CHROM. 13,117

QUANTITATIVE CHROMATOGRAPHIC ANALYSIS OF LIPIDS IN FOODS

EUGENE W. HAMMOND

Unilever Research Colworth, Sharnbrook, Bedford MK44 1LQ (Great Britain)

SUMMARY

The characterisation and quantitative analysis of lipids in foods relies on the combination of gas–liquid chromatography (GLC) and adsorption chromatography. Despite the exceptional degree to which GLC has been developed, quantitative problems still exist, particularly in the analysis of triglycerides and diglycerides. Quantitative analysis of lipids on thin-layer chromatograms has been tried by densitometric scanning, with mixed success. Flame ionisation scanning of "Chromarods" using the Iatroscan THIO yields better results, but considerable errors still exist. It is preferable to substitute high-performance liquid chromatography for thin-layer chromatography where accuracy is required. However, the lack of a suitable, commercially available detector system is restrictive. Nevertheless, excellent results are obtained using the Pye Unicam LCM2 transport detector.

INTRODUCTION

The term "lipid" describes all those food components which are broadly soluble in organic solvents. With respect to foods we are concerned mainly with the analysis of triglycerides (TG), 1:3 and 1:2 diglycerides (DG), free fatty acids (FFA), monoglycerides (MG) and lecithins (PC). We have recently reviewed the broad area of the determination of lipids in foods[1].

To obtain sufficient information we must combine the tecnhiques of gas–liquid chromatography (GLC) and silica adsorption chromatography. Normally, quantitative totals of TG, DG, MG, FFA and PC are required. Adsorption chromatography lends itself as the most rapid method and the quantitative problems arising from this technique are discussed later. A further and most important application of adsorption chromatography is in the analysis of saturated and unsaturated triglycerides by inclusion of silver nitrate in the adsorbent.

GLC is utilised widely for the analysis of fatty acid methyl esters (FAME), but is increasingly being used for the analysis of intact TG and maybe DG. While there are few quantitative problems in the GLC analysis of FAME, considerable problems can arise in the GLC of TG and DG.

This paper describes some of our experiences and problems, in the quantitative analysis of some food lipids, using GLC, thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC).

GAS–LIQUID CHROMATOGRAPHY

We have used a variety of instruments (*e.g.* Packard/Becker series 430, Pye Unicam series 104 and 204), all fitted with flame ionisation detectors (FID). In our experience few quantitative problems exist with any of these instruments in the analysis of FAME. However, we have found some evidence to suggest that quantitative errors may arise in the analysis of FAME, from lipids containing high levels of the shorter chain fatty acids[2]. Such lipids (*e.g.* coconut fat and palm kernel fat) contain major peaks in the C_8, C_{10} and C_{12} region of the chromatogram. These peaks have a high elution velocity, and thus a high concentration in the carrier gas. Using calibration standards that mimic palm kernel fatty acids, we find that the normal hydrogen flow-rate, which is set for optimum sensitivity, must be increased by *ca.* 30 % for optimum accuracy. This evidence was based on work done using a Packard/Becker model 430 and calibration mixtures obtained from Nu Check Prep. Inc., Elysian, MN, U.S.A.

Direct analysis of TG by GLC is of increasing importance to food manufacturers. Reviews of this application have been published[3–5]. We use 0.5 m × 4 mm I.D. packed columns, with on-column injection of sample in chloroform. Comparing a number of stationary phases, 3 % OV-1 on 100–120 mesh Supelcoport or Gas-Chrom Q was the most consistent. We rarely experience problems due to poor peak resolution. Fig. 1 demonstrates typical separations of palm kernel oil and cocoa butter. However, we can experience problems of quantitative recovery, particularly of the higher carbon number TG (*i.e.* > C_{50}). The method requires that the temperature at the point of injection is maintained at 360°C over the entire column-oven temperature programme. Many instruments display this temperature, but in our experience the actual injection point temperature may be up to 10°C lower. This temperature is critical for good chromatography and quantitative recovery of TG.
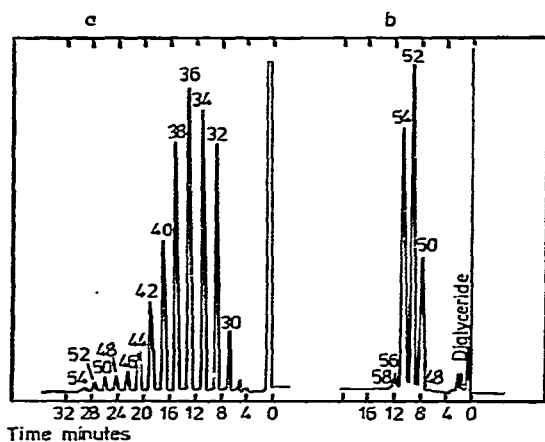

