HPLC Separation of Natural Oil Triglycerides into Fractions with the Same Carbon Number and Numbers of Double Bonds

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ABSTRACT

A high performance liquid chromatographic method was developed and tested on palm oil, low-erucic-acid rapeseed oil and cocoa butter. A 100-cm long column was used, packed with Nucleosil 5 C18 and using acetonitrile/acetone, 60:40 (v/v) as mobile phase. Using this system, the oils could be separated directly into fractions specified by having the same carbon number and number of double bonds. Earlier, the same fractionation could be obtained only by a 2-step chromatographic separation. The fractions have been found to be very pure and to have simple triglyceride composition with only one totally dominating triglyceride type in almost all cases.

INTRODUCTION

No single separation technique can adequately separate the very complex mixture of individual triglycerides (TG) in natural oils and fats. However, with certain restrictions, a combination of different modern chromatographic techniques can normally give sufficient information to enable an estimation of the TG composition.

A TG molecule has 2 distinct, chromatographically available parameters, i.e., the number of double bonds and the number of carbon atoms it contains. The established techniques for the determination of these are, respectively, argentation thin layer chromatography (Ag-TLC) and carbon number gas chromatography (CN-GC). Reversed-phase high performance liquid chromatography (RP-HPLC) separates normally according to partition number, which is a function of both the number of carbon atoms and number of double bonds.

Several papers have been published recently concerning TG analysis based on different combinations of the chromatographic techniques just given together with fatty acid analysis of the fractions obtained. Two-step fractionation with Ag-TLC followed by CN-GC has been reported for palm kernel oil (1,2) and rat adipose tissue (3). Ag-TLC/RP-HPLC has been used for black cod (4) and cottonseed oil (5), and in reversed mode for palm oil (6). Of all the techniques, RP-HPLC and, to a lesser degree, Ag-TLC (7) are well suited for 2-step fractionations. Fractions from CN-GC with high carbon numbers are difficult to recover and often are contaminated (3).

One-step fractionation followed by analyses of the fractions by a second technique plus fatty acid determination have been performed on rat adipose tissue (7) and peanut oil (8) by Ag-TLC/CN-GC. RP-HPLC/CN-GC has been used to investigate beef lipids (9), soybean oil (10), black cod (11) and low-erucic-acid rapeseed oil (12). The estimation of the TG composition by one-step fractionation is, in principle, more uncertain as the fractions are more complex. In practice, satisfactory results are obtained as long as no TG isomers are to be estimated. The main advantage of 2-step separations is that fractions are obtained containing TG with the same number of double bonds and the same carbon numbers.

An attempt has also been made to use only RP-HPLC followed by fatty acid determinations, but it was not possible to get precise TG compositions for peanut or cottonseed oils (5).

In a previous investigation with RP-HPLC of TG, some separations within the partition numbers also were observed (13) when a 25-cm Nucleosil 5 C18 column was used with acetonitrile/acetone (50:50). This paper reports efforts to develop an RP-HPLC system which completely separates natural vegetable oils within the partition numbers giving fractions containing TG with carbon numbers and numbers of double bonds defined.

Terminology and Definitions

We defined our terms as: carbon number (CN): sum of the carbon atoms of the fatty acids in a triglyceride (TG); number of double bonds (NDB): sum of the double bonds of the fatty acids in a triglyceride; partition number (PN): PN = CN - 2 × NDB; triglyceride class: triglycerides having the same CN and NDB; triglyceride type: triglycerides with known fatty acids but unknown positional distribution.

EXPERIMENTAL

Materials

Trilauroylglycerol (purity ≥ 99%) was purchased from Larodan, Malmö, Sweden.

Lobra oil (rapeseed oil with ca. 0.5% erucic acid) and palm oil were taken directly from the final refining step at AB Karlshamns Oljefabriker, Karlshamn, Sweden. Cocoa butter was purchased from Nordchoklad, Kalmar, Sweden. The TG from these oils were isolated by silicic acid column chromatography (14). The purity of the TG fractions were checked by TLC.

The solvents used, acetonitrile, acetone and chloroform (Merck), were of analytical grade and were used without further purification.

High Performance Liquid Chromatography (HPLC)

Apparatus. An LDC Minipump 711 was used with an extra pulse-dampener. We also used an LDC Refracto Monitor Model 1107, a Varian CDS 111 electronic integrator and a Rheodyne loop injector Model 7120 (10 μL).

