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RAPID ANALYSIS OF TRIACYLGLYCEROLS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH LIGHT SCATTERING DETECTION

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SUMMARY

This paper details a high-performance liquid chromatography (HPLC) method for the separation of triacylglycerols, using a 3-μm, 15 cm × 4.6 mm I.D. Spherisorb ODS column and gradient elution with dichloromethane and acetonitrile. The triacylglycerols are detected using a light scattering detector (mass detector). Separations of a number of different edible oils and fats are reported. The procedure offers a possible method for determining cocoa butter equivalents and the adulteration of edible oils and fats by other non-generic fats and oils.

INTRODUCTION

Since Pei *et al.*[1] applied reversed-phase high-performance liquid chromatography (HPLC) to the separation of triacylglycerols a number of reversed-phase systems have been developed as rapid and efficient resolution of complex triacylglycerol mixtures can be achieved. El-Hamdy and Perkins[2] and Plattner[3] evaluated several packing materials and concluded that reversed-phase C_{18} columns of small particle size gave the most efficient separations. Most authors[4,5] have used 5 μm, 25 cm columns, often two in series, to obtain detailed triacylglycerol profiles.

A number of papers [4,6–8] have detailed isocratic systems combined with refractive index (RI) detectors, often with acetonitrile–acetone mobile phases. Such solvent systems continued to be used even though the lack of solubility of triacylglycerols with carbon numbers greater than 46 in this mobile phase has been noted[9]. Solvent gradients which would be required for optimum separations of complex triacylglycerol mixtures are not compatible with RI detection. Ultraviolet (UV) detectors have also been used[10–12] but the range of mobile phases is limited since triacylglycerols absorb only in the far UV range.

Detectors compatible with gradient elution systems have been used. Mass spectrometric (MS) detection has been used with solvent gradients of acetone–acetonitrile and propionitrile–acetonitrile[13,14]. Phillips *et al.*[5,15] used their own design flame ion-

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isation detection (FID) system with solvent gradients of methylene chloride and acetonitrile. Different gradients were used for different oils. More recently Herslöf and Kindmark[16] used a light scattering detector with gradients of acetonitrile–ethanol–hexane. Both of these systems produced efficient separations of complex triacylglycerol mixtures but retention times were long. There has been no report of one gradient system that can be used to separate all known natural triacylglycerol mixtures regardless of their carbon number distribution.

This work was carried out to separate triacylglycerols by HPLC using a 3-μm, 15 cm × 4.6 mm I.D. reversed-phase column and light scattering detection. A rapid separation using a single gradient system was sought to enable the method to be adopted for routine analytical work, for identification of oils and possible detection of adulteration.

MATERIALS AND METHODS

Analysis of triacylglycerols using HPLC

The HPLC system consisted of a Spectra Physics SP8700 extended range pump with a Spherisorb ODS-2 column, 150 × 4.6 mm I.D., 3 μm particle size and 12% carbon loading, obtained from Phase Separations (Queensferry, U.K.). Chromatography was carried out using a stepped gradient elution system of 30–70% dichloromethane in acetonitrile over 60 min, with 45% dichloromethane after 20 min and 70% after 60 min. The flow-rate was 0.6 ml/min. The detector used was an Applied Chromatograhpy Systems mass detector and peak areas were measured using a Spectra Physics SP4270 integrator.

Standard triacylglycerol and sample preparation

Triacylglycerol standards were stated as 99% pure (carbon numbers and units of unsaturation are indicated in brackets): tricaprylin (24:0), trinonanoin (27:0), tricaprin (30:0), trilaurin (36:0), trimyristin (42:0), tripalmitin (48:0), tristearin (54:0), triolein (54:3), trilinolenin (54:9) and trinonadecanoin (57:0) were obtained from Sigma Chemical (Poole, U.K.), triundecanoin (33:0) from Fluka (Buchs, Switzerland), *rac*-glycerol-1,3-palmitate-2-oleate (50:1) from PL Biochemicals (WI, U.S.A.) and *rac*-glycerol-1-palmitate-2-oleate-3-stearate (52:1) and *rac*-glycerol-1,3-stearate-2-oleate (54:1) from Supelco (Bellefonte, PA, U.S.A.).

