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SIMPLE SEPARATION OF PRODUCTS OF GLYCEROLYSIS BY LOW-PRESSURE COLUMN CHROMATOGRAPHY

LAJOS BALOGH
Institute of Applied Chemistry, Kossuth Lajos University, P.O. Box 10, H-4010 Debrecen (Hungary)

SUMMARY

A method and apparatus are described for the separation of non-polar lipids into classes by means of low-pressure column chromatography. Baseline separations were achieved on a Lobar® Si-60 glass column. The components were eluted isocratically with methanol–chloroform mixtures and monitored by infrared absorption at 1750 cm^{-1}. The evaluation was carried out by area ratio measurement. This simple method is applicable for routine analysis of mono-, di- and triglycerides and free fatty acids without any preliminary esterification.

INTRODUCTION

The final products of glycerolysis contain various amounts of saturated and unsaturated tri-, di- and monoglyceride isomers, free glycerol and free fatty acids depending on the method of preparation and subsequent concentration treatment. The determination of the free glycerol content is easy by the method of quantitative oxidation[1], but in the case when the glycerolysis has been carried out in the present of an excess of glycerol, the change in the concentration of glycerol is insignificant.

The cheap classical column chromatographic methods separate lipid classes but are time-consuming[2,3]. The separations were carried out most successfully by dual[4] or single[5] gradient high-performance liquid chromatographic (HPLC) systems, but the equipment is rather expensive and may therefore be inaccessible to some workers.

I have now developed a simple low-pressure LC apparatus working in the isocratic mode and which utilizes infrared (IR) detection. This apparatus combines the advantages of the above two analytical procedures and gives satisfactory results for the quantitation of glyceride classes and free fatty acids.

EXPERIMENTAL

LC apparatus

A diagram of the apparatus is shown in Fig. 1. The eluent was delivered to the column (7) by a DV-96 (Labor MIM, Hungary) metering pump (3) equipped with a home-made pneumatic pulse-dampening device (5). A six-port injection valve (6)

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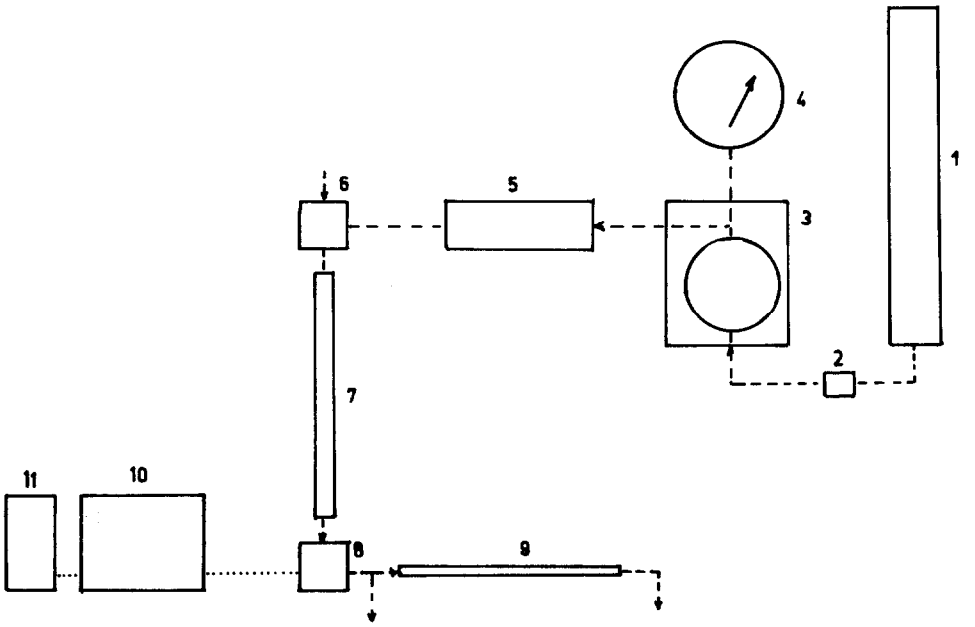

Fig. 1. Diagram of the apparatus: 1 = solvent reservoir; 2 = bubble-trap; 3 = metering pump; 4 = pressure control; 5 = pulse-dampening device; 6 = injection valve; 7 = column; 8 = detector; 9 = bubble-timer; 10 = recorder; 11 = integrator.

was connected to the top of the column. The loop volumes were 250 μl for analytical and 2.5 cm^3 for preparative chromatography. The separations were achieved on Lobar® glass columns, purchased from Merck. The eluent leaving the column passed through a 20-μl flow cell and a bubble-timer (9). The detector (8) was a slightly modified IR spectrophotometer (Spectromom Type 2000; MOM, Hungary; wavenumber accuracy, ±5 cm^{-1}, reproducibility, 1% transmission): the shaft of the phase-sensitive motor and a helical potentiometer which was fed by stabilized direct current had been coupled. This photometer can work as a variable-wavelength IR detector. The chromatograms were recorded by a OH-814/1 recorder (10) (Radelkis, Hungary) and integrated by an EDI 71 integrator (11) and an area report was printed.

