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NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF TRIACYLGLYCEROLS

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SUMMARY

Triacylglycerols have been separated by normal-phase high-performance liquid chromatography (HPLC) on silica utilising a solvent system consisting of dry acetonitrile–half water saturated hexane (0.7:99.3). This solvent system is UV transparent allowing detection at 200 nm and affords a separation in which retention is primarily dependent on the number of constituent double bonds. There is also a slight separation on chainlength, the longer chainlengths being eluted first. The system is therefore complementary to currently used reversed-phase HPLC systems. Chromatograms for some polyunsaturated fats and oils are given, and the most polyunsaturated triacylglycerols from linseed oil are analysed in more detail. Data are given for the separation and quantitation of the pentafluorobenzyl esters of constituent fatty acids from these triacylglycerols by a similar normal-phase HPLC system.

INTRODUCTION

There have been a considerable number of publications on the high-performance liquid chromatographic (HPLC) separation of triacylglycerols in the reversed-phase mode[1–6], most of which have used mixtures containing acetonitrile and acetone[1,3–6]. In these systems the separation of critical pairs (that is a pair of triacylglycerols in which the carbon number minus twice the number of double bonds is equal) can be difficult and time consuming[3,7] although improvements have certainly been made recently[8,9]. The addition of silver ions to the mobile phase does alter the selectivity[5] but not sufficiently to widely separate critical pairs. Triacylglycerols have also been separated by argentation HPLC[10] and as mercury(II) acetate adducts on silica gel sintered rods[11], but in both cases the peak shape was somewhat unsatisfactory.

Our work on the normal-phase HPLC separation of the pentafluorobenzyl (PFB) esters of fatty acids[12,13] according to the number of double bonds that they contain led us to suspect that this separation could be obtained if the ester functional group was sufficiently masked. More recent experience[14] with the separation of estradiol-17β-fatty acid esters as 3-pentafluorobenzyl ethers by normal-phase HPLC confirmed this view. Since the fatty acyl groups of triacylglycerols should also mask

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the ester functional groups on the glycerol, it seemed likely that a separation according to the number of constituent double bonds might also be possible on normal-phase HPLC.

We wished to utilise the relatively high sensitivity of UV detection at low wavelengths since these normal phase systems[12–14] are susceptible to overloading. This necessitated the investigation of new solvent systems since the dichloromethane used previously[12–14] absorbs below about 235 nm. This paper, then, presents such a normal-phase system in which critical pairs are very widely separated and in which the triacylglycerols are primarily separated according to the number of constituent double bonds.

EXPERIMENTAL

Materials

The following triacylglycerol standards were purchased from Sigma (St. Louis, MO, U.S.A.): tristearoylglycerol, tripalmitoleoylglycerol, trioleoylglycerol, trilinoleoylglycerol, trilinolenoylglycerol and tri-11-eicosenoylglycerol. Some domestically used fats and oils were obtained from local retail outlets: Bartolli olive oil, Lucca, Italy; Eta sunflower oil, polyunsaturated, Vegetable Oils (Vic) P/L, West Footscray, Australia; safflower oil, E.O.I. P/L, Marrickville, Australia; Meadow Lea polyunsaturated margarine (P/S > 2:1), Vegetable Oils P/L, Mascot, Australia, and Glendale raw linseed oil, Glendale Chemical Products P/L, Alexandria, Australia. The pentafluorobenzyl bromide was obtained from Fluka (Buchs, Switzerland).

Flax seeds were purchased from a local produce store and 2.5 g were ground in a mortar in 20 ml hexane and the solution filtered through a sinter. This gave a final concentration of oil of 30 mg/ml.

Instrumentation

HPLC was performed on Waters (Milford, MA, U.S.A.) equipment consisting of an M510 pump and a U6K injector. The column effluent flowed to a Hewlett-Packard (Waldbronn, F.R.G.) diode array detector (1040M) which was interfaced to an HP 300 series computer, a 9133 disc drive, a 7470A plotter and a Thinkjet printer for the manipulation and presentation of data.

