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Note

Fatty acids

XIX*. A quantitative treatment of saturated triglycerides by reversed-phase high-performance liquid chromatography

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Plattner *et al.*[2,3] reported the separation of triglycerides by high-performance liquid chromatography (HPLC) with reversed-phase packings containing octadecylsilane groups chemically bonded to a permanent stationary support[4,5]. Scholfield[6] also studied the behaviour of series of methyl esters of long chain fatty acids by this technique, and on both occasions a linear relationship was reported for the plots of the carbon numbers (obtained by addition of the number of carbon atoms in the acyl chains of the triglyceride, thus the carbon number for trilaurin, trimyristin and tripalmitin is 36, 42 and 48 respectively) *versus* the logarithmic value of the retention volume (void volume excluded) of these homologues. This linear relationship was reconfirmed by our studies, but no report has yet indicated the precise relationship between peak height or peak area with the actual or absolute quantity (mass) of triglyceride sample under investigation. This report emphasizes the quantitative aspects of the reversed-phase HPLC analysis of triglycerides using a differential refractometer as the detection system.

EXPERIMENTAL

Chromatographic procedures

Chromatographic runs were performed with a Waters Assoc. liquid chromatograph equipped with a 30 × 0.4 cm I.D. stainless-steel μBondapak C_{18} column. Samples were injected as toluene solutions by means of a U6K septumless loop injector. A Waters R401 differential refractometer was used as the detector. Samples were run isocratically using a mixture of acetonitrile–acetone (2:1) at ambient temperature. A flow-rate of 2.0 ml/min was used. The retention volume was measured from the midpoint of the solvent peak to the maximum of the recorded peak for each of the eluted components.

Triglyceride standards

Saturated triglyceride standards of carbon numbers 28, 30, 32, 34 and 36 were

* For part XVIII, see ref. 1.

synthesized by well-established methods[7], *viz.*, either by the reaction of acyl chlorides of appropriate fatty acids with glycerol in the case of tridecanoin and trilaurin, or by esterification of monoglycerides with acyl chlorides of fatty acids to give the required mixed triglyceride molecules. Trimyristin was isolated from nutmeg and purified by repeated recrystallization from acetone[8,9].

Triglyceride standards used in this study are listed in Table I together with their melting points. Coconut oil was extracted from the endocarp of the coconut with light petroleum (b.p. 60–80°). Solutions of known (*ca.* 2%) concentrations of triglycerides in toluene were prepared and a 25-μl microsyringe was used to inject volumes of 5, 10, 15, 20 and 25 μl for HPLC analysis.

TABLE I

SYNTHETIC TRIGLYCERIDES USED FOR HPLC ANALYSIS AND THEIR MELTING POINTS

$CH_2OCO(CH_2)_xCH_3$
|
$CH–OCO(CH_2)_yCH_3$
|
$CH_2OCO(CH_2)_zCH_3$

Carbon No.	*Triglyceride*			*Melting point (°C) (uncorrected)*
	x	*y*	*z*	
28	6	6	10	27.0
30	8	8	8	31.5
32	8	8	10	36.5
34	8	8	12	41.0
36	10	10	10	46.5
42*	12	12	12	54.0

* From nutmeg.

RESULTS AND DISCUSSION

The initial exercise was to determine the response of the differential refractometer towards different quantities of two triglyceride molecules of differing molecular weights, and to compare the peak height and peak area ratios of the eluted components. Fig. 1 shows the plots of trilaurin and trimyristin concentration *versus* height and area of the corresponding peaks. Trilaurin exhibited a greater peak height as well as a larger peak area by this refractive index detection method than that observed for trimyristin for the same quantity (mass) of triglyceride injected. It was therefore assumed that triglycerides of increasing molecular weights tend to lower the peak height and area response due to their increasing retention volumes.

Accurate known amounts of triglycerides were subsequently analyzed and calibration curves obtained from the results of five injections for each triglyceride sample. Figs. 2 and 3 present curves relating concentration of triglyceride sample with peak height and peak area respectively. All plots were acceptably linear. Furthermore the peak height response was largest for triglyceride molecules with the lowest

