was washed (at 8 ml/min) with 1 l of 8N HNO_3, 1 l distilled water, 1 l distilled water with 40 g $NaNO_3$ (a 100% excess calculated from 3.6 meq SO_3H per gram of resin) and then again with 1 l of distilled water. The Na^+ form of the resin was then washed with 1.5 l of distilled water containing 60 g of $AgNO_3$ (50% excess), 2 l of distilled water, then by 2 l of a gradually increasing percentage CH_3OH in distilled water.

DISCUSSION

Fractionation of fish oil methyl esters or acids and fish oil concentrates by silver resin chromatography provides a simple and convenient approach to isolation of fractions containing ca. 70% 20:5 and 22:6 and 88 to >95% total ω3 fatty acids. Samples of fish oil esters can be enriched rapidly, and oxidation and decomposition can be minimized by using a nitrogen gas blanket. While samples of isomerized methyl arachidonate (18) and other fats have been separated by the number of *trans* double bonds, this is the first example where silver resin chromatography has been used to separate a mixture of fatty acid methyl esters into fractions each composed of a group of fats with the same number of *cis* double bonds. Acetonitrile (ACN)/acetone is a better eluting solvent than ACN/methanol for the separation of fatty acids, because the latter can result in the production of traces of methyl esters. The formation of these esters is catalyzed by sulfonic acid groups on the resin, and the formation of these esters occurs even if the sodium ion form of the resin is prepared before silver ion incorporation (4).

We cannot explain the loss of resolution which occurred once when rapid changes in solvent composition were made. Resin volume does change with solvent polarity, but FAMEs were resolved as well on the regenerated resin as on the original. Thus, fracturing and loss of sulfonic acid groups can be ruled out. Washing the column with 50% ACN in acetone did not regenerate the resin. However, this problem does not seem to occur if solvents are changed slowly.

This study was conducted with mostly 100% Ag^+/Na^+ resin columns and mixed solvent systems. A 2.8 g sample of PUFAs was also enriched on a 42% Ag^+/Na^+ column with acetone as solvent. However, the use of partial argentation

TABLE III

Composition of Chromatographic Fractions[a]

Fatty acid components	FOC methyl esters[b]								FOC methyl esters[c]			FOC acids			Menhaden methyl esters			Menhaden acids[d]			
	1	2	3	4	5	6	7	8	1	2	3	1	2	3	1	2	3	1	2	3	4
Saturates																					
12:0	6.2	0.6							0.3			0.2			–			–			
14:0	32.6	4.5							8.3			8.4			10.8			12.2	6.8		
15:0	9.3	2.2							3.7			2.7			1.0			1.1	0.6		
16:0	22.6	3.2							5.7			5.4			29.2			32.0	14.6		
16:0 Br	0.5								1.0			0.2			–						
17:0	8.2	5.4							2.2			1.8	1.0		0.4			0.2	1.3		
18:0	2.3	1.1							0.1			0.1			5.0			7.0	2.3		
20:0	0.7	11.7							0.9			0.3			0.3				1.6		
24:0	–	1.9							0.3			0.1						0.3			
Monoenes																					
14:1	1.4	2.8	1.1	1.3					0.6			3.8			0.1			0.4			
16:1	13.8	16.8	40.2	4.9					24.7			24.9	0.8		15.3			14.3	17.8		
17:1		0.1	0.8	3.0					0.5			2.3			–						
18:1		4.4	32.9	1.9					20.9			20.3			22.6	17.8		21.9	19.4	19.5	
20:1		7.2	12.7	0.3					10.4			10.3			2.7	3.2		3.3	1.9		
22:1		23.8	3.9						6.2			6.9	1.2		0.3			1.0			
24:1		1.2							0.5			0.3			0.3			1.2			
Dienes																					
16:2 ω4				28.2					2.0			0.1	1.2		2.4			0.6	6.5		
18:2			0.1	47.6	0.7				4.0	0.4		4.1	2.1		1.7	2.5		0.7	4.6	1.4	
20:2			2.0	0.9	2.4				1.5	1.0			1.1	1.0	0.5	1.0		0.2	1.0	1.0	
Trienes																					
16:3 ω4				0.7	37.3					1.4			18.2	0.7	2.3				5.2	10.0	
18:3 ω6				3.8	15.0					0.3			0.2	0.2		0.3	0.2		0.3	0.5	
18:3 ω3				0.1	32.4					1.2			19.9	0.3	1.1	16.9			7.3	0.7	0.2
20:3 ω3				0.3	3.1					0.1	0.2		0.3	0.2		0.5	0.3		0.2	0.4	0.3
Tetraenes																					
16:4 ω4					0.2	1.4				0.2			0.6				2.0				1.5
16:4 ω1					0.4	4.3	4.9			4.4	0.2		12.0	9.5			10.1				3.6
18:4 ω3						78.4	0.4			26.5	0.4		10.5	12.3		10.6	0.1			19.1	9.3
20:4 ω6						2.4	0.3			0.9			9.5	0.5		11.5	0.8		1.2	11.5	0.6
20:4 ω3						4.6				1.9			9.7	1.1		20.2	2.4		1.9	22.9	2.5
22:4 ω3							3.0			2.0				1.7		1.9	2.2				2.6
Pentaenes																					
18:5 ω3							0.1				1.0			0.6							
20:5 ω3						2.2	87.7	1.5		53.3	7.6		3.0	42.1			48.0			2.1	52.3
22:5 ω3						1.2	2.5			2.0			2.1	1.4		2.9	8.3			5.6	8.6
Hexaenes																					
22:6 ω3								95.4		2.6	90.2		1.6	27.4		1.2	20.4			1.51	17.4
24:6 ω3								0.2			0.3			0.8							
Σ ω 3				0.4	35.5	88.8	93.7	97.1		89.6	99.7		47.1	88.4		54.2	81.7		9.4	44.7	93.2
Σ PUFA			2.1	81.6	91.5	94.5	98.9	97.1	7.5	97.3	99.9	4.2	92.0	99.8	8.0	69.5	94.8	1.5	28.2	76.7	98.9
Fraction weight (gm)									1.00	2.05	1.12	1.49	0.30	3.47	6.0	0.5	2.7	4.2	1.3	0.3	2.1

[a]Determined by GC of methyl esters.
[b]Figure 1.
[c]Figure 2.
[d]Figure 3.