The columns were silica spheri-5 (250 × 4.6 mm, 5 μm, Brownlee Labs, Santa Clara, CA, U.S.A.) or Waters silica Semiprep (300 × 7.8 mm, 10 μm). HPLC solvents were Chromar Grade from Mallinckrodt, Aust. P/L (Putney, Australia).

The gas chromatography–mass spectrometry (GC–MS) equipment was as described previously[15], the column being 1.5 m × 2 mm packed with 1.5% OV-1 on Gas Chrom Q (100–120 mesh).

Chromatography

In order to optimise the system for UV detection we investigated solvent modifiers to replace the dichloromethane that had been used previously[12,13]. Tetrahydrofuran (1.6%) in half water saturated hexane gave satisfactory separations but absorbed in the low UV, thus reducing sensitivity. However, 0.7% acetonitrile in half water saturated hexane was UV transparent and give adequate retention of the triacylglycerol mixtures. It was made up by dissolving 7 ml of dry (molecular sieve)

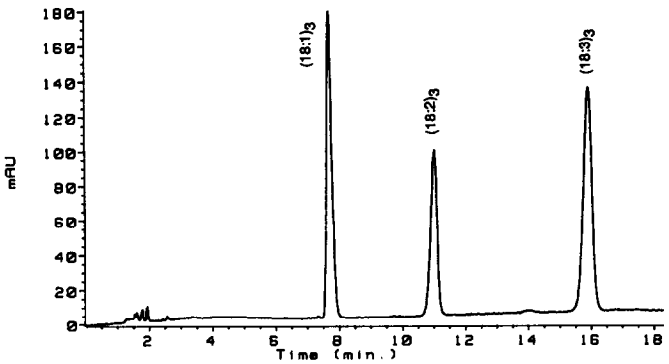

Fig. 1. Triacylglycerol standards (trioleoylglycerol, trilinoleoylglycerol and trilinolenoylglycerol). Normal-phase HPLC on 5-μm silica using dry acetonitrile–half water saturated hexane (0.7:99.3) at 2 ml/min. Detection at 200 nm, bandwidth, 4 nm.

acetonitrile in 496.5 ml of water saturated hexane by vigorous shaking and then making up to 1 l with dry (molecular sieve) hexane. Approximately 1% acetonitrile is the maximum that is soluble under these conditions. The flow-rates were 2 ml/min for the Brownlee column and 4 ml/min for the Waters. The diode array detector was set at 200 nm with a band width of 4 nm for peak detection.

Appropiate fractions were collected from the chromatograph and saponified and derivatised with PFB bromide in a one pot reaction as described previously[13]. They were then either injected directly into the GC–MS system or rechromatographed on the Waters column in 0.18% acetonitrile in half water saturated hexane with detection at 200 nm and 263 nm (bandwidth of 4 nm in both cases) and the resulting peaks checked by GC–MS.

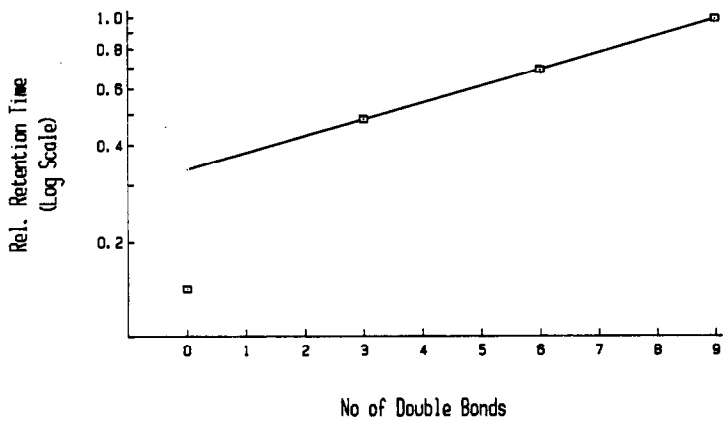

Fig. 2. Log relative retention time *versus* number of constituent double bonds for standards in Fig. 1 plus tristearoylglycerol.

