Note

High-performance liquid chromatographic technique for the separation of lipid classes

A. S. RITCHIE* and M. H. JEE

Cadbury Schweppes PLC, The Lord Zuckerman Research Centre, The University, Whiteknights, Reading (U.K.)

(Received April 17th, 1985)

Separation of the simple lipid classes (triglycerides, diglycerides, monoglycerides and free fatty acids) can readily be carried out using thin-layer chromatographic (TLC) techniques[1]. However, quantification by TLC necessitates either scanning densitometry, which is only semi-quantitative, or extraction of components followed by quantification, either gravimetrically or by gas chromatography using an internal standard.

High-performance liquid chromatography (HPLC) is potentially superior to TLC, but relatively little work has been published in the literature describing this type of separation by HPLC. Hammond[2] used the now unavailable Pye Unicam LCM2 transport detector, separating lipid classes on a silica column using a solvent gradient from toluene–hexane (50:50) to toluene–ethyl acetate–formic acid (75:25:1.2). Payne-Wahl *et al.*[3] utilised a Du Pont infrared detector, but this detector placed limitations on solvent suitability. It was necessary, using their system, to esterify the free fatty acids to avoid peak tailing and obtain adequate resolution of free acids from diglycerides. Greenspan and Schroeder[4] succeeded in separating certain lipid classes on silica using an isocratic solvent system (isooctane–tetrahydrofuran–formic acid). Their method resolved triglycerides, diglycerides, free fatty acids, sterols and sterol esters, but they did not study monoglycerides.

A literature search revealed no method which resolved triglycerides, diglycerides, monoglycerides and free fatty acids, using a readily available detector, without derivatisation.

The three types of detector which were potentially suitable were: ultraviolet (UV), infrared (IR) and refractive index (RI).

Short-wavelength (200–215 nm) UV detection has been successfully used for detection of both saturated and unsaturated triglycerides and has proved the basis of an isocratic method for the separation of constituent triglycerides in natural fats[5]. The major advantage of UV detection over RI detection is that it permits solvent programming. However, where solvent programs are employed, severe problems can be encountered at short UV wavelengths with regard to baseline stability and spurious peaks from solvent contaminants[6]. In addition, according to Greenspan and Schroeder[4], short-wavelength UV detection is unsuitable for saturated free fatty acids, as they exhibit almost no absorbance in this region.

Free acids are generally difficult to resolve by liquid chromatography[3]. In order to solve this problem it is usually necessary either to derivatise the free acids as demonstrated by Payne-Wahl et al.[3], or to use solvent containing a small amount (ca. 1%) of an organic acid, e.g. formic or acetic acid. The latter approach is suitable for RI detection, but not UV or IR detection systems.

The work described here was carried out to develop a system which would separate and quantify monoglycerides in addition to other lipid components.

EXPERIMENTAL

Equipment

All analyses were carried out using a Du Pont HPLC system comprising an 8800 gradient controller, a 870 pump and a Du Pont oven together with a Waters RI detector. Solvents were degassed using a stirred vacuum degasser. For quantitative work peaks were integrated using a Spectra-Physics 4100 integrator.

All analyses were carried out using a 25 cm × 4.6 mm I.D. Zorbax silica column. The mobile phase was isooctane–tetrahydrofuran–formic acid [90:10:0.5 (ref. 4) or 80:20:0.5]. For the former mixture, analyses were carried out at ambient temperature, with a 1.5 ml/min initial flow-rate. For the latter mixture, column temperature was elevated to 35°C with a 1.0 ml/min initial flow-rate. In some cases, the flow-rate was increased during the run to accelerate the elution of the later peaks.

Materials

All lipid standards were obtained from Sigma. Triglycerides, diglycerides (mixtures of isomers), monoglycerides and free acids of palmitic, stearic and oleic acyl residues, triarachidin, trielaidin, cholesterol, cholesterol oleate and methyl palmitate were studied. Methyl ricinoleate, ricinoleic acid, and ricinoleyl alcohol were investigated as internal standards. A cocoa butter and a superglycerinated partially hardened rape seed oil were also studied. The latter was made by heating the oil with glycerol which results in a mixture with a lipid class profile similar to commercial glyceryl mono-stearate (*i.e.* approximately equal proportions of triglyceride, diglyceride and monoglyceride).

