Silver-Complexation Liquid Chromatography for Fast, High-Resolution Separations of Triacylglycerols

B.S.J. Jeffrey
Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford MK44 1LQ U.K.

A high-speed, high-resolution approach to silver-complexation chromatography of triacylglycerols in vegetable fats and cocoa butter equivalents is described. Trisaturated and major isomeric unsaturated species with up to five double bonds are eluted within 12 min. A simple change of solvent composition enables elution of species with up to nine double bonds within the same time-frame. Throughput is three samples/hr (70 samples/24 hr with an automatic system). Short-term reproducibility of analytical data for the system gives standard deviations similar to those obtained in practice for high-resolution capillary gas chromatography of triacylglycerols, and quantitative accuracy is good. Details of column preparation, solvent gradient and system operation are given.

KEY WORDS: High performance liquid chromatography, quantitation, silver-complexation, transport-FID detection, triacylglycerol isomers.

The ability to separate triacylglycerols (TAG) on the basis of degree of unsaturation by silver-complexation (argentation) chromatography has proven an indispensable tool for the lipid analyst. As long ago as 1962, de Vries (1) carried out the separation of glycerides on columns of silica impregnated with silver nitrate. However, well into the 1970's the preferred approach to silver-complexation chromatography was to use silver-loaded thin-layer chromatography (TLC) plates. Barrett *et al.* (2) used this approach to separate glycerides with 0–6 double bonds. These workers also demonstrated another powerful aspect of the technique—the ability to separate positional isomers of TAG containing the same number of double bonds in the molecule (e.g., StOSt was separated from OStSt. See Fig. 1 for abbreviations). Silver-nitrate TLC became a routine analytical tool, particularly useful in the field of confectionery fats (3), although the technique does present some problems of quantitation (4). In an attempt to overcome such problems, workers turned their attention to the possibilities of argentation high performance liquid chromatography (HPLC). Smith *et al.* (5) reported the use of benzene as mobile phase with 5-micron Partisil (impregnated with silver nitrate) to separate TAG according to position and number of double bonds. Optimum resolution was achieved at 7°C, and detection was refractometric. Aitzetmuller (6) briefly reported the results of a collaborative study between groups at this Laboratory and the Unilever Research Laboratory at Vlaardingen (The Netherlands). He showed TAG separations by gradient elution from a silver nitrate-impregnated silica HPLC column with transport-flame ionization detection (FID). A concave gradient from hexane/toluene/ethyl acetate (72:26:2, v/v/v) to toluene/ethyl acetate (90:10, v/v) in 30 min was used to give good resolution of positional isomers at room temperature. Hammond and Irwin (7) reported this approach in more detail at a later date. Thus, 250 mm × 5 mm columns packed with 5-micron silica that had been impregnated with 10% (w/w) of silver nitrate resolved major positional isomers of TAG within run times of 45 min. More recently, Takano and Kondoh (8) described a combination of argentation and non-aqueous reversed-phase (NARP) chromatography for the analysis of TAG. They used two columns, 150 mm × 4.6 mm packed with 10% silver nitrate on 3-micron silica, coupled together. Benzene was used as the mobile phase at 20°C, with infra-red detection. Good separations of isomeric TAG were achieved in analysis times of up to one hour. Christie (9,10) has reported the preparation of silver-loaded ion-exchange HPLC columns (based on Nucleosil 5SA) and their application to the separation of molecular species of TAG. Using solvent systems based on 1,2-dichloroethane, dichloromethane, acetone and acetonitrile, column retention behavior was reproducible and stable over long time periods. No separation of positional isomers was reported, and analysis times were typically 30–40 min. Thus columns packed with 5-micron silicas and 250–300 mm in length have been the standard configuration adopted for analysis of TAG. In practical terms, such columns require analysis times of 35–45 min per sample if sufficient resolution is to be obtained. The availability of high-efficiency 3-micron column packings has, however, opened the way to the application of shorter columns producing equivalent or superior resolution within much shorter analysis times. We now report an approach that is routinely used in this laboratory. Under the operating conditions described, major positional isomers are well resolved, and TAG with five double bonds are eluted within 12 min. Injection to injection times are within 20 min. With autosampler injection, it is possible to analyze 70 samples in a 24-hour period.

EXPERIMENTAL PROCEDURES

All solvents used are HPLC Grade (FSA Laboratory Supplies, U.K.).