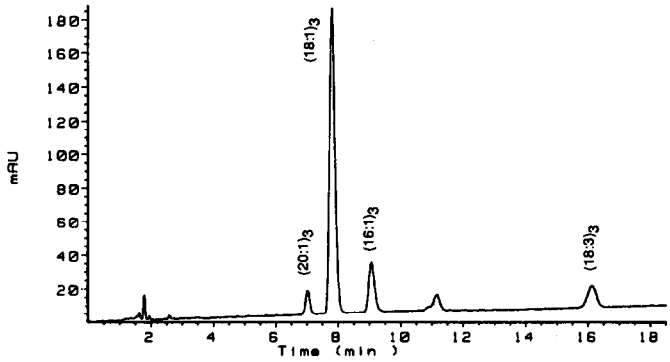

Fig. 3. Triacylglycerol standards (tri-11-eicosenoylglycerol, trioleoylglycerol, tripalmitoleoylglycerol and trilinolenoylglycerol). Conditions as for Fig. 1.

RESULTS AND DISCUSSION

Fig. 1 shows the separation of the three standard triacylglycerols containing C_{18} fatty acids. It can be seen that there is a large separation between the three peaks containing 3, 6 and 9 double bonds respectively. Fig. 2 shows that a linear relationship is obtained when log relative retention time is plotted against the number of double bonds, except that tristearoylglycerol does not lie on the line. This differs from the pentafluorobenzyl esters where, on the log t_R *versus* number of double bonds graph, PFB 18:0 was colinear with PFB 18:1, PFB 18:2 and PFB 18:3 (ref. 12). We suspect that, with triacylycerols, the first double bond in any given chain has a larger effect on t_R than subsequent double bonds in the same chain, each chain interacting independently: but this point requires further investigation. It was also found that, like the system used for the PFB esters[12], this system gave a small separation on chainlength. This is illustrated for tripalmitoleoylglycerol, trioleoylglycerol and tri-11-eicosenoylglycerol in Fig. 3. The separations of some domestically utilised fats and oils are illustrated in Figs. 4–8. It can be seen that the system described here is

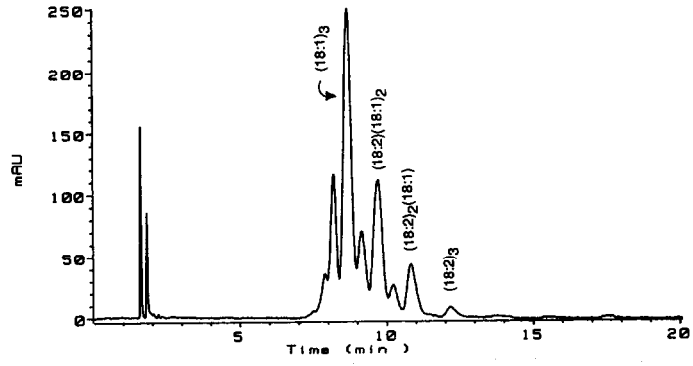

Fig. 4. Olive oil triacylglycerols. Approximately 5 μg total. Conditions as for Fig. 1.

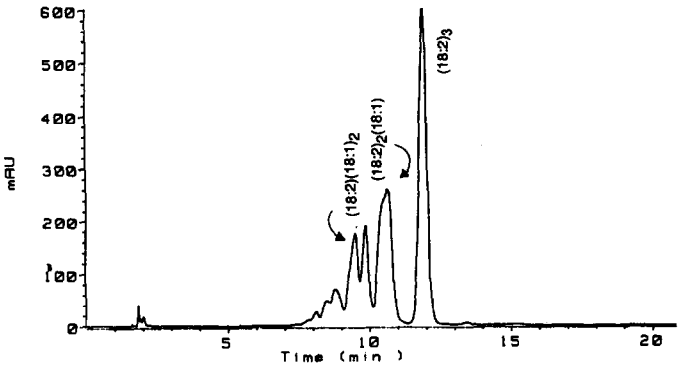

Fig. 5. Sunflower oil triacylglycerols. Conditions as for Fig. 4.