Preparation of monoglyceride samples

The change in composition of glycerides during glycerolysis in pyridine[6] was studied. It was found that the crude products generally comprised only diglycerides (DGs), monoglycerides (MGs), free fatty acids (FFAs) and free glycerol and that the triglyceride (TG) content was negligible. However, triglycerides were present in the samples coming from earlier stages of the reaction.

The samples were stirred with an equal volume of dichloromethane, washed by dilute HCl and distilled water to remove pyridine and unreacted glycerol and then dried over anhydrous sodium sulphate. The products contained 0.05–0.20 g/cm^3 mixed glycerides and free fatty acids.

The LC method

The conditions for the analytical separations were as follows: column, 250 × 10 mm I.D., 40–63 μm LiChroprep® Si 60; solvent flow-rate, 3.0 cm^3/min; maximum column inlet pressure, < 2.0 bar. The conditions for the preparative runs were: column, 310 × 25 mm I.D., 40–63 μm LiChroprep Si 60; solvent flow-rate, 6.0 cm^3/min; maximum column inlet pressure, < 12.0 bar. To separate triglycerides plus diglycerides, free fatty acids and monoglycerides methanol–chloroform (2.5:97.5), 50% water-saturated, was used, when the 2-MGs which could be eluted had large retention volumes and isomerized on the adsorbents[3]. For the separation of TGs, 1,3-DGs and 1,2-DGs, methanol–chloroform (0.4:99.6), 50% water-saturated, was applied. The solvents were purified by the usual procedures.

The components were monitored by IR absorption[7] at 1750 cm^{-1} where chloroform and methanol have virtually no absorbance.

RESULTS AND DISCUSSION

Fig. 2 illustrates that the apparatus was able to separate isocratically the glycerides listed in Table I. The band of free fatty acids exhibited tailing but was not overlapped by either DGs or MGs; thus the previous esterification of the FAs was

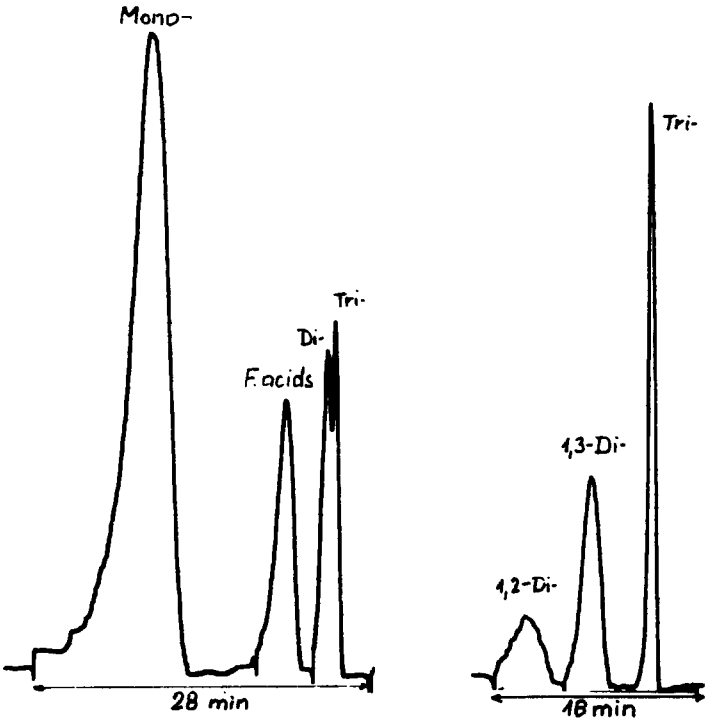

Fig. 2. Separations of glyceride mixtures. Left: methanol–chloroform (2.5:97.5); right: methanol–chloroform (0.4:99.6). Mono-, Di-, 1,2-Di, 1,3-Di- and Tri- = monoglycerides, diglycerides, 1,2-diglycerides, 1,3-diglycerides and triglycerides, respectively; F-acids = free fatty acids.

TABLE I

RETENTION VOLUMES (V_R) AND CAPACITY RATIOS (k')

Eluent	*Compound*	V_R *(cm³)*	k'
Methanol–chloroform (2.5:97.5)	TG	8.9	0.11
	DG	10.9	0.36
(50% water-saturated)	FA	18.5	1.31
	MG	56.7	6.08
Methanol–chloroform (0.4:99.6)	TG	9.0	0.12
	1,3-DG	20.9	1.61
(50% water-saturated)	1,2-DG	46.6	3.57

not necessary. The evaluation of chromatograms was performed by means of area ratio measurements. Because randomization takes place during the reaction[8], one can calculate and use the average molecular weights if the fatty acids composition is known. The fatty acid composition of the soy bean oil employed here was determined by gas chromatography[9] after a preparative analysis of some final products.