Method

All samples and standards were dissolved in the elution solvent to minimise solvent peak signal. Aliquots containing 0.2–5 mg total lipid were injected onto the column. Where necessary, fractions were collected and purity checked by TLC analysis on silica gel 60. Plates were developed in hexane–diethyl ether–formic acid (60:40:1.5) and components detected by spraying the plate with 7.5% ammonium hydrogen sulphate in 50% aqueous ethanol, followed by drying for 10 min at 100°C and charring for 1 h at 180°C.

RESULTS AND DISCUSSION

A solvent mixture of heptane–tetrahydrofuran–formic acid (90:10:0.5)[4] eluted all components other than monoglyceride within about 20 min with baseline resolution at a flow-rate of 1.5 ml/min. However, even when the flow-rate was increased

to 3 ml/min after elution of the second diglyceride peak, over 1 h was required to elute the monoglyceride which appeared as a broad, asymmetric peak. Also, the diglyceride peak shape was poor. Using a solvent mixture of heptane–tetrahydrofuran–formic acid (80:20:0.5) and a column temperature of 35°C resulted in monoglyceride eluting in about 30 min, with considerable improvement in peak shape of both monoglyceride and diglyceride, even at 1 ml/min. With some mono-glyceride standards, notably monoolein, a second peak due to the 2-isomer was ob-served. Monoglyceride peak shape was further improved by increasing the flow-rate to 3 ml/min after elution of the second diglyceride, which reduced total run time to about 15 min. Identity and purity of all components was confirmed by TLC. Though this method offered excellent preparative separation, it was unsuitable for quantita-tive work due to the change in flow-rate (except where integrators can be set up to integrate with respect to volume eluted instead of time). A flow-rate of 2.0 ml/min was found experimentally to offer the best compromise between separation of the earlier eluting peaks and obtaining reasonable shaped monoglyceride peaks. A typical chromatogram, using palmitic acid lipid standards, is shown in Fig. 1.

Several suitable internal standards are suggested in the literature. Payne-Wahl et al.[3] employed methyl 9,10-dihydroxystearate; Hammond[2] used ricinoleyl alcohol; Hitchcock and Hammond[7] found stearyl alcohol most suitable. With our solvent system, ricinoleic acid was shown to be superior to ricinoleyl alcohol in terms of peak shape, and was clearly resolved from the lipid classes of interest. Methyl ricinoleate eluted very close to 1,3-diglyceride and the literature suggested that stearyl alcohol would not be likely to be well resolved from the lipid classes studied.
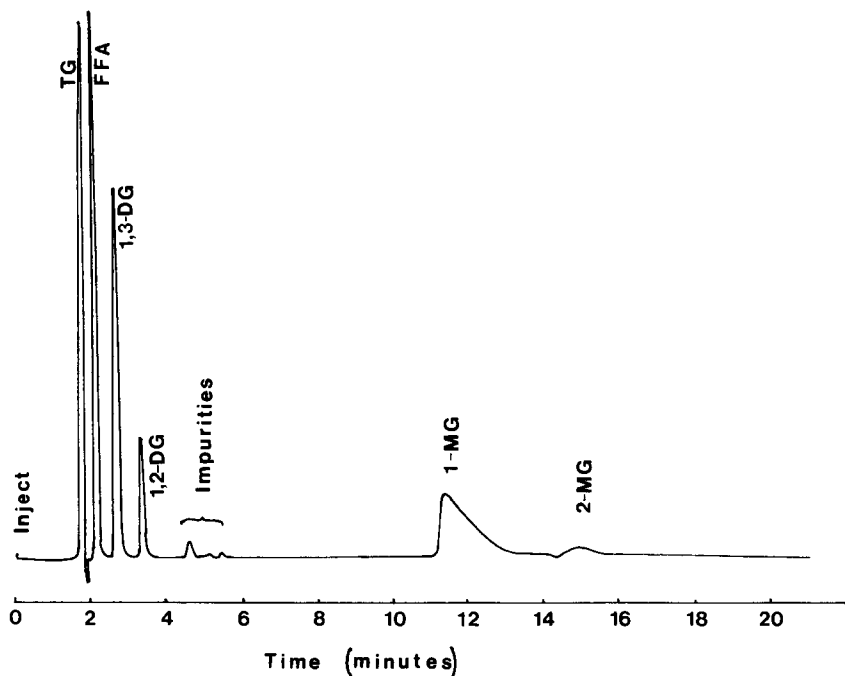

Fig. 1. Separation of lipid classes containing only palmitic acid. TG = Triglyceride; DG = diglyceride; MG = monoglyceride; FFA = free fatty acid.