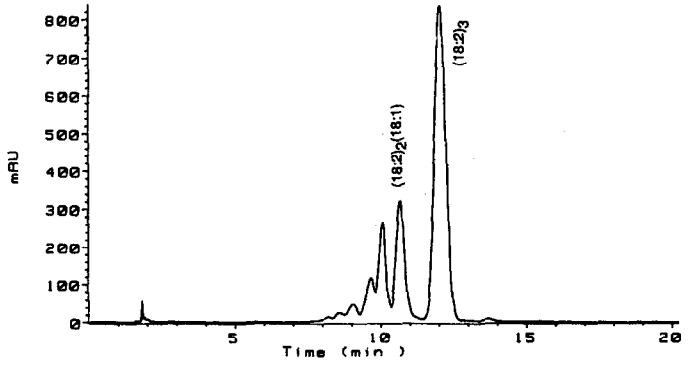

Fig. 6. Safflower oil triacylglycerols. Conditions as for Fig. 4.

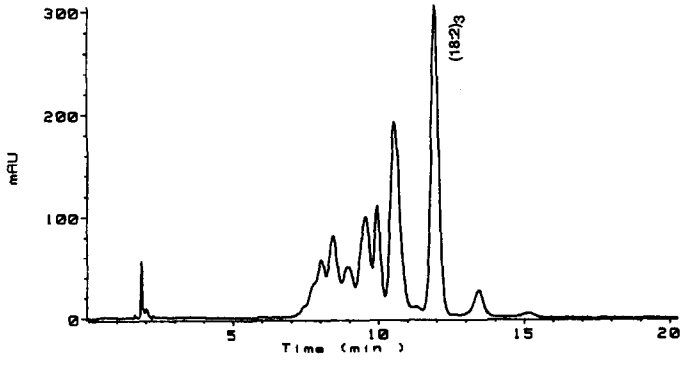

Fig. 7. Polyunsaturated margarine triacylglycerols. Conditions as for Fig. 4.

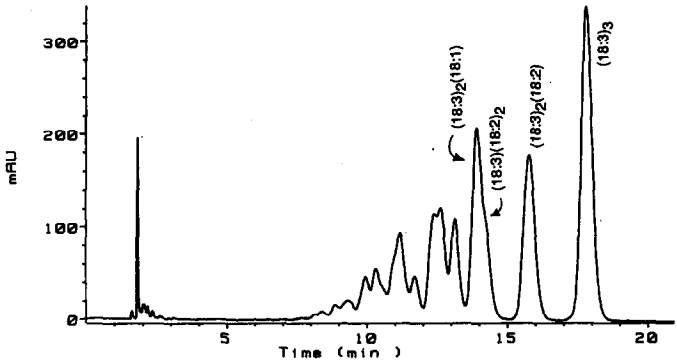

Fig. 8. Linseed oil triacylglycerols. Conditions as for Fig. 4.

particularly suitable for separating out the polyunsaturated triacylglycerols. This is primarily because the number of carbon atoms in the acyl groups of the more unsaturated triacylglycerols are usually restricted to eighteen.

The solvent system described here can take some considerable time to equilibrate with the column and can give retention times that vary slightly with the solvent batch. Thus the standards (Figs. 1 and 3), which were chromatographed on a different day to the fats and oils (Figs. 4–8), gave retention times of some two minutes less for $(18:3)_3$ and some one minute less for $(18:2)_3$. However, when the fats and oils (Figs, 4–8) were coinjected with an internal standard (trilinolenoylglycerol) the relative retention times were reproducible to approximately 3 parts in 1000. Using a log relative retention time *versus* number of double bonds graph, this allowed a preliminary identification of some of the later eluting peaks in the chromatograms. This is the origin of the labels in Figs. 4–8. We examined the later eluting peaks from linseed oil in more detail. After saponification and derivativisation with PFB bromide, subsamples were analysed by GC–MS in the negative ion chemical ionisation (NICI) mode. The $[M - PFB]^-$ ions, which are almost the sole ions in these mass spectra, indicated that the PFB esters from the last peak from linseed oil (Fig. 8) contained 18:3, from the second last peak, 18:2 and 18:3 (in approximately the expected ratios of 1:2), and from the third last, 18:1, 18:2 and 18:3. However, there were various other minor peaks that appeared to correspond to derivativised fatty acids. We therefore used HPLC to re-examine the commercial linseed oil and also the fresh oil extracted from flax seeds. Samples from both these oils were run on the Waters column and the last three peaks collected, saponified and esterified with PFB bromide. Each of the six samples was then reinjected into the HPLC using 0.18% acetonitrile in hexane with detection at 200 nm for maximal sensitivity.