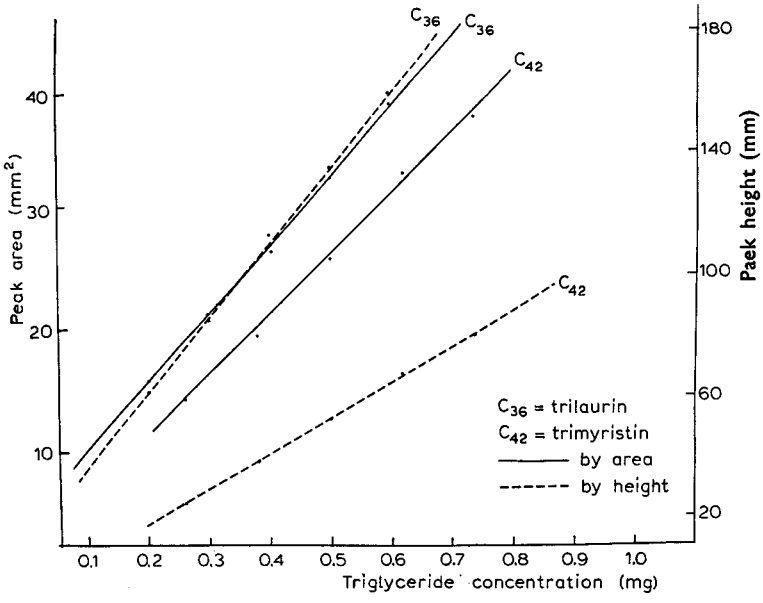

Fig. 1. Detector responses (peak height and peak area) of trilaurin and trimyristin on a μBondapak C_{18} column. Eluent: acetonitrile–acetone (2:1); flow-rate, 2 ml/min. Attenuation, $\times 4$.

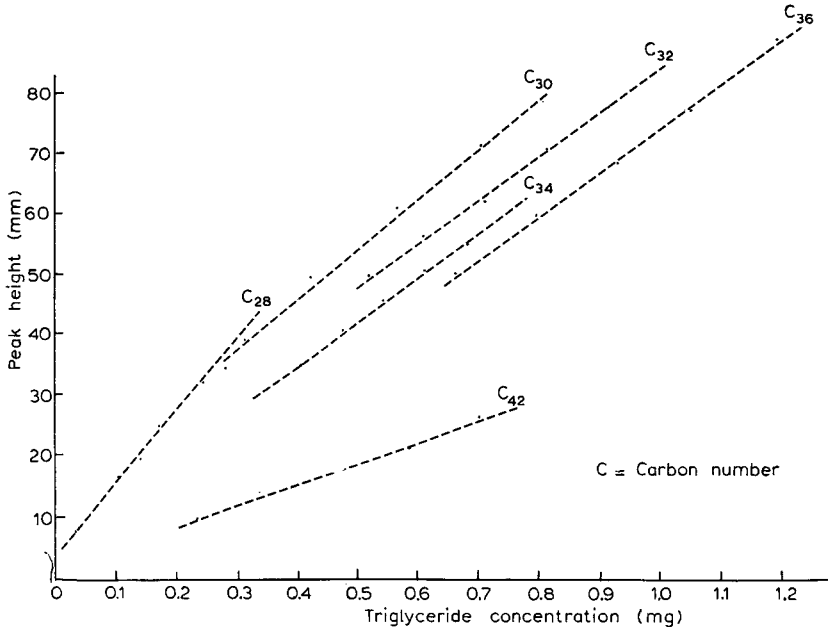

Fig. 2. Calibration curves of triglyceride concentration *versus* peak height for components of various carbon numbers. Conditions as in Fig. 1.

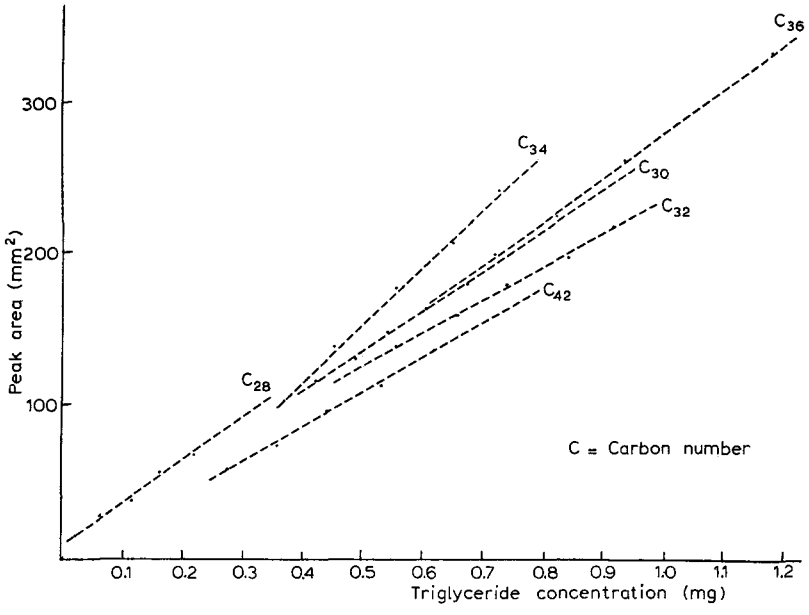

Fig. 3. Calibration curves of triglyceride concentration *versus* peak area for components of various carbon numbers. Conditions as in Fig. 1.

carbon number, and smallest for triglycerides with the highest carbon number under investigation. This trend thus substantiates the observed response behaviour of trilaurin and trimyristin. These results infer that peak height is an acceptable linear function for the quantification of triglycerides, provided that suitable reference standards are available in view of the differing detector responses observed.