Fig. 1. GLC separation of triglycerides on a 50 cm × 4 mm I.D., 3 % OV-1 column. (a) Palm kernel oil (programme 250–350°C at 6°C/min). (b) Cocoa butter (programme 300–350°C at 4°C/min). Carbon number equals the sum of the total fatty acid carbons (tristearin = C_{54}).

With the instrument set correctly, the main and most difficult quantitative problems emanate from the column itself. Careful recording of column behaviour over a two-year period, has resulted in a column conditioning procedure that is 75% successful. We do not fully understand the reasons for poor quantitative recoveries from columns. Table I shows the results for six columns, all packed by the same technique from the same homogeneous batch of packing. The columns were all conditioned in the same way.

TABLE I

COMPARISON OF THE QUANTITATIVE BEHAVIOUR OF ESSENTIALLY IDENTICAL OV-1 COLUMNS IN THE GLC OF TRIGLYCERIDES (TG)

The TG are given as percentage.

	TG carbon number				
	C_{30}	C_{36}	C_{42}	C_{48}	C_{54}
Nu Check 51A actual	20	20	20	20	20
Column 1	20.4	20.1	19.9	19.7	19.9
Column 2	20.1	20.2	20.0	19.8	19.9
Column 3	20.2	20.0	20.0	19.9	19.9
Column 4	21.0	20.5	20.4	19.7	18.4
Column 5	20.9	20.6	20.6	19.8	18.1
Column 6	20.2	20.0	19.9	19.9	20.0

It is apparent from these results that only columns 1, 2, 3 and 6 are suitable for true quantitative analysis. Columns 4 and 5 were discarded as they did not reach specification within five days of use.

The conditioning procedure we presently use is as follows. The column is purged with nitrogen (60 ml/min) at 50°C for 10 min. The column flow rate is then reduced to 5 ml/min of nitrogen and temperature programmed to 370°C at 1°C/min rise, and with the injector heater on. The final condition is held for 36 h. The column temperature is then reduced to 300°C and the nitrogen flow-rate increased to 80 ml/min. A single injection of 500 μg of cocoa butter in chloroform is made, and the column temperature programmed at 4°C/min to 355°C. The column is then calibrated with standards and is usually ready for use in 24 h.

THIN-LAYER CHROMATOGRAPHY

Stahl[6], in 1969, published a treatise on the practice of TLC. Excellent qualitative separations of lipids can be achieved. However, quantitative analysis has always posed a problem. The problems experienced in such techniques as densitometry are rarely functions of the instrumentation, but emanate from the thin layers. Touchstone et al.[7] and Privett[8] have shown that good results can be achieved from densitometry with very careful work, but generally errors in excess of ±20% are not unusual.

Two alternatives to densitometry, using FID have been used with some success. Mangold and Mukherjee[9] reviewed tubular TLC in 1975. This is a pyrolysis technique. Padley[10] in 1967 pioneered a different approach, where a silica-coated quartz rod is passed directly through an FID. This was commercially developed in Japan as the