The column—preparation and conditioning. Columns are 100 mm × 4.6 mm i.d., and are packed with 3-micron spherical silica (Nucleosil 100-3, Machery-Nagel, Düren, Germany), loaded with 10% (w/w) of silver nitrate A.R. (Sigma Chemicals, St. Louis, MD). To prepare the packing, silver Nitrate (0.2 g) is dissolved in acetonitrile (5 mL) in a 250-mL round-bottom flask fitted with a PTFE (greaseless) tap assembly. Methanol (15 mL) is added and mixed in to give 20 mL total volume. If desired, the flask can be wrapped in aluminum foil to exclude light. Microparticulate silica (2.0 g) is added to the flask, taking adequate precautions to avoid the silica dust. The tap assembly is replaced onto the flask, and the contents are swirled by hand to ensure thorough mixing. Removal of the solvent is simply accomplished by attaching the flask (via a solvent trap) to moderate vacuum in a water-bath (not exceeding 50°C) and agitating by hand only until the silica becomes a free-flowing powder once more. It is important to avoid excessive agitation (which leads to loss of silica into the vacuum line), and it is undesirable to overheat or overdry the prepared phase, which should re-

main perfectly white in appearance. To pack the phase, it is first slurried in carbon tetrachloride (the volume required will depend upon the packing device used). Limited ultrasonication may be used (30 sec is normally sufficient), after which the slurry is immediately transferred to the packing bomb. The column is packed (initially by upward displacement) for 4 hr at 5000 psi (350 bar) with toluene as the fluid. This approach produces columns that show no voiding during their useful lifetime.

The newly-packed column requires conditioning. This step is extremely important as it produces stable columns with reproducible retention behavior—both on a sample-to-sample and column-to-column basis. To condition, toluene (1.5 mL/min) is pumped through the column and two injections of formic acid (AR, 98% v/v, 20 μL) are made at 5-min intervals with the detector disconnected. A blank gradient is run through the column, making it ready for use. Note that in order to maintain the required column condition, a low level of formic acid is included in solvent "C", used for column regeneration between samples (Table 1).

Chromatography—equipment and solvent gradient. The system consists of a Varian 5560 ternary liquid chromatograph with a Varian 8300 autosampler (Varian Associates, U.K.) and a Tracor 945 transport-FID detector (Tracor Instruments Inc., Austin, TX). The solvent gradient is a multilinear combination of three solvent mixtures; the gradient "set points" and solvent compositions are given in Table 1.

The detector. Aitzetmuller (6) discussed the basic requirements of liquid chromatographic detectors for the analysis of lipids in some detail. Our operating conditions for the Tracor 945 detector are as follows: Block heater, 180°C; FID hydrogen, 160 mL/min; air, 400 mL/min; cleaner hydrogen, 500 mL/min; oxygen, 260 mL/min. Our experience has been that operating the cleaner flame hydrogen at the manufacturer's specification (600 mL/min) will severely shorten the operating lifetime of the quartz belt to as little as two days in some cases. Also, the condition of the belt has a marked effect on the quantitation and resolution of the overall chromatographic system, and requires replacement every 3–6 months.

Sample preparation. Sample preparation is critically important when short columns are used. Free fatty acids and diacylglycerols, which elute with unsaturated TAG, must be removed before analysis. Removal of both species is accomplished by pre-treatment on a small "solid phase extraction" (SPE) column, employing alkaline alumina. The sample (0.1 g) is dissolved in dichloromethane (1 mL, AR grade), and is transferred onto an SPE column containing alkaline alumina (1 g, Brockmann grade 1). Non-polar components are eluted from the column with another 2 mL of dichloromethane and are recovered by evaporation of the solvent under nitrogen. For analysis on the silver-loaded column the recoverd TAG is dissolved in toluene to give a solution in the range of 3–10 mg/mL, and 20 μL of this solution is injected.

RESULTS AND DISCUSSION

The importance of correct column conditioning was discussed above. Our approach gives excellent column-to-column reproducibility in terms of separation, retention and elution order. On conditioned columns, SLS is eluted before SOO with good peak shape; on an unconditioned column, this elution order is initially reversed and the SLS peak is very broad and misshapen. In use, the unconditioned column shows changing retention for SOO/SLS, such that the peaks may co-elute for a time before eventually reaching an elution order with SLS preceding.