Columns. Stainless steel tubes (4.6 mm id, 25, 75 and 100 cm long) were packed with Nucleosil 5 C18, Macherey-Nagel. Slurry (chloroform) was packed at 400 bar by a Haskel pneumatic pump (15). Mobil phase flow was 1.8 mL/min; column temperature was 23 C.

Gas Chromatography (GC)

Apparatus. We used a Varian Model 1400 gas chromatograph with FID and an Autolab System IV electronic integrator.

Column. Glass tubes (2 mm id, 2 m long) were packed with 6% BDS on Anakrom ABS (100/110 mesh). Column temperature was 190 C, whereas carrier gas was nitrogen at 25 mL/min.

Performance

Separation on all 3 HPLC columns was tested with 2 mobile phases (acetonitrile/acetone, 50:50 and 60:40, v/v). The fractions, however, were only taken from the 75- and 100-

cm columns with acetonitrile/acetone (50:50) and from the 100-cm column with acetonitrile/acetone (60:40) as mobile phase.

Each oil (200 μg) was injected as a chloroform solution (10 μL). The collected fractions were evaporated under nitrogen to dryness using a Rotavapor, redissolved in hexane, transferred to 3-mL vials with screw caps and then stored in a refrigerator until analyzed for fatty acid composition by GC.

For the fatty acid analysis, the fractions were transesterified by dimethylcarbonate/sodium methylate after evaporation using a micro procedure based on a published method (16). By this method, even the smallest fractions of about 10 μg TG gave usable GC results within the linear range of the GC system used.

RESULTS AND DISCUSSION

During earlier HPLC work on natural oils and fats, some tendency was observed for splitting of the peaks within the identified partition number when using a 25-cm column and acetonitrile/acetone (1:1, v/v) as the mobile phase (13). This phenomenon could not be seen with, e.g., methanol/acetone (60:40, v/v) as the mobile phase. Theoretically, this splitting could be increased by an increase in the number of theoretical plates, or by using a modified mobile phase, or a combination of both.

The packed 25-, 75- and 100-cm HPLC columns had, respectively 4,300, 10,000 and 15,500 theoretical plates based on trilauroylglycerol (acetonitrile/acetone, 50:50, v/v).

The efficiency of the separation in the experimental work was followed in 2 ways: (a) by using the quantitative results, as presented by Tables IA, IIA and IIIA together with complementary Tables, IB, IIB and IIIB.

The A tables show how the calculated values (from fatty acid analyses) of carbon numbers and numbers of double bonds successively approach integer numbers as the more powerful separation system is used. When a satisfactory value was achieved, no further fractions were taken. With the 100-cm column and acetonitrile/acetone (60:40, v/v) mobile phase, these values indicate the actual TG classes with acceptable confidence. Knowing the integer CN and NDB, the partition number can be calculated using

TABLE I

A: Calculated Carbon Numbers and Number of Double Bonds of TG Fractions of Lobra Oil

				Calculated values					
				System 1[b]		System 2[b]		System 3[b]	
Fraction[a]	PN	TG class	Area %	CN	NDB	CN	NDB	CN	NDB
1	38	54:8	0.8	56.4	3.8	53.4	5.3	53.8	7.0
2	40	54:7	0.8	59.7	3.6	54.0	6.4	53.9	6.7
3		54:7	3.0	53.9	3.9	56.9	4.3	54.0	6.8
4		54:6	0.6	Not visible		53.4	5.1	53.8	5.6
5	42	54:6	7.7	59.0	4.3	53.9	5.9		
6		52:5	1.3	56.7	4.5	52.3	4.5	52.1	4.7
7		54:5	5.4	54.0	2.5	53.9	4.9		
8	44	54:5	14.7	55.0	3.5	53.9	5.0		
9		52:4	2.9	53.6	1.2	52.4	4.1		
10	46	54:4	19.7	57.2	3.5	54.0	4.1		
11		52:3	5.1	51.1	2.0	52.2	3.1		
12	48	54:3	25.2	54.0	3.0				
13		52:2	6.8	58.7	3.8	52.4	2.2		
14	50	56:3	0.8	55.8	4.1	55.7	3.0		
15		54:2	1.7	54.0	1.9				