Vegetable oils were obtained from consignments imported into the U.K. Samples of cocoa butter were obtained from British Cocoa Mills (Hull, U.K.) and a cocoa butter equivalent was obtained from Loders and Nucoline plc. Solutions of 0.05% (w/v) of each standard triacylglycerol and 1% (w/v) oil and fat samples in dichloromethane–acetonitrile (1:1) were filtered through a 0.45-μm filter and injected onto the HPLC system using a 20-μl injection loop.

Argentation TLC

Argentation TLC was carried out according to the IOCCC Method No. 111/B-1985[17], with the following amendment: 20 × 20 cm glass plates coated with 0.25 mm silica gel were dipped in a 10% aqueous solution of silver nitrate and dried at 100°C in the dark.

Capillary gas chromatography (GC)

Fatty acid methyl esters were prepared by the IUPAC Method No. 2.301[18]. Capillary GC was carried out using a Carlo Erba Vega gas chromatograph fitted with a 50 m × 0.32 mm I.D. WCOT Silar-5 column and a flame ionisation detector.

RESULTS AND DISCUSSION

A rapid method for separating a standard mixture of triacylglycerols by HPLC, based on the procedure of Phillips *et al.*[15], was devised. Resolution of the standard triacylglycerols was achieved with a linear gradient of 30–70% dichloromethane in acetonitrile with a flow-rate of 0.6 ml/min but optimum resolution of the complex triacylglycerol mixtures present in natural oils and fats requires a step in this gradient. Using the stepped gradient the resolution and retention times of these standards were found to be reproducible. The method was then applied to a range of edible oils and fats. Trinonadecanoin was used, at a level of 0.01%, as an internal standard. This compound was chosen as it is not found naturally in the oils and it elutes after all the other triacylglycerols. Since trinonadecanoin had been subjected to all the conditions of the gradient, reproducible relative retention times for each triacylglycerol were obtained. Figs. 1–3 show examples of triacylglycerol profiles obtained by this method. Triacylglycerols elute from this system in order of increasing carbon number. The presence of double bounds reduces the retention time of the triacylglycerol, *e.g.* , triolein elutes before tristearin and trilinolein before triolein.

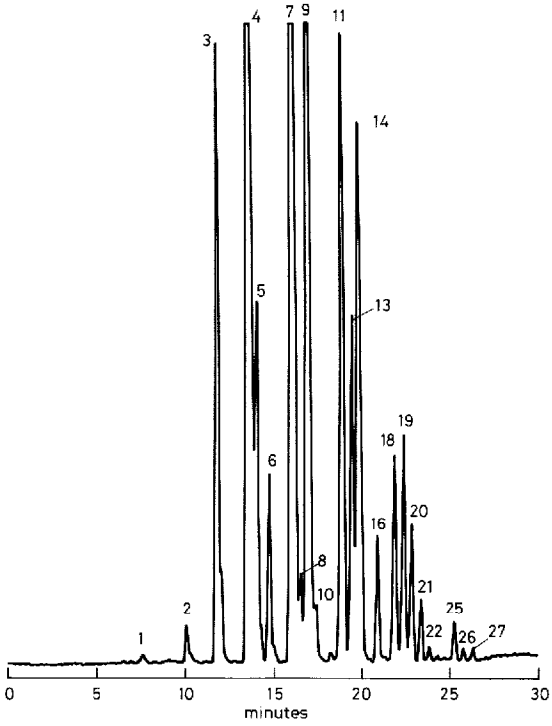

Fig. 1. Triacylglycerol profile of soyabean oil. For peak identification see Table I.