On the other hand, although the calibration curves obtained by peak area measurements (Fig. 3) were also linear, an erratic detector response was observed. The triglyceride of carbon number 34 furnished the highest area response, while that of carbon number 28 gave a higher response value than that of 36, which in turn showed a higher response value than the triglyceride of carbon number 30.

It is therefore possible to conclude from these results that the measurements of the peak heights or peak areas of the eluted components may be confidently used to determine the quantity of triglyceride molecules using the calibration plots obtained for an identical or reference standard of the same carbon number. It must be stressed that a single internal or external reference standard does not suffice for quantitative work on a mixture of triglycerides.

In application of these results, the triglyceride composition of six components in coconut oil was reexamined. Using the available calibration curves for the triglycerides of carbon numbers 28, 30, 32, 34, 36 and 42, the weight percentages of these components in coconut oil were determined by peak height and peak area measurements of the corresponding triglyceride components. These results were compared with the gas–liquid chromatographic (GLC) data on the composition of coconut oil previously reported by Bezard and Bugaut[10] and Kuksis *et al.*[11] (Table II). A very good relationship exists between the values obtained by peak height measurements of

TABLE II

WEIGHT PERCENTAGES OF SIX GROUPS OF TRIGLYCERIDE COMPONENTS IN COCONUT OIL BY HPLC ANALYSIS

Group	*Carbon No.*	*HPLC (wt.%)*		*GLC*	
		Peak height	*Peak area*	*wt.% (ref. 10)*	*mole % (ref. 11)*
1	28	1.2	1.5	0.9	0.6
2	30	3.4	3.7	3.6	3.1
3	32	14.5	15.5	14.3	13.2
4	34	17.1	15.0	17.9	17.9
5	36	19.8	19.6	20.7	20.8
6	42	7.5	10.4	7.2	7.0

the six components by HPLC analysis. Weight percentages obtained by peak area measurements were less reliable as the separation of most of these components on HPLC were not baseline separations, making triangulation of the peaks for measurement a difficult und unreliable task.

Assuming that the GLC data reported by other workers[10,11] were a correct reflection of the triglyceride composition of coconut oil and that GLC and HPLC separations of triglyceride molecules into groups with similar carbon numbers were identical, then the height measurements of peaks in an HPLC chromatogram (using appropriate external standards) could provide a very accurate quantitative measurement of the triglyceride component under investigation. The only drawback would be the provision of an array of triglyceride standards. The use of coconut oil as a set of reference standards for medium sized triglycerides is recommended as the GLC and HPLC data on its composition are quite similar.

Using the present solvent system (acetonitrile–acetone, 2:1) it has been found that triglyceride molecules of carbon numbers higher than 46 are difficult to elute and tend to crystallize out in the column. It is therefore possible to infer that some "loss or retention" of material on the column occurs during HPLC analysis of molecules of low solubility in the mobile phase. This phenomenon will certainly affect the detector response for such compounds, such as the analysis of tristearin under the above conditions.

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REFERENCES

1 M. S. F. Lie Ken Jie and G. Tsang, *J. Chromatogr.*, 138 (1977) 446.
2 R. D. Plattner, G. F. Spencer and R. Kleiman, *J. Amer. Oil Chem. Soc.*, 54 (1977) 511.
3 R. D. Plattner, K. Wade and R. Kleiman, *J. Amer. Oil Chem. Soc.*, 55 (1977) 381.
4 H. D. Durst, M. Milano, E. J. Kikta, S. A. Connely and E. Grushka, *Anal. Chem.*, 47 (1975) 1797.
5 R. F. Borch, *Anal. Chem.*, 47 (1975) 2437.

6 C. R. Scholfield, *J. Amer. Oil Chem. Soc.*, 52 (1975) 36.
7 K. S. Markley, *Fatty Acids—Their Chemistry, Properties, Production and Uses, Part 2*, Interscience, New York, 1961, pp. 797–826.
8 G. D. Beal, *Org. Syn.*, Coll. Vol. 1 (1941) 538.
9 T. P. Hilditch and P. N. Williams, *The Chemical Constitution of Natural Fats*, Chapman and Hall, London, 4th ed., 1964.
10 J. Bezard and M. Bugaut, *J. Chromatogr. Sci.*, 7 (1969) 639.
11 A. Kuksis, M. J. McCarthy and J. M. R. Beveridge, *J. Amer. Oil Chem. Soc.*, 41 (1964) 201.