B: Main TG Types and Main Fatty Acid Contents of TG Fractions of Lobra Oil

TG class	Area %	Main TG type	Main fatty acids (mol %)		
54:8	0.8	L-Ln-Ln	L-27.5	Ln-54.0	
54:7	0.8	L-L-Ln	L-62.7	Ln-31.6	
54:7	3.0	O-Ln-Ln	O-33.5	Ln-63.1	
54:6	0.6	L-L-L	L-88.1		
54:6	7.7	O-L-Ln	O-33.3	L-32.5	Ln-32.5
52:5	1.3	P-L-Ln	P-28.3	L-27.7	Ln-29.5
54:5	5.4	O-L-L	O-34.1	L-61.6	
54:5	14.7	O-O-Ln	O-62.8	Ln-31.3	
52:4	2.9	P-O-Ln	P-27.2	O-31.7	Ln-31.6
54:4	19.7	O-O-L	O-64.9	L-33.0	
52:3	5.1	P-O-L	P-28.8	O-33.2	L-32.8
54:3	25.2	O-O-O	O-96.6		
52:2	6.8	P-O-O	P-27.3	O-61.6	
56:3	0.8	O-O-C20:1	O-59.2	20:1-32.1	
54:2	1.7	St-O-O	St-23.9	O-52.1	

[a]Numbered in order of elution from the HPLC column.

[b]System 1: column 75 cm; acetonitrile/acetone, 50:50; system 2: column 100 cm; acetonitrile/acetone, 50:50; system 3: column 100 cm; acetonitrile/acetone, 60:40.

PN = partition number; CN = carbon number; NDB = number of double bonds; M = myristic acid, 14:0; P = palmitic acid, 16:0; ST = stearic acid, 18:0; O = oleic acid, 18:1; L = linoleic acid, 18:2; Ln = linolenic acid, 18:3.

TABLE II

A: Calculated Carbon Numbers and Number of Double Bonds of TG Fractions of Cocoa Butter

Fraction[a]	PN[a]	TG class	Area %	Calculated values					
				System 1[a]		System 2[a]		System 3[a]	
				CN	NDB	CN	NDB	CN	NDB
1	46	52:3	0.5	Not visible		50.3	2.4	52.0	2.6
2		50:2	1.7	50.7	2.1	49.2	1.8	50.4	1.9
3		54:3	0.5	53.5	2.8	52.9	2.7	54.3	3.1
4	48	52:2	4.8	51.9	2.0				
5		50:1	19.8	50.5	1.7	50.2	1.2		
6	50	54:2	6.0	51.5	2.9	53.4	1.9	53.9	2.0
7		52:1	40.4	52.1	1.0				
8	52	54:1	26.3	54.0	0.9				

B: Main TG Types and Main Fatty Acid Contents of TG Fractions of Cocoa Butter

TG class	Area %	Main TG type	Main fatty acids (mol %)		
52:3	0.5	P-O-L	P-28.3	O-34.1	L-24.1
50:2	1.7	P-P-L	P-57.6	L-28.6	
54:3	0.5	O-O-O	O-63.0		
52:2	4.8	P-O-O	P-32.4	O-67.6	
50:1	19.8	P-P-O	P-59.0	O-29.7	
54:2	6.0	St-O-O	St-32.7	O-65.4	
52:1	40.4	P-St-O	P-28.7	St-37.8	O-30.6
54:1	26.3	St-St-O	St-65.5	O-30.4	

[a]See footnotes to Table I.

TABLE III

A: Calculated Carbon Numbers and Number of Double Bonds of TG Fractions of Palm Oil

Fraction[a]	PN[a]	TG class	Area %	Calculated values					
				System 1[a]		System 2[a]		System 3[a]	
				CN	NDB	CN	NDB	CN	NDB
1	44	54:5	0.4	52.2	1.4	54.1	4.5	53.7	4.7
2		52:4	2.2	51.7	3.3	51.9	3.9		
3		50:3	0.6	50.1	2.9				
4	46	54:4	2.1	53.1	3.1	53.8	3.7	53.9	3.9
5		52:3	10.8	52.1	3.0				
6		50:2	10.3	50.0	2.0				
7		48:1	0.5	48.1	0.8				
8	48	54:3	4.2	53.6	2.8	53.6	2.9	53.9	2.9
9		52:2	24.7	52.1	2.0				
10		50:1	30.5	50.2	1.1				
11		48:0	4.6	48.3	0.2	48.2	0.0		
12	50	54:2	3.0	53.4	1.9	53.8	2.4	53.8	2.0
13		52:1	4.9	52.1	1.1				
14		50:0	1.2	50.3	0.4	50.1	0.2		