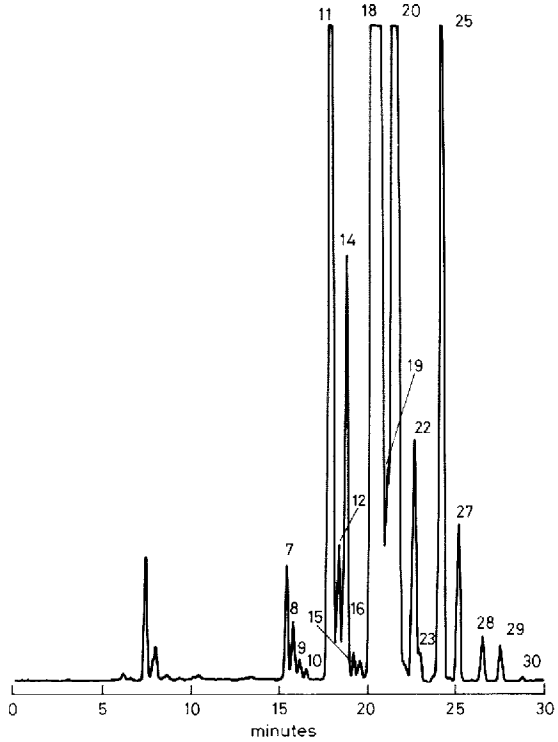

Fig. 2. Triacylglycerol profile of olive oil. For peak identification see Table I.

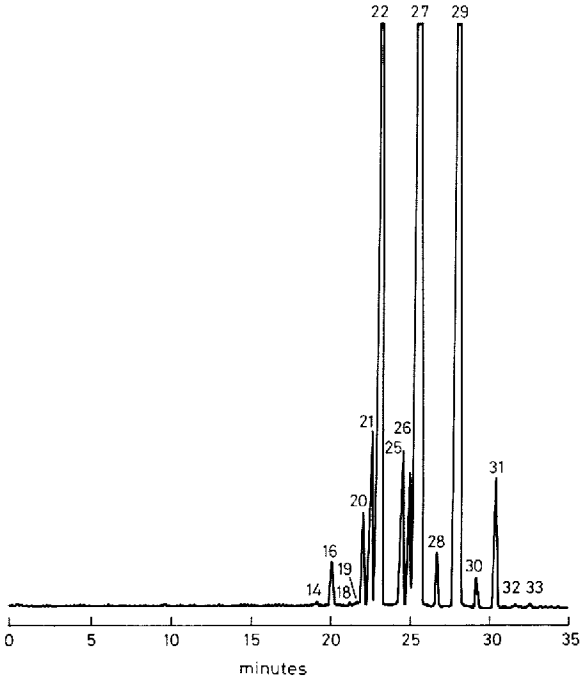

Fig. 3. Triacylglycerol profile of cocoa butter. For peak identification see Table I.

The triacylglycerol profile of cocoa butter (Fig. 3) shows sixteen triacylglycerols being separated. Cocoa butter is known to consist of three major triacylglycerols with carbon numbers 50, 52 and 54. Standards of glycerol-1,3-palmitate-2-oleate (POP) (50), glycerol-1-palmitate-2-oleate-3-stearate (POS) (52) and glycerol-1,3-stearate-2-oleate (SOS) (54) were chromatographed separately under the same conditions in order to identify the major peaks by comparison of relative retention times. As this method does not differentiate positional isomers such as glycerol-1,3-palmitate-2-oleate and glycerol-1,2-palmitate-3-oleate, the position of unsaturation in the cocoa butter triacylglycerols was confirmed to be in the "2" position using argentation TLC[17].

This information was initally used for identifying the triacylglycerol peaks along with the triacylglycerol labels used by Phillips *et al.*[5]. For confirmation, the eluent containing each component triacylglycerol was collected from the HPLC system using a stream splitter. Capillary GC of the fatty acid methyl esters prepared from each of the collected fractions was used to confirm their identity (*e.g.*, methyl palmitate and methyl oleate in a ratio of 2:1 are obtained from the POP fraction of cocoa butter). The peaks of the triacylglycerol profiles (Figs. 1–6) are numbered, the peak identification is shown in Table I. The early eluting components in Figs. 2 and 4–6 are diacylglycerols.