The chromatogram for the fraction containing seven double bonds from the flax seed extract is given in Fig. 9. Examination of the UV spectra of the peaks eluting after 8 min suggested that only those eluting between 9.5 and 14.0 min were PFB esters. Comparison of retention times with standards suggested that these three peaks were PFB 18:1, PFB 18:2 and PFB 18:3 respectively, and that other PFB esters were absent. NICI-MS confirmed the identity of the C_{18} peaks and failed to show the presence of any other PFB fatty acid esters. Similar results were obtained from the

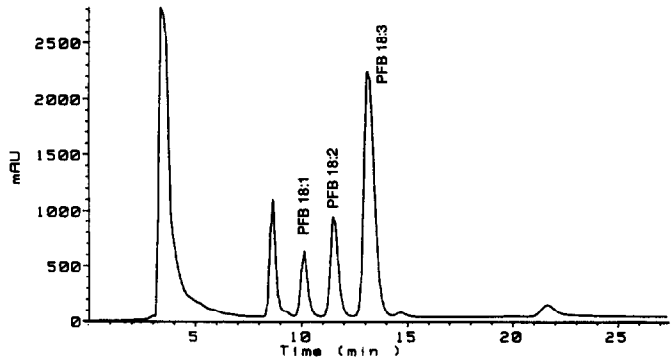

Fig. 9. PFB esters from saponified and esterified 3rd last peak of flax seed extract [corresponds to $(18:3)_2$-(18:1) plus (18:3)-$(18:2)_2$ in Fig. 8]. Total 3rd last peak from the injection of appprox. 3.3 mg flax seed extract onto a 300 × 7.8 mm column (10 μm) was collected, saponified and esterified with PFB bromide and the PFB esters rechromatographed on the same column in dry acetonitrile–half water saturated hexane (0.18:99.82) at 4 ml/min with detection at 200 nm, bandwidth 4 nm.

fraction containing seven double bonds from the commercial linseed oil. The NICI-MS and a detailed spectral analysis of the PFB 18:2 peaks from both the commercial linseed oil and from the flax seed extract respectively, showed that both of these esters were contaminated with a minor component. In the case of the flax seed extract this was apparently insufficient to affect the composition of the esters from this fraction. Integration of the chromatogram corresponding to Fig. 9 at 263 nm gave 22.1% PFB 18:1, 22.4% PFB 18:2 and 55.5% PFB 18:3. The two expected triacylglycerols from this fraction with seven double bonds are $(18:3)_2$-(18:1) and (18:3)-$(18:2)_2$. Table I shows the proportion of fatty acid in each triacylglycerol that this implies. It can be seen that there is excellent agreement between the proportion of PFB 18:3 measured from the chromatogram and that calculated from the triaclglycerols expected. The estimate of 66% $(18:3)_2$-(18:1) and 34% (18:3)-$(18:2)_2$ suggest that the shoulder at the rear of the peak containing seven double bonds (Fig. 8) is due to a slight separation between these two triacylglycerols, (18:3)-$(18:2)_2$ being eluted after $(18:3)_2$-(18:1). A similar calculation from the results for the commercial linseed oil gave 72% $(18:3)_2$-(18:1) and 29% (18:3)-$(18:2)_2$, while Hilditch and Williams[16] gave a figure of approximately 83% $(18:3)_2$-(18:1) for these two triacylglycerols from linseed oil.

