HPLC of Triglycerides

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ABSTRACT

Triglyceride separation was investigated on a reverse phase high performance liquid chromatography (HPLC) column using two different solvent systems. Complete separation of model compounds differing by two methylene groups was achieved. Partial or complete separation was also observed in critical pairs; for example, the different types of triglycerides consisting of palmitic and oleic acids. This observation was confirmed on natural oils (coconut oil, palm kernel oil).

INTRODUCTION

The recent rapid development in high performance liquid chromatography (HPLC) has been of great importance in many areas, but its potential for lipid analyses is still to be fully attained. Separation of triglycerides has been a general problem for many years (1). At present they are carried out more or less by a routine GC procedure. However, the GC method gives insufficient information to provide a complete triglyceride composition of a complex mixture (for instance, a vegetable oil), even when combined with the total fatty acid composition or distribution.

To increase the amount of information obtainable about triglyceride composition, types of separation other than the carbon number distribution are necessary. Argentation chromatography provides such a system. It has been used by many workers in combination with GC and enzymatic methods. However, these procedures are rather tedious. Reverse phase liquid chromatography is another system which can be combined with carbon number separation. Furthermore, with the modern reverse phase HPLC technique this system has become more accessible in many laboratories.

In recent years a few papers on HPLC separation of triglycerides have appeared in the literature, all of which deal with reverse phase chromatography.

The first paper on triglyceride HPLC was published in 1975 by Pei et al. (2). Triglycerides were separated on a VYDAC reverse phase (35-44 μ) column and eluted with methanol/water (9:1). Wada et al. (3) performed the analysis on a μ-Bondapac C18 column (Waters) and separated soybean oil into six peaks with methanol/chloroform (9:1). Fractions were collected for GC analysis. In a recent paper (4), the same authors report similar work on beef triglycerides. Plattner et al. (5) present the results of extensive work in which different solvent mixtures are tested and separations performed on μ-Bondapac C-18 columns (Waters). According to Plattner et al., methanol/acetone mixtures gave complex separations while acetonitrile-containing systems gave more easily interpreted chromatograms. In other work, Plattner et al. (6) used the same technique for the direct analysis of trivernolin.

A refractometer detector was used in the above studies. Parris (7) found that an infrared detector was suitable for triglyceride analysis. The separation was carried out on a Zorbax ODS column, and the eluent mixtures consisted of methylene chloride, tetrahydrofuran and acetonitrile. A comparison between the two types of detectors showed similar sensitivities.

In our laboratory we are working on a general program for triglyceride composition analysis. As a part of this program we have been investigating HPLC methods, and this paper reports some of our results in this field.

EXPERIMENTAL PROCEDURES

HPLC was carried out on the following equipment: LDC Instrument Minipump 711 with dampener; Rheodyne loop injector Model 7120; LDC Refracto-Monitor; 250 mm column 4.6 mm ID; Nucleosil 5 μ C18 spherical particles (Macherey-Nagel). The column was slurry-packed with a Haskel pneumatic pump at a pressure of 380 bar, according to the procedure described by Bristow (7). The particles were slurried in chloroform and the pump was charged with acetone. The amount of injected triglyceride sample varied between 0.1 and 1.0 mg in a volume of 10 μl. Chromatograms were run with the column thermostated at 23 C.

The diacid triglycerides used were synthesized for other purposes, as reported elsewhere (8,9). The monoacid triglycerides were obtained from commercial sources (Fluka AG; Nu-Chek Prep). The purity of all compounds was 99% or better (GC).

RESULTS AND DISCUSSIONS

The purpose of this work was to use model compounds in a systematic investigation to establish the best conditions for triglyceride separations. Three different columns were tested, one of which was packed at our laboratory as described above. The other two were commercial columns: μ-Bondapac C18, 10 μ, 300 mm x 4.0 mm ID (Waters); Spherisorb S50DS, 5 μ, 250 mm x 4.6 mm ID (Phase Sep Ltd). The column packed by us was superior to the commercial ones in observed separation of triglycerides as well as in measured parameters (Table I). These calculations were based on trilauroylglycerol (LaLaLa), a triglyceride within our series of model compounds. Normally other

TABLE I

Column Parameters (Calculated on Trilaurin in Methanol/Acetone 3:2 and a 10 μl loop Injection)

Column	N[a]	H[b] (mm)	h[c]
μ Bondapac C18	1150	0.261	26.1
Spherosorb 50DS	2300	0.109	21.8
Nucleosil 5 C18	3950	0.063	12.6

[a]N = Theoretical plate no.
[b]H = Plate height.
[c]h = Reduced plate height (H/particle diam).