"Chromarod" and the "Iatroscan" (Namba et al.[11]). We have found the chromatographic properties of the "Chromarod" to be excellent (Fig. 2). However, when scanning the rods in the Iatroscan, quantitative errors in excess of $\pm 10\%$ were experienced. The technique has attracted increasing applications in the lipid field[12-14], but similar levels of error to that stated above have been reported by Sipos and Ackman[13] and Christie and Hunter[14]. We believe that the quantitative problems are a function of the FID design, so that quantitative application of the current Iatroscan is somewhat limited.
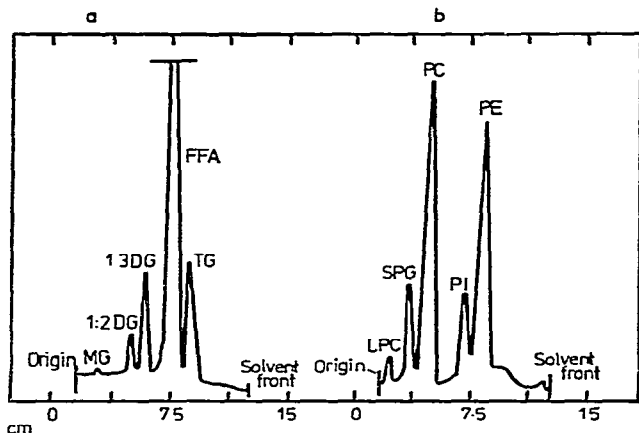

Fig. 2. Separation of (a) neutral lipids and (b) polar lipids on "Chromarod" S. Detection by Iatroscan TH10. LPC, lysophosphatidycholine; SPG, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.

In the analysis of unsaturated TG by argentation TLC the errors in densitometry are aggravated by the difficulty in quantitatively charring the TG to carbon. However, Dallas and Padley[15] have demonstrated that this is possible. Hammond and Walker[16] adopted a fluorescence scanning technique using a Zeiss KM3 chromatogram spectrometer as the scanning detector. Fig. 3 shows typical scans for cocoa butter and palm oil. In this technique, phloxin fluorescent indicator is incorporated into the thin layer. Secondary fluorescence is excited at 312 nm. The method produces valid results, since the silver nitrate quenches background fluorescence, which normally interferes in this type of system. It is possible to achieve absolute errors of $\pm 2\%$ or less, but the technique relies on rigorous standardisation of each TLC plate. We have successfully applied this technique to the analysis of some 1000 TG mixtures.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

It is evident from the problems associated with quantitative TLC of lipids, that a move to HPLC is preferable. Saxby[17] has described the general application of HPLC in analysis, and Aitzetmuller[18] has comprehensively reviewed its application to lipid analysis. Recently, Porter et al.[19] compared the use of refractive index (RI) and UV detection at 215 nm in the HPLC of lecithins. Silver nitrate has also been
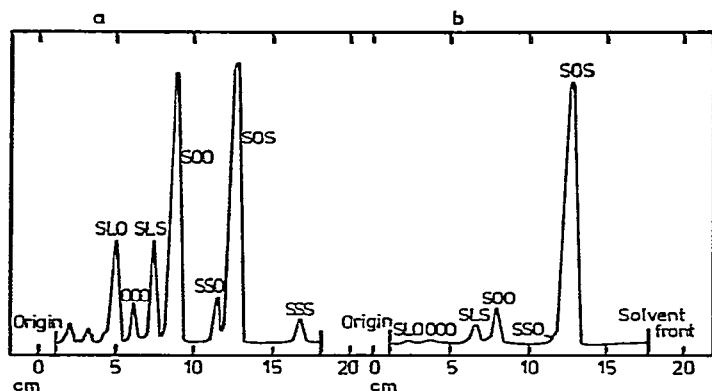
Fig. 3. 5% Silver nitrate TLC separation of (a) palm oil triglycerides and (b) cocoa butter triglycerides. Detection by fluorescence scanning using a Zeiss KM3. S, saturated; O, oleate; L, linoleate. Solvent: two developments in 75% chloroform in cyclohexane.

exploited in HPLC, as silver-impregnated silica[20-22], silver-loaded resins[23] or as an addition to the mobile phase in reversed-phase HPLC[24-26].