Table I shows the retention times of the lipid classes of interest to us (triglyceride, diglyceride, monoglyceride and free fatty acid), of the internal standards investigated (ricinoleic acid, methyl ricinoleate and ricinoleyl alcohol) and of methyl ester (palmitate), a sterol (cholesterol), and a sterol ester (cholesteryl oleate). Most systems cause methyl esters to elute before triglycerides (*e.g.* the system of Payne-Wahl *et al.*[3]). Our system, however, eluted triglycerides before methyl esters, though they were not completely resolved. Cholesteryl oleate was not resolved at all from triglyceride, but, as Greenspan and Schroeder[4] showed, they could be resolved using a solvent with only 10% tetrahydrofuran. Cholesterol was clearly resolved from all other lipid classes.

TABLE I

RETENTION TIME OF LIPID CLASSES

Lipid	Typical retention time (min)
Triglyceride	1.73
Free fatty acid	2.20
1,3-Diglyceride	2.65
1,2-Diglyceride	3.37
1-Monoglyceride	11.30
2-Monoglyceride	14.98
Methyl ester	1.82
Cholesterol	4.00
Cholesteryl ester	1.69
Ricinoleic acid	3.80
Methyl ricinoleate	2.89
Ricinoleyl alcohol	6.86

The identity of the fatty acid was found to have a slight, but detectable effect on the retention time of certain lipid classes. The effect was most noticeable for free fatty acids, and was not observed at all for triglycerides. Fig. 2 shows the chromatogram obtained for a mixture of oleic and linoleic acids. Although the peak is split, the components were not resolved as Greenspan and Schroeder observed. Fig. 3 shows a superglycerinated partially hydrogenated rape seed oil, which consists of glycerides containing fatty acids ranging from C_{14} to C_{22}, containing 0–3 double bonds, and significant amounts of *trans*-isomers of unsaturated lipids. Peak broadening (*cf.* Fig. 1) was noticeable on most peaks, and shoulders were observed on partial glyceride peaks.

In order to determine response factors and method repeatability, mixtures were analysed which contained approximately equal quantities of each lipid class of interest and of internal standard; all classes in any one run comprised only one acyl residue, either palmitic, stearic or oleic acid. As other workers had found[3,4], difficulties were encountered in setting integrator parameters so as to give consistent results, because of interfering negative peaks due to solvent. It was necessary to program the integrator to ignore the parts of the chromatogram where these negative peaks occurred. This was achieved with the Spectra-Physics integrator using a combination of the "integrator inhibit" and horizontal baseline projection facilities. In
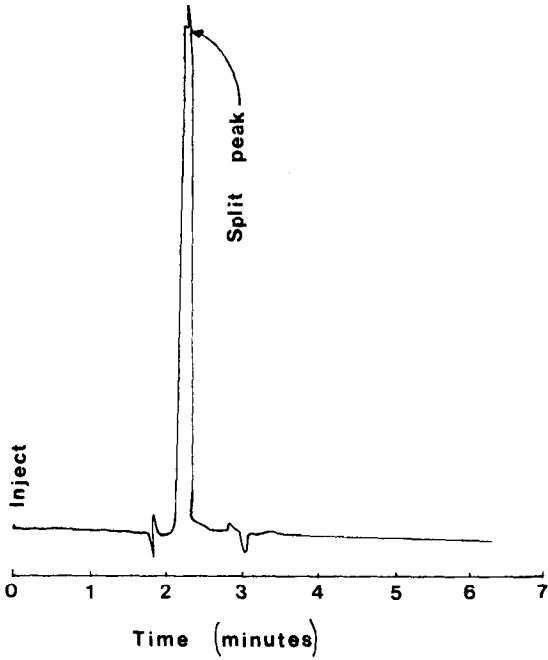
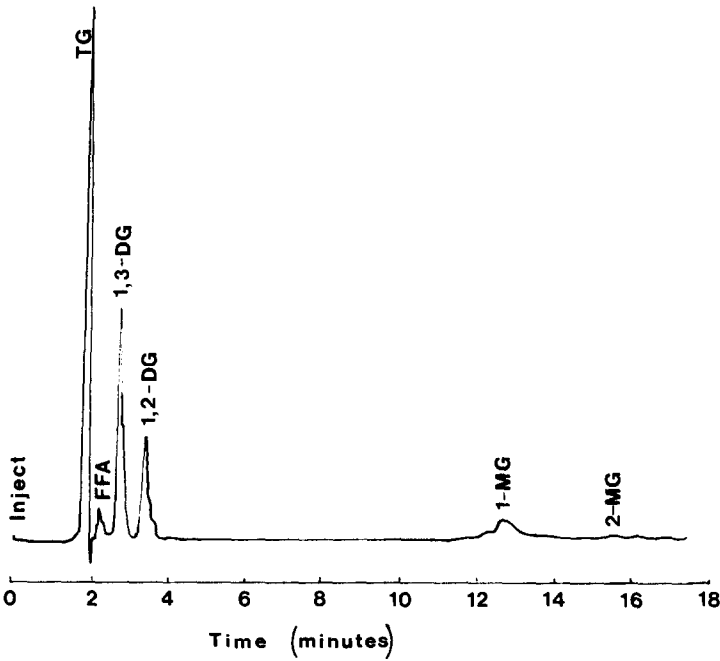Fig. 2. Separation of oleic and linoleic acids.