TABLE II

Retention Times[a] (Min) of Model Triglycerides

Compound[b]	Carbon number	t_1[c]	t_2[d]
sn HHM	26	1.40	1.50
LaLaLa	36	4.75	4.35
sn-LaLaM	38	6.20	5.45
sn-LaLaP	40	7.95	7.00
MMM	42	10.50	8.75
sn-PPM	46	17.95	13.75
PPP	48	23.45	17.45
sn-PPO		22.00	16.40
sn-OOP		20.25	16.35
OOO		19.25	16.30

[a]Corrected by subtracting column void volume.
[b]H: Hexanoic, M: Myristic, La: Lauric, P: Palmitic, O: Oleic acid.
[c]Solvent A: acetonitrile/acetone 1:1, 1.82 ml/min; 23 C.
[d]Solvent B: methanol/acetone 3:2, 1.48 ml/min; 23 C.

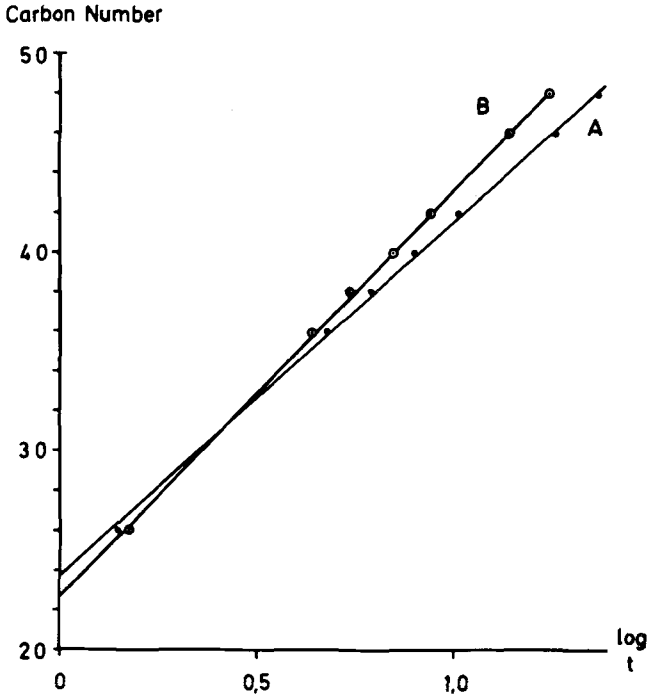

FIG. 1. Plot of retention times (log) vs. carbon number. Found equation A. y = 17.926 x + 23.654; r = 0.9996. Found equation B: y = 20.581 x + 22.620; r = 0.9997.

TABLE III

Equivalent Carbon Numbers of P-O Triglycerides in the Different Solvent Mixtures A and B (see text)

Compound	Equivalent Carbon Number A	B
PPP	48	48
sn-PPO	47.71	47.62
sn-OOP	47.07	47.60
OOO	46.67	47.57

types of substances are used to measure theoretical plates, but we chose the triglyceride even if this meant a smaller plate number. The following results are based upon chromatography on our Nucleosil column.

Separation and Solvents

Complete separation of saturated triglycerides differing by two methylene units was achieved. Two solvent mixtures were investigated. A: acetonitrile/acetone 1:1, flowrate 1,82 ml/min. B: methanol/acetone 3:2, flowrate 1.48 ml/min. The choice of eluents was made to reflect the difference between methanol and acetonitrile reported by Plattner (5). The corresponding mixture ratios with acetone and flowrates were experimentally found to give good separations and a similar retention time (from injection) of trilauroylglycerol.

We used two pairs of triglycerides to calculate the resolution (difference in retention times/mean peak base width): 1) LaLaLa/Sn-LaLaM, carbon number 36:38; 2) sn-PPM/PPP, carbon number 46:48.

The separation of these pairs was studied in the two solvent mixtures used. The calculated values of resolution are: A 1.5 36:38, 2.8 46:48, B 1.1 36:38, 2.2 46:48. This indicates that the separation is better in solvent A. These values can of course be changed. A lower flowrate and/or a larger amount of acetonitrile or methanol in the solvent mixtures will improve the resolution values up to a certain point. However, the conditions chosen seemed to us to be