All our present applications are normal adsorbent or silver-loaded silica HPLC. These require solvent gradients for elution of the lipids and thus we are restricted to some rapidly ageing Pye Unicam LCM2 transport detectors. The absence of any commercial alternative may be a severe problem for the future. However, some recent work by Jeffrey[27] in our own laboratories, has demonstrated promising separations of TG, DG and FFA using flow programming as an alternative to solvent gradient programming. This system might therefore use RI as a means of detection.

Fig. 4a shows a typical HPLC chromatogram for the quantitative analysis of mixtures of TG, FFA, 1:3 DG, 1:2 DG and MG. For accuracy we always include an internal standard and apply relative response factor correction. Using the LCM2 we obtain day-to-day relative errors of between 5 and 10% over a linear response range of 1000. Maximum working sensitivity for good signal to noise is equivalent to *ca.* 10 μg eluted sample. The column used for this analysis is 10 cm × 4 mm I.D. stainless steel, packed with LiChrosorb Si-60 (5 μm) (Merck, Darmstadt, G.F.R.). Figure 4a also displays the gradient plot. It is important to note the steep rise in gradient; this is necessary to maintain good resolution and peak shape, particularly of DG and MG.

We are in the process of transferring our argentation TLC method for TG to silver-loaded silica HPLC. Our initial work[21] has centred on an isocratic separation (10% AgNO₃ by weight on Whatman Partisil 5 μm) using toluene solvent at reduced temperature (7°C). During this work we found that column behaviour changed with the method of coating the silica with silver nitrate (precipitation from acetonitrile–methanol or aqueous methanol)[28]. *K'* of the linoleate containing TG particularly, changed markedly. Column stability and reproducibility were much improved if 10% aqueous methanol was used as the silver nitrate solvent. Good resolution of trisaturated (SSS), 2-monounsaturated (SOS) and 3-monounsaturated (SSO) triglycerides is achieved (Fig. 4b). 2,3-Diunsaturated (SOO) and 2-linoleo TG (SLS) are also resolved but peak shape is poor and therefore also quantitation. Internal standards of trilaurin
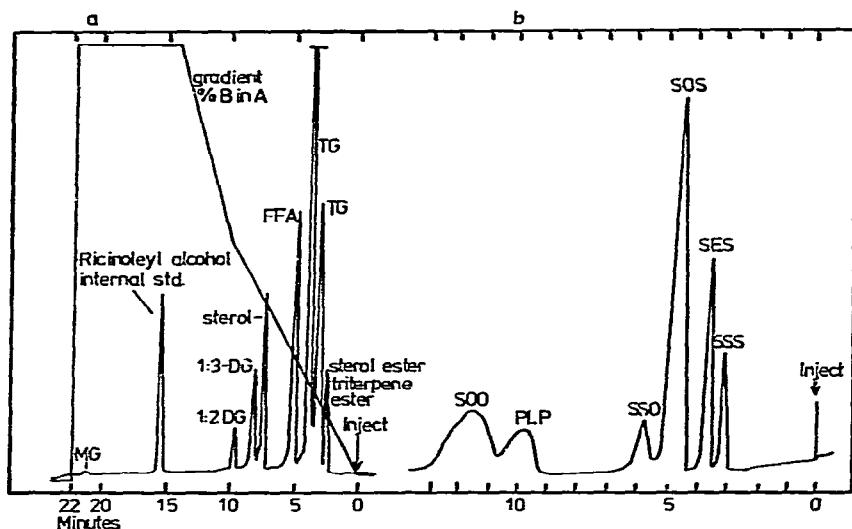
Fig. 4. (a) Silica HPLC of neutral lipids. Column: 10 cm × 4 mm I.D. of LiChrosorb Si6O (5 μm). Solvent: A, toluene–hexane 1:1; B, toluene–ethyl acetate 3:1 plus 1.2% formic acid. Flow-rate: 1.5 ml/min. Detector: Pye LCM2. (b) Separation of TG by AgNO₃ HPLC. Column: 25 cm × 4 mm I.D. of 10% AgNO₃ on Partisil (5 μm). Solvent: toluene at 6°C and 1 ml/min flow-rate. Detector: RI.

and 2-elaido-1,3-distearin (SES) have been used with equal success. This particular application lends itself well as a method of preparing pure TG species for further analysis. The next stage of this work is aimed at producing quantitative data for the completed range of TG species, to at least SLO and OOO, using solvent gradient programming or perhaps flow programming.