Fig. 3. Separation of superglycerinated rape seed oil.

TABLE II

RELATIVE RESPONSE FACTORS OF LIPID TYPES

Lipid	Response factor	
	Relative to triglyceride	Relative to internal standard
Palmitic lipids		
Tripalmitin	1.000	1.189
Dipalmitin	0.891	1.059
Monopalmitin	0.912	1.084
Palmitic acid	1.073	1.276
Stearic lipids		
Tristearin	1.000	1.076
Distearin	1.047	1.127
Monostearin	1.151	1.238
Stearic acid	1.152	1.240
Oleic lipids		
Triolein	1.000	1.175
Diolein	0.833	0.979
Monoolein	1.054	1.113
Oleic acid	0.971	1.141
Elaidic lipid		
Trielaidin	1.000	1.209

this way, consistent results were obtained. Table II shows the response factors for each lipid class for palmitic, stearic and oleic acyl residues, and for trielaidin, relative to the appropriate triglyceride (column 1) and to the internal standard (column 2). It is unlikely that this method would be accurate for quantification of the individual positional isomers of the partial glycerides, because of the probability of some on-column intramolecular acyl migration. Thus, for the purpose of determining response factors, data for individual isomers were summed. All response factors lay in the range 1.05–1.28 relative to the internal standard, and between 0.83 and 1.15 relative to the appropriate triglyceride. Thus, response was reasonably consistent for all lipid classes of interest, though sensitivity to the internal standard was markedly lower than to the lipids studied. The response factor for trielaidin (glycerol tri-*trans*-octadecenoate) was similar to triolein (tri-*cis* isomer), indicating that double bond configuration has little effect on instrument response.

Table III shows the coefficients of variations between runs for each lipid type. The apparent slight differences between the errors for different fatty acids were due to day-to-day variations in baseline stability caused by detector noise.

Although it had been shown that the method was suitable for analysis of mixtures in which all lipid classes were present at similar concentrations, many natural fats contain >95% triglyceride, and <5% other lipid classes. Table IV shows that appreciable errors occur when weight percentages are calculated from raw chromatographic data for standard mixtures similar to fats of this type. However, when the results are calculated using an internal standard present at approximately the

TABLE III

COEFFICIENTS OF VARIATION FOR RUN-TO-RUN VARIABILITY

TG = Triglyceride; DG = diglyceride; MG = monoglyceride; FFA = free fatty acid; I.S. = internal standard.