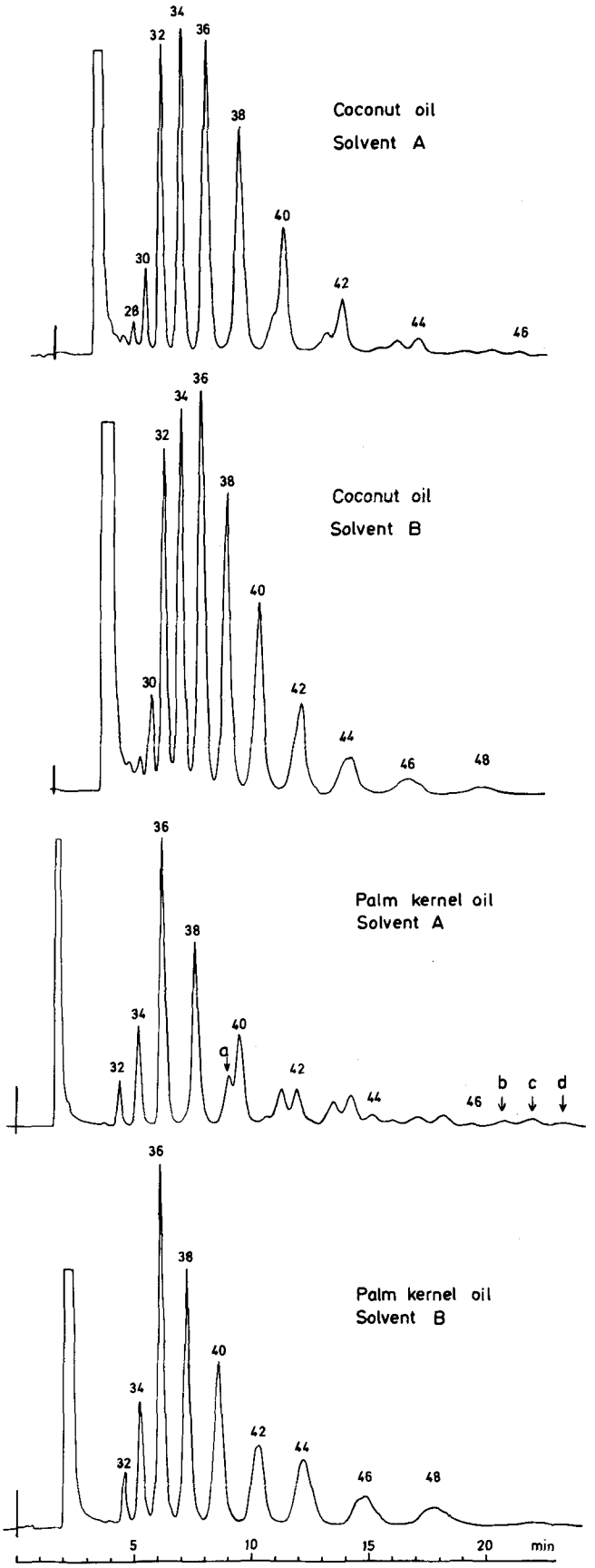

FIG. 2. Chromatogram of vegetable oils. The separation in solvent A reflects the degree of unsaturation. For example, in Palm kernel oil A, peak a represents monounsaturated triglycerides (40:1), while peak 40 is saturated (this was confirmed by fractionation and GC analysis). Peaks b, c and d correspond in retention time to the model compounds, OOO, sn-OOP, sn-PPO, respectively (see Table II).

suitable for separating triglycerides of the most common carbon numbers.

Retention Times

Retention times were measured on model triglycerides. The results are listed in Table II. Retention times are significantly shorter in solvent B in spite of a lower acetone ratio and flowrate. Both sets of times fit very well when plotted as log values against the carbon number and submitted to linear regression calculations (Fig. 1). The intercepts of the plots give a rough idea of the carbon number at which triglyceride separation starts in practice (X = 0 at retention time 1).

"Equivalent carbon numbers" (ECN) can be calculated from retention times of unsaturated compounds and the resulting equations. These ECN values are listed in Table III for triglycerides consisting of palmitic and oleic acids. Relatively large differences are observed between the solvent systems. In A the double bond has a pronounced effect on the ECN values. In B, on the other hand, almost no difference between the triglycerides containing oleic acid is observed. This is another reflection of the ability of the solvent systems to separate the glycerides, and again this favors system A.

Application

Chromatograms of coconut and palm kernel oils were run (Fig. 2) to apply our results to the separation of a natural triglyceride mixture. Differences between the two solvent systems are very clearly observable. Chromatograms run in solvent A tell definitely more about the triglyceride composition.

Our observations are in some cases contradictory to those of Plattner et al. (5). However, this is most certainly due to the differences in the column material used. With the development of even more efficient HPLC columns, separations could be further improved. An important advantage of HPLC is that fractions can be collected, which means that a specific triglyceride fraction can be submitted to further separation in other solvent systems. We will continue our work along these lines.

The response of the RI detector is greater for saturated glycerides than the unsaturated ones depending on the difference in refractive index of the specific compounds. This is a disadvantage in the quantitative evaluation, which has to be considered further.

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[Received March 8, 1978]