The fraction containing eight double bonds from the flax seed extract would, of course, be expected to contain 67% PFB 18:3 and 33% PFB 18:2. Integration of

TABLE I

Triacylglycerol	*Constituent fatty acids (%)*			
	18:3	*18:2*	*18:1*	*Total*
$(18:3)_2$-(18:1)	44.2	–	22.1	66.3
(18:3)-$(18:2)_2$	11.2	22.4	–	33.6
Total	55.4	22.4	22.1	

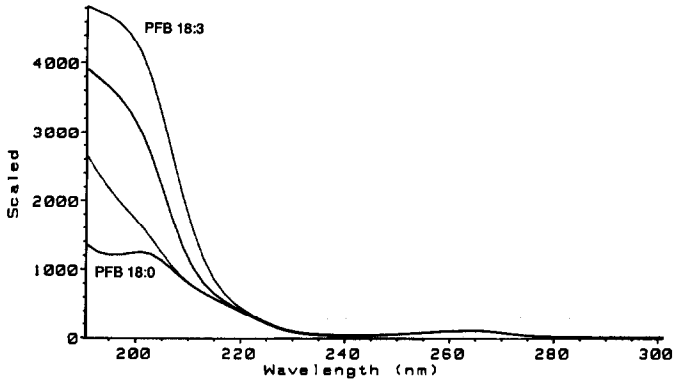

Fig. 10. Ultraviolet spectra of the PFB estes of 18:0, 18:1, 18:2 and 18:3. All spectra normalised to 100 mAU at 263 nm showing effect of double bonds on extinction in far UV.

the chromatogram at 263 nm gave 62% PFB 18:2 and 38% PFB 18:2, indicating that a UV absorbing contaminant was present in PFB 18:2 and this was confirmed by a spectral analysis of this peak. The contaminant, however, did not give a response in NICI. Integration of the chromatogram at 200 nm, followed by correction for the absorbance of the double bonds using the data given below, gives 33.5% PFB 18:2 and 66.5% PFB 18:3. Similar results were obtained for the commercial linseed oil sample.

The fraction containing nine double bonds, from both the flax seed extracts and the commercial linseed oil sample, as well as giving large peaks of PFB 18:3, gave small peaks corresponding in t_R to PFB 18:1 and PFB 18:2. These, however, did not have spectra that corresponded to PFB esters and did not give a response in NICI. The latter of these two peaks presumably corresponded to that interfering with PFB 18:2 in the fraction containing eight double bonds.

Fig. 10 gives the UV spectra, in 0.18% acetonitrile in hexane, of PFB 18:0, PFB 18:1, PFB 18:2 and PFB 18:3 all normalised to 100 mAU at 263 nm. It can be seen that operation in the low UV range markedly increases the sensitivity. Thus at 200 nm the sensitivities are increased approximately 12.2×, 17.4×, 31.8× and 42.8× for PFB 18:0, PFB 18:1, PFB 18:2 and PFB 18:3 respectively. Thus for applications that require high sensitivity this new solvent system offers considerable advantages over that containing dichloromethane described previously[12,13].

In our earlier publication[12] we sought to explain the separation of the fatty acid PFB esters according to the number of double bonds that they contain by reference to Scott's[17] model of the silica surface. That is, under the conditions used[12], the silica surface is covered by a bilayer of water surmounted by a monolayer of dichloromethane. Separation was due to competition between the double bonds in the PFB esters and the dichloromethane molecules for the aqueous surface and the small separation on chainlength was due to the inductive effect reducing the polarity of the carbonyl group. Since the properties of the separations reported here are exactly analagous, we believe that the same physico-chemical principals underlie them and that, therefore, in this system the surface of the silica is covered in a bilayer of water and the separation depends primarily on competition between the double bonds and acetonitrile molecules.

Finally, it seems to us that the separation system described here is ideally suited for the LC–MS of triacylglycerols and would be complementary to the systems reviewed by Kuksis and co-workers[18,19] utilising GC–MS and reversed-phase LC–MS that both separate primarily on chainlength.

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