Glyceride	Coefficient of variation (%)		
	Palmitic	Stearic	Oleic
TG	1.4	0.4	2.4
1,3-DG	2.2	0.8	4.3
1,2-DG	0.6	0.8	1.1
Total DG	0.7	0.4	2.3
1-MG	—*	—*	5.9
2-MG	—*	—*	45.3**
Total MG	0.9*	0.3*	3.8
FFA	0.5	0.5	0.5
I.S.	0.7	0.5	2.0

    * Only 1-isomer present in standard mixture.
    ** Very small quantity present.

same level as the minor components, assuming the sample contains no components retained on the column, these minor components can be quantified accurately, and major component(s) calculated by deduction (as shown in the right hand column of Table IV). The reason for this can be seen in Fig. 4; response is linear only to about 0.5 mg of triglyceride, and it was necessary to inject more than this (ca. 5 mg) in order to quantify minor components. Thus the major component would give a low result in the absence of an internal standard, minor components being reported correspondingly high. It may be necessary, when using the method with the internal standard, to carry out a preliminary analysis on samples without the added standard, to ascertain the approximate levels of minor components.

TABLE IV

ANALYSIS OF A SYNTHETIC LIPID MIXTURE IN PROPORTIONS TYPICAL OF NATURAL FATS

Lipid	Percentage			
	Actual	Area %	Wt. % (response corrected)	Calculated (from I.S.)
TG	93.62 (95.77)*	85.79	86.92	—**
1,3-DG	1.51 (1.54)	3.62	3.26	1.63
1,2-DG	1.00 (1.02)	2.48	2.23	1.12
Total DG	2.51 (2.56)	6.10	5.49	2.75
FFA	1.62 (1.66)	2.86	3.10	1.55
I.S.	2.24 —	5.26	4.48	—

    * Values in parentheses represent percentages excluding I.S.
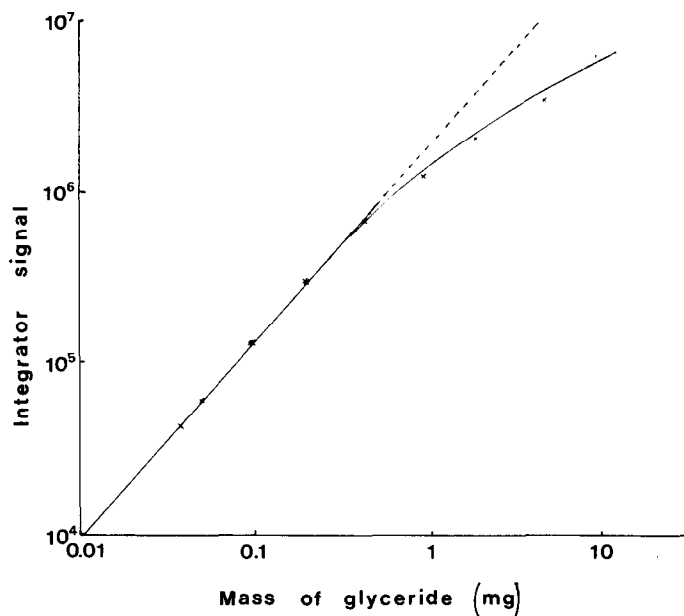   ** Triglyceride calculated by deduction = 95.70%.

Fig. 4. Linearity of response.

As with the method of Greenspan and Schroeder[4], this method is only suitable for separation of neutral lipids and free acids. Samples with large proportions of polar lipids would probably rapidly block the column as they would not be eluted. For such samples, a clean-up such as they describe may be required.

REFERENCES

1 W. W. Christie, *Lipid Analysis*, Pergamon, Elmsford, Oxford, 1973.
2 E. W. Hammond, *J. Chromatogr.*, 203 (1981) 397–403.
3 K. Payne-Wahl, G. F. Spencer, R. D. Plattner and R. O. Butterfield, *J. Chromatogr.*, 209 (1981) 61–66.
4 M. D. Greenspan and E. A. Schroeder, *Anal. Biochem.*, 127 (1982) 441–448.
5 V. K. S. Shukla, W. Schiotz Neilson and W. Batsberg, *Fette, Seifen, Anstrichm.*, 85 (7) (1983) 274–278.
6 S. M. McCown, B. E. Morrison and D. L. Southern, *Int. Lab.*, June (1984) 76–82.
7 C. H. S. Hitchcock and E. W. Hammond, in R. D. King (Editor), *Developments in Food Analysis Techniques*, Vol. 2, Applied Science Publ., Barking, 1980, pp. 185–224.