CONCLUSIONS

The quantitative analysis of food lipids relies heavily on the accurate setting up and calibration of instrumentation. Otherwise excellent results can be obtained from GLC analysis. In view of the difficulties experienced in obtaining accurate data from TLC techniques, it is preferable to use HPLC. However, the continued use of HPLC in lipid analysis very much rests on the production of alternative detector systems to the Pye LCM2 transport detector. These alternatives should retain the relatively good quantitative behaviour of the LCM2, but hopefully will increase the absolute sensitivity.

ACKNOWLEDGEMENTS

REFERENCES

1 C. H. S. Hitchock and E. W. Hammond, *The Determination of Lipids in Foods*, in R. D. King (Editor), *Developments in Food Analysis Techniques*, Vol. 2, Applied Science Publ., Barking, 1980, p. 185.
2 E. W. Hammond and B. Dobson, unpublished results.
3 C. Litchfield, *Analysis of Triglycerides*, Academic Press, London, 1972.
4 A. Kuksis in G. V. Marinetti (Editor), *Lipid Chromatographic Analysis*, Vol. 1, Edward Arnold, London, 1967, p. 239.
5 R. J. Hamilton and R. G. Ackman, *J. Chromatogr. Sci.*, 13 (1975) 471.
6 E. Stahl (Editor), *Thin Layer Chromatography*, George Allen and Unwin, London, Springer, Berlin, 2nd ed., 1969.
7 J. C. Touchstone (Editor), *Quantitative Thin Layer Chromatography*, Wiley-Interscience, New York, 1973.
8 O. S. Privett, in J. C. Touchstone (Editor), *Quantitative Thin Layer Chromatography*, Wiley-Interscience, New York, 1973, p. 57.
9 H. V. Mangold and K. D. Mukherjee, *J. Chromatogr. Sci.*, 13 (1975) 398.
10 F. B. Padley, *Chem. Ind. (London)*, (1967) 874.
11 T. Namba, J. M. Newman and S. Yoskioka, *Lab. Equip. Digest*, January (1976) 14.
12 E. Gantois, F. Mordret and N. Le Barbanchon, *Rev. Fr. Corps Gras*, 24 (1977) 167.
13 J. C. Sipos and R. G. Ackman, *J. Chromatogr. Sci.*, 16 (1978) 443.
14 W. W. Christie and M. L. Hunter, *J. Chromatogr.*, 171 (1979) 517.
15 M. S. J. Dallas and F. B. Padley, *Lebens. Wiss. Technol.*, 10 (1977) 328.
16 E. W. Hammond and D. J. Walker, in preparation.
17 M. J. Saxby, in R. D. King (Editor), *Developments in Food Analysis Techniques*, Vol. 1, Applied Science Publ., London, 1978, p. 125.
18 K. Aitzetmüller, *J. Chromatogr.*, 113 (1975) 231.
19 N. A. Porter, R. A. Wolf and J. R. Nixon, *Lipids*, 14 (1979) 20.
20 C. R. Scholfield, *J. Amer. Oil Chem. Soc.*, 56 (1979) 510.
21 E. C. Smith, A. D. Jones and E. W. Hammond, *J. Chromatogr.*, 188 (1980) 205.
22 M. Özcimder and W. E. Hammers, *J. Chromatogr.*, 187 (1980) 307.
23 C. R. Scholfield and T. L. Mounts, *J. Amer. Oil Chem. Soc.*, 54 (1977) 319.
24 H. W. S. Chan and G. Levett, *Chem. Ind. (London)*, (1978) 578.
25 R. R. Health, J. H. Tumlinson and R. E. Doolittle, *J. Chromatogr. Sci.*, 15 (1977) 10.
26 B. Vonach and G. Schomburg, *J. Chromatogr.*, 149 (1978) 417.
27 B. Jeffrey, personal communication.
28 E. W. Hammond and G. Jones, unpublished results.