B: Main TG Types and Main Fatty Acid Contents of TG Fractions of Palm Oil

TG class	Area %	Main TG type	Main fatty acids (mol %)		
54:5	0.4	O-L-L	O-34.1	L-59.6	
52:4	2.2	P-L-L	P-29.2	L-58.6	
50:3	0.6	M-D-L	M-35.4	O-20.2	L-14.1
54:4	2.1	O-O-L	O-66.3	L-32.2	
52:3	10.8	P-O-L	P-30.7	O-33.8	L-32.0
50:2	10.3	P-P-L	P-60.1	L-31.2	
48:1	0.5	M-P-O	M-20.4	P-48.9	O-21.7
54:3	4.2	O-O-O	O-96.0		
52:2	24.7	P-O-O	P-31.8	O-64.7	
50:1	30.5	P-P-O	P-63.4	O-33.3	
48:0	4.6	P-P-P	P-96.9		
54:2	3.0	St-O-O	St-28.8	O-61.2	
52:1	4.9	P-St-O	P-32.8	St-31.6	O-33.6
50:0	1.2	P-P-St	P-62.0	St-31.1	

[a]See footnotes to Table I.

the known equation PN = CN - 2 x NDB. The partition numbers agree with earlier results for Lobra (12) and for palm oil (6) from this laboratory and with literature data for cocoa butter (17). The boundaries between the partition numbers can be seen both in the chromatograms (Figs. 1-3) and calculated as above. The partition numbers increase with the retention time. Within one partition number, the higher unsaturated fractions elute sooner than the more saturated. An interesting observation is that, in the case of Lobra oil, it was possible at 3 occasions to separate components within one class. This was verified by the fatty acid analysis (see Table IB). The B tables show the main TG type in each fraction together with the percentage of the main fatty acids. The figures indicate that even the smallest fractions are, in almost all cases, very pure and contain only one totally dominating TG type.

The second efficiency check was done (b) visually, as shown for Lobra oil in Fig. 1. The various sequences in the figure show how the separation between and within the partition numbers increases. It can be seen, for all 3 oils, that the groups of peaks belonging to each partition num-