In earlier work[19] the author has looked at the natural variation within six cocoa butters of different origins. Normalising the percentages of the three major cocoa butter peaks (POP, POS, SOS) gives a very similar composition for all the cocoa butters analysed, the range from the mean percentage being 1.4 for POP, 1.7 for POS

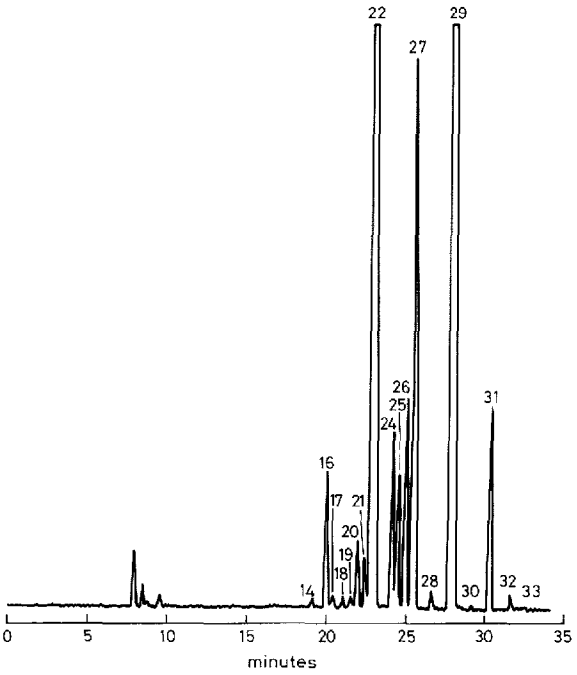

Fig. 4. Triacylglycerol profile of cocoa butter equivalent. For peak identification see Table I.

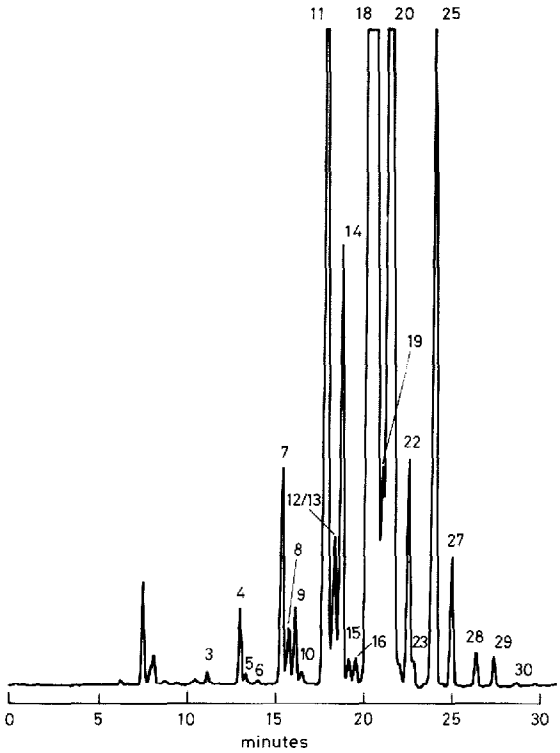

Fig. 5. Triacylglycerol profile of 5% soyabean oil in olive oil.

and 1.1 for SOS. The triacylglycerol composition of a cocoa butter equivalent (Fig. 4) shows a marked difference from that of cocoa butter. This would probably enable the addition of 10% of this cocoa butter equivalent to be easily detected. Using the triacylglycerol composition for detecting this cocoa butter equivalent offers an alternative to using fatty acid profiles which are often used for the identification of oils and fats. There are no significant differences between the fatty acid profiles of this cocoa butter equivalent and cocoa butter[19].

The triacylglycerol profiles for soyabean oil (Fig. 1) and olive oil (Fig. 2) are significantly different. Olive oil contains no triacylglycerols eluting before LLO whereas soyabean oil contains a large proportion of triacylglycerols eluting before LLO. This difference could be used to detect the addition of low levels of soyabean oil in olive oil. Figs. 5 and 6 illustrate the effect of blending 5 and 10% of soyabean oil with olive oil. The changes in the composition of olive oil with the addition of soyabean oil are detailed in Table II. Soyabean oil has been used to illustrate the possibilities for the method to detect adulteration of olive oil. Any oil containing a significant percentage of early eluting triacylglycerols, *e.g.*, zero erucic rapeseed oil could be detected in the same manner.