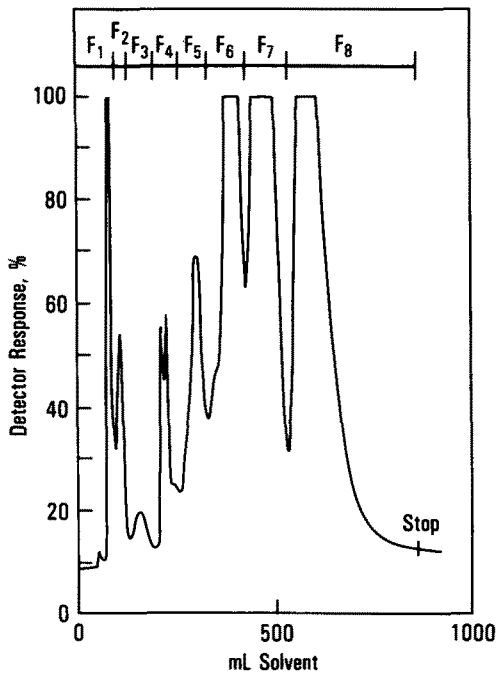

FIG. 1. Fractionation of FOC methyl esters on column A (100% Ag^+/Na^+). Flow rate = 7.5 ml/min; programmed from 0 to 30% ACN in methanol over 1 hr; sample size: 100 mg. See Table III for composition of fractions.

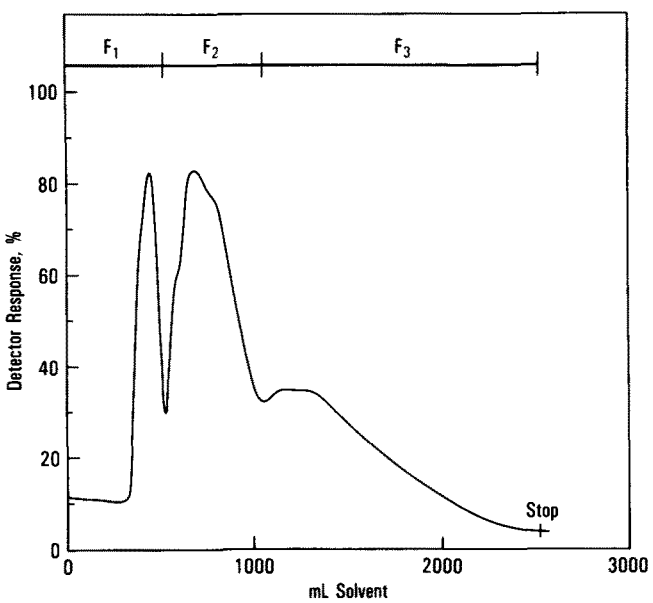

FIG. 2. Fractionation of FOC methyl esters on column B (100% Ag^+/Na^+). Flow rate: 8.5 ml 30% ACN in acetone/min; sample size: 4.15 g. See Table III for composition of fractions.

resin chromatography would require the use of smaller samples due to less capacity per given volume of resin (unpublished results). The 100% Ag^+/Na^+ columns were easier to prepare and, with mixed solvent systems, samples of $\omega 3$ enriched PUFAs and PUFA methyl esters were rapidly isolated.

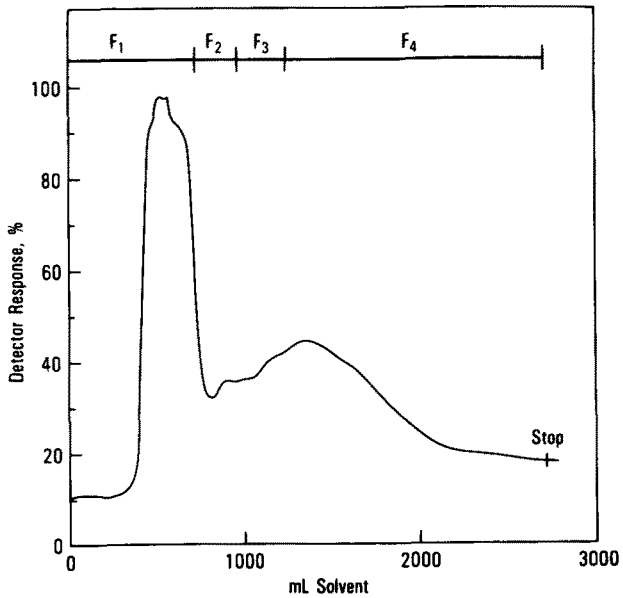

FIG. 3. Fractionation of menhaden oil acids on column B (100% Ag^+/Na^+). Flow rate: 8.0 ml 45% ACN in acetone/min; sample size: 8.10 g. See Table III for composition of fractions.

ACKNOWLEDGMENT

W.K. Rohwedder and S.M. Duval provided GC/MS analysis.

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[Received May 30, 1985]