The following expressions were applied

$$W_i = A_i \times \mathrm{MW}_i \times \frac{1}{f_i}$$

where W_i = weight of the individual component TG, DG, FFA and MG, A_i = area of the corresponding peak, MW_i = average molecular weight (for TGs: 870.5, DGs: 611.0, MGs: 351.5, FFA: 277.5) and f_i = response factor (3,2,1,1), and

$$W_i / \sum_{i=\mathrm{TG}}^{\mathrm{MG}} W_i = g_i \text{ (in \%, w/w)}$$

Since the detection was based on similar absorbances due to fatty acid carbonyl groups, the molar response factors had to be taken into account; in the present approximation these were the theoretical ones.

TABLE II

REPRODUCIBILITY

"Soy bean diglyceride", "fatty acid" and "monoglyceride" were obtained in a preparative analysis. Composition of the standard sample: 16% DG; 16% FFA; 68% MG. The values quoted are the means from three determinations.

Concentration of the sample (mg/cm³)	*"Diglyceride" (%)*	*"Fatty acid" (%)*	*"Monoglyceride" (%)*
150.16	16.4 ± 2.1	16.9 ± 1.9	66.7 ± 0.98
99.63	16.7 ± 2.3	15.4 ± 4.0	67.9 ± 0.40
49.80	16.6 ± 11.7	14.2 ± 7.0	69.2 ± 1.30

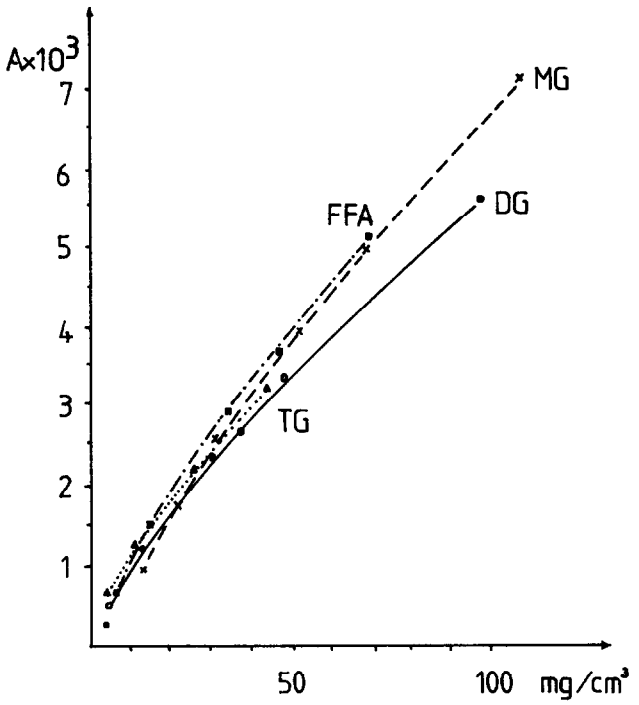

Fig. 3. Absolute calibration curves. Plots of peak area *versus* concentration of component.

To determine whether the apparatus and method were as applicable as was originally hoped, the reproducibility and accuracy were investigated. The same sample was injected at several concentrations (Table II). It was found that the reproducibility of peak areas decreased with decreasing sample size and that the detection limit depended on the extent of peak broadening. Consequently, there are various optimal ranges for detection and quantitation of the individual components of the reaction product.

TABLE III

COMPARISON OF RESULTS

Reaction No.	*Weight per cent on the basis of*					
	Absolute calibration curves			*Area ratio measurement*		
	DG	*FA*	*MG*	*DG*	*FA*	*MG*
827	6.4	20.6	73.0	7.1	19.5	73.4
828	9.6	23.8	66.6	9.7	22.1	68.2
841	4.9	20.7	74.4	5.5	19.2	75.2
856	8.4	6.3	85.3	8.3	6.9	84.7
858	9.0	13.0	78.0	8.3	12.9	78.8

Since the use of an internal standard method was not possible because this column had too few theoretical plates (about 250), an absolute calibration was carried out (Fig. 3). The results obtained from the two kinds of evaluations were in good agreement (Table III). This is remarkable, because technical grade monoglycerides may contain unknown amounts of water and glycerol, and it is difficult to weigh a sample exactly, although this would be necessary for using the absolute calibration method. However, if triglycerides are present then a second analysis would be needed.

In conclusion, it has been established that this cheap apparatus and simple method are applicable to routine analysis of mixtures of tri-, di- and monoglycerides and free fatty acids.

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