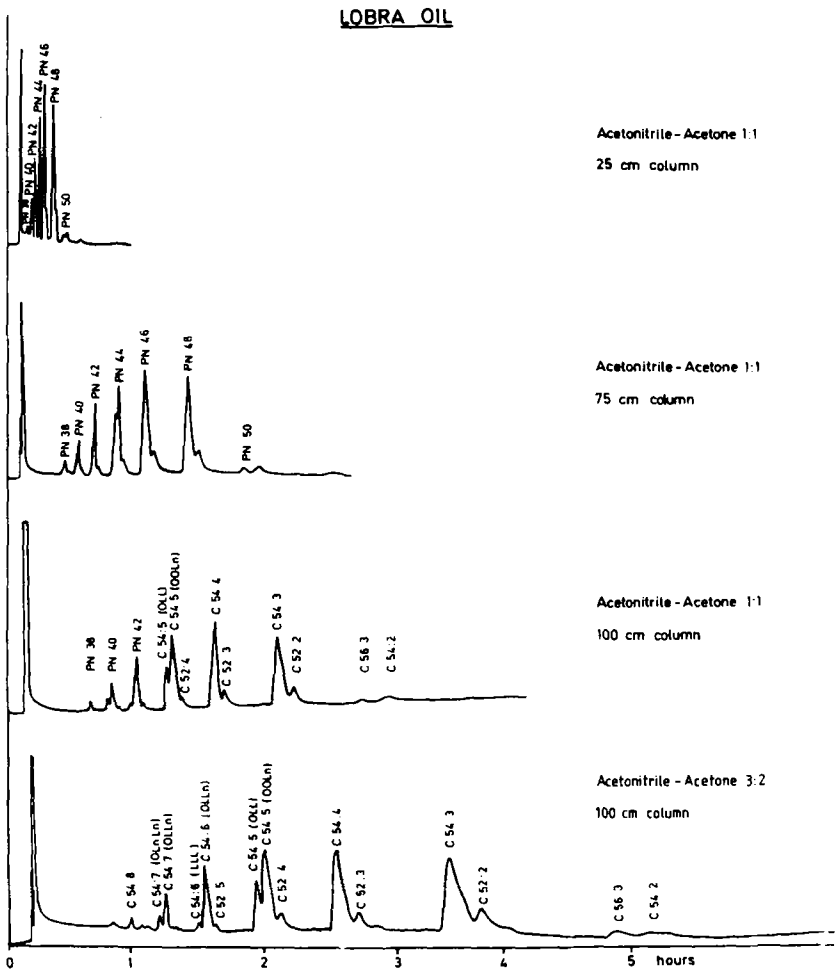

FIG. 1. HPLC separations of Lobra oil at varying experimental conditions.

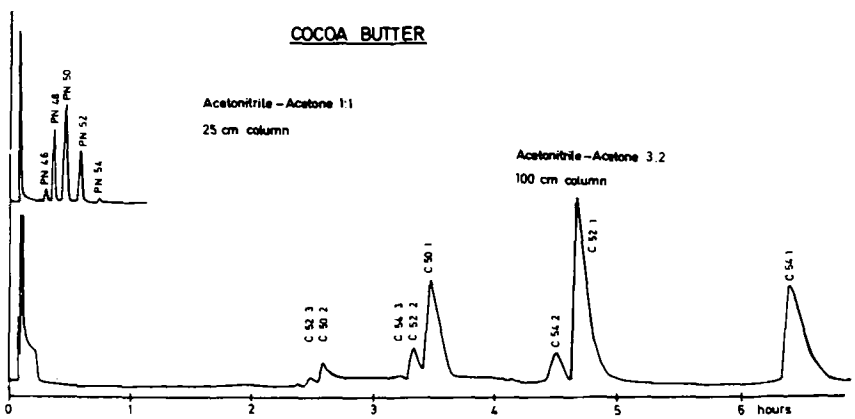

FIG. 2. HPLC separations of cocoa butter at 2 different experimental conditions.

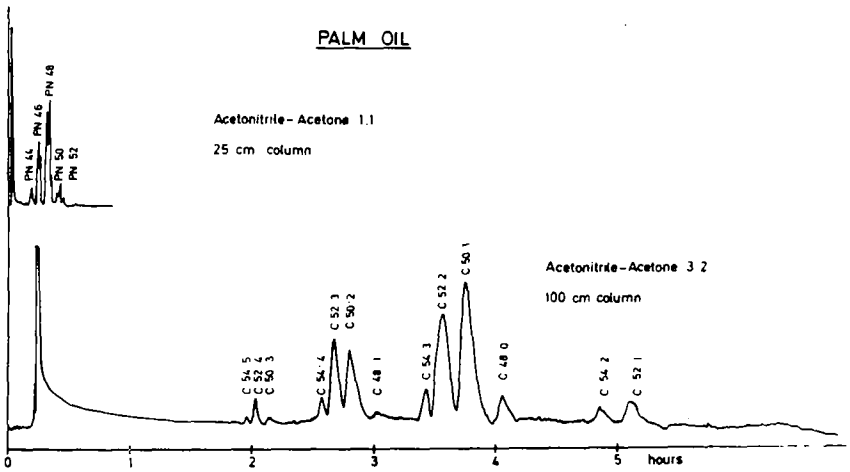

FIG. 3. HPLC separations of palm oil at 2 different experimental conditions.

ber are well separated. The interference between the most saturated fraction of one partition number and the most unsaturated from the following one is negligible. Figures 2 and 3 show the corresponding first and last chromatogram for palm oil and cocoa butter, respectively.

The oils used for this study were chosen so as to cover a broad range of partition numbers and different complexities in their triglyceride compositions. It is shown that a 100-cm column in combination with acetonitrile/acetone (60:40, v/v) mobile phase for these oils gives a generally acceptable separation. In special cases, however, it is possible to use a less powerful mobile phase and obtain results in a shorter time without substantial loss of efficiency (e.g., palm oil).

With the described method, it was thus possible to separate the 3 oils studied, directly into TG classes using only a one-step HPLC fractionation, which, to our knowledge, has not been reported before.

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[Received January 8, 1981]