Detailed work on the natural variation in triacylglycerol composition of a range of oils is required before very low levels of adulteration could be accurately assessed.

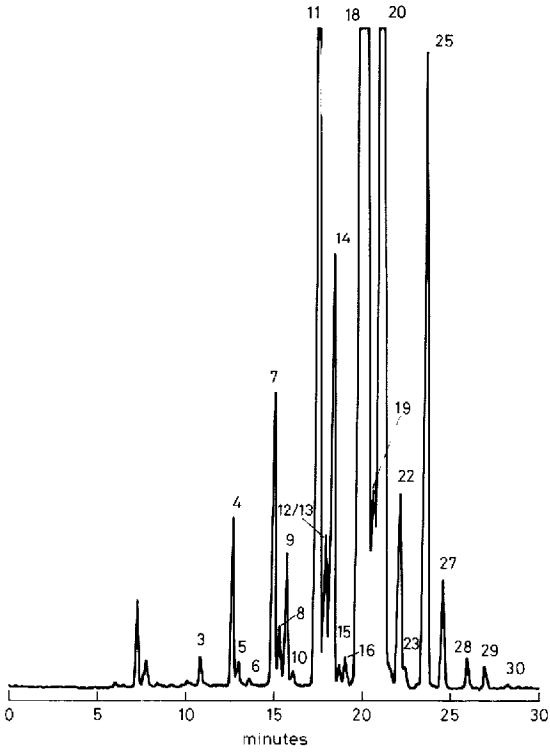

Fig. 6. Triacylglycerol profile of 10% soyabean oil in olive oil.

TABLE I

IDENTIFICATION OF TRIACYLGLYCEROL PEAKS FOR FIGS. 1–6

P = palmitic acid, Pa = palmitoleic acid, S = stearic acid, O = oleic acid, L = linoleic acid, Ln = linolenic acid, A = arachidic acid.

Peak No.	*Triacylglycerol*	*Peak No.*	*Triacylglycerol*
1	LnLnLn	18	OOO
2	LLnLn	19	OLS
3	LLnL	20	OOP
4	LLL	21	PLS
5	LLnO	22	POP
6	LLnP	23	PPaS
7	LLO	24	PPP
8	OLnO	25	OOS
9	LLP	26	SLS
10	OLnP	27	POS
11	OLO	28	PPS
12	OPaO	29	SOS
13	LLS	30	SPS
14	OLP	31	SOA
15	OPaP	32	SSS
16	PLP	33	SSA
17	SLnP		

TABLE II

TRIACYLGLYCEROL COMPOSITIONS SHOWING THE CHANGE IN OLIVE OIL COMPOSITION WITH THE ADDITION OF SOYABEAN OIL

Each composition is the mean of 3 determinations.

Peak No.	*Triacylglycerol*	*Triacylglycerol composition*			
		100% Olive oil	*5% Soya in olive oil*	*10% Soya in olive oil*	*100% Soyabean oil*
1	LnLnLn	–	–	–	0.1
2	LLnLn	–	–	–	0.4
3	LLnL	–	0.1	0.2	7.3
4	LLL	–	0.5	1.3	18.9
5	LLnO	–	0.1	0.2	5.4
6	LLnP	–	0.1	0.1	2.1
7	LLO	0.8	1.8	2.6	18.8
8	OLnO	0.5	0.5	0.6	1.0
9	LLP	0.2	0.6	1.2	14.2
10	OLnP	0.1	0.2	0.1	0.7
11	OLO	11.0	10.8	10.9	9.0
12	OPaO	1.2	1.4[a]	1.5[a]	–
13	LLS	–			3.8
14	OLP	4.4	4.8	4.8	9.2
15	OPaP	0.2	0.2	0.2	–
16	PLP	0.2	0.3	0.3	1.3
18	OOO	41.1	39.8	39.0	2.2
19	OLS	1.6	1.8	1.4	2.6
20	OOP	25.2	24.6	24.1	1.6
21	PLS	–	–	–	0.7
22	POP	2.6	2.5	2.2	0.1
23	PPaS	0.2	0.2	0.2	–
25	OOS	8.6	8.0	7.5	0.4
26	SLS	–	–	–	0.1
27	POS	1.3	1.1	1.0	0.1
28	PPS	0.4	0.3	0.3	–
29	SOS	0.3	0.2	0.2	–
30	SPS	0.1	0.1	0.1	–

[a] Combination of OPaO and LLS.

It should be noted that the detector does not respond equally for all triacylglycerols and is not quantitative[20]. Therefore the compositions quoted in this paper are not absolute compositions. However reproducible responses were obtained for this work so the compositions can be used for comparative purposes.

CONCLUSIONS

The method described is a rapid method utilising a simple solvent regime. The component triacylglycerols of a range of oils and fats are separated within 35 min. The method is reproducible and capable of providing detailed information on the triacylglycerol composition of oils and fats containing triacylglycerols of carbon numbers 12 to 57.

The short 3-μm, 15 cm column used gives rapid separations with no loss in resolution of individual triacylglycerols when compared to the separations of Phillips *et al.*[5], where two 5-μm, 25 cm columns in series were used giving 80-min elution times.

The method offers the possibility of detecting and determining adulteration of oils and fats, particularly in cases where the triacylglycerol profiles of two oils show greater differences than their fatty acid profiles.

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REFERENCES

1 P. Pei, R. Henly and S. Ramachandran, *Lipids*, 10 (1975) 152.
2 A. H. El-Hamdy and E. G. Perkins, *J. Am. Oil Chem. Soc.*, 58 (1981) 49.
3 R. D. Plattner, *J. Am. Oil Chem. Soc.*, 58 (1981) 638.
4 G. W. Jensen, *J. Chromatogr.*, 204 (1981) 407.
5 F. C. Phillips, W. L. Erdahl, J. A. Schmitt and O. S. Privett, *Lipids*, 19 (1984) 880.
6 B. Herslöf, O. Podlaha and B. Töregård, *J. Am. Oil Chem. Soc.*, 56 (1979) 864.
7 B. Petersson, O. Podlaha and B. Töregård, *J. Am. Oil Chem. Soc.*, 58 (1981) 1005.
8 R. D. Plattner, G. F. Spencer and R. Kleimann, *J. Am. Oil Chem. Soc.*, 54 (1978) 511.
9 M. S. F. Lie Ken Jie, *J. Chromatogr.*, 192 (1980) 457.
10 W. P. Edwards, *J. Liq. Chromatogr.*, 8 (1985) 2625.
11 B. Herslöf, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 4 (1981) 471.
12 V. K. S. Shukla, W. Nielsen and W. Batsburg, *Fette, Siefen, Anstrichm.*, 85 (1983) 274.
13 L. Marai, J. J. Myher and A. Kuksis, *Can. J. Biochem. Cell Biol.*, 61 (1983) 840.
14 A. Kuksis, L. Marai and J. J. Myher, *J. Chromatogr.*, 273 (1983) 43.
15 F. C. Phillips, W. L. Erdahl, J. D. Nadenicek, L. J. Nutter, J. A. Schmitt and O. S. Privett, *Lipids*, 19 (1984) 142.
16 B. Herslöf and G. Kindmark, *Lipids*, 20 (1985) 783.
17 *Methods of Analysis of the International Office of Cocoa, Chocolate and Sugar Confectionery.*
18 *IUPAC, Standard Methods for the Analysis of Oils, Fats and Derivatives*, Pergamon Press, Oxford, 6th ed., 1979.
19 F. J. Lovelace (married now Palmer), *Undergraduate Project for Council for National Academic Awards*, London, 1986.
20 J. L. Robinson, M. Tsimidou and R. Macrae, *J. Chromatogr.*, 324 (1985) 35.