TABLE 1

Solvent Gradient for Silver-Phase Chromatography (Flow Rate is Set at 1.5 mL/min)

Time (min)	Solvent composition[a] %A	%B	%C	Comment
o	94	6	0	Inject
2.5	92	8	0	
7.0	75	25	0	
10.0	10	90	0	
10.1	0	0	100	Regenerate
15.0	0	0	100	Column
15.1	94	6	0	
18.0	94	6	0	Next injection

[a]Solvent A, Toluene/hexane (1:1, v/v); solvent B, toluene/ethyl acetate (9:1, v/v); solvent C, toluene/98% formic acid (500/mL:40 μL).

Our column/gradient system gives good separations of TAG with up to five double bonds within total retention times of around 12 min [see Fig. 1, a fully randomized palm oil, and Fig. 2, an isometric plot comparing cocoa butter and Coberine, a cocoa butter equivalent (CBE)]. Symmetric and assymetric TAG within most unsaturated series can be resolved (e.g., SOS from OSS, SLS from SSL, SOO from OSO, SLO from OSL). In Figure 2, the assymetric TAG are present at comparatively low levels. For chromatographing more liquid oils (i.e., those containing more unsaturated TAG), it is necessary to increase the polarity of solvent mixture B by increasing the ethyl acetate content to 25% v/v. In this way, TAG with up to nine double bonds can be eluted with the gradient profile given above, and within the same time-frame (see Fig. 3, soybean oil).

TAG of different carbon number, but with the same degree of unsaturation, are separated to a limited extent. We observe partial resolution of stearic and palmitic acid-based TAG (e.g., StOSt/POP, StStSt/PPP, StLSt/PLP) which can have the undesirable effect of broadening peaks and reducing overall resolution in the case of fat blends, such as CBE (Fig. 2). However, the difference in carbon number between StStSt/PPP and trilaurin, LaLaLa, is such that these TAG are baseline resolved (Fig. 4). Trilaurin is readily available in high purity, and makes an excellent internal standard for the determination of low-level trisaturated TAG in fats. Figure 5 plots the relative responses of PPP and LaLaLa. Linearity of response is good, and relative response for these components can be taken as 1.00. Trilaurin cannot be used where the sample contains short to medium-chain or *trans*-fatty acids combined in the TAG, as some species are eluted between the SSS and SOS peaks.

Reproducibility and quantitative accuracy achieved are illustrated in Tables 2–4. Reproducibility data are given for a sample of palm oil, together with calculated (11) and determined compositional data for a randomized palm oil and a gravimetric mixture of pure synthetic TAG. Standard deviations are similar to those obtained in our

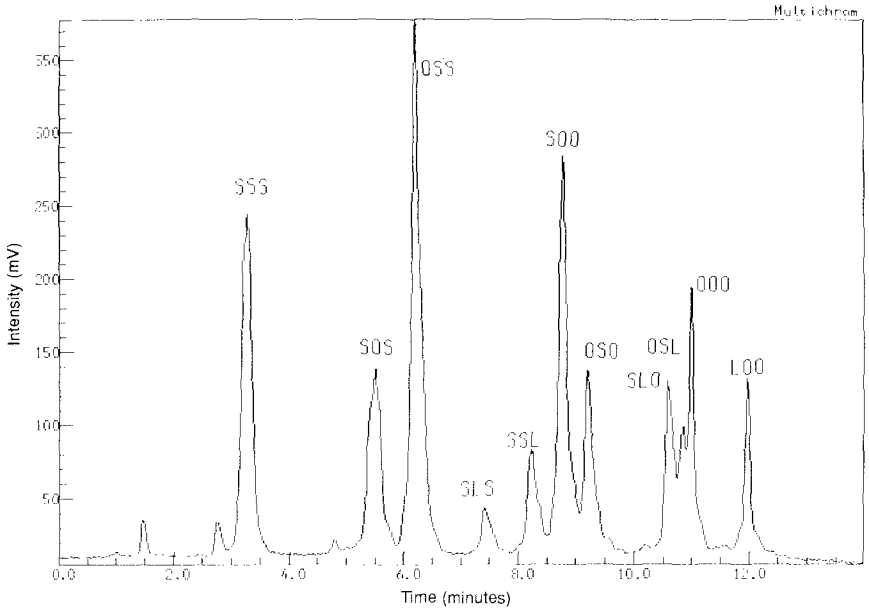

FIG. 1. Silver-complexation separation of TAG from fully randomized palm oil. For conditions see text. Acyl group derivations used in TAG nomenclature are as follows: P from palmitic acid (C16:0), St from stearic acid (C18:0), La from lauric acid (C12:0), S from any long-chain saturated fatty acid, O from oleic acid (C18:1), L from linoleic acid (C18:2), Le from linolenic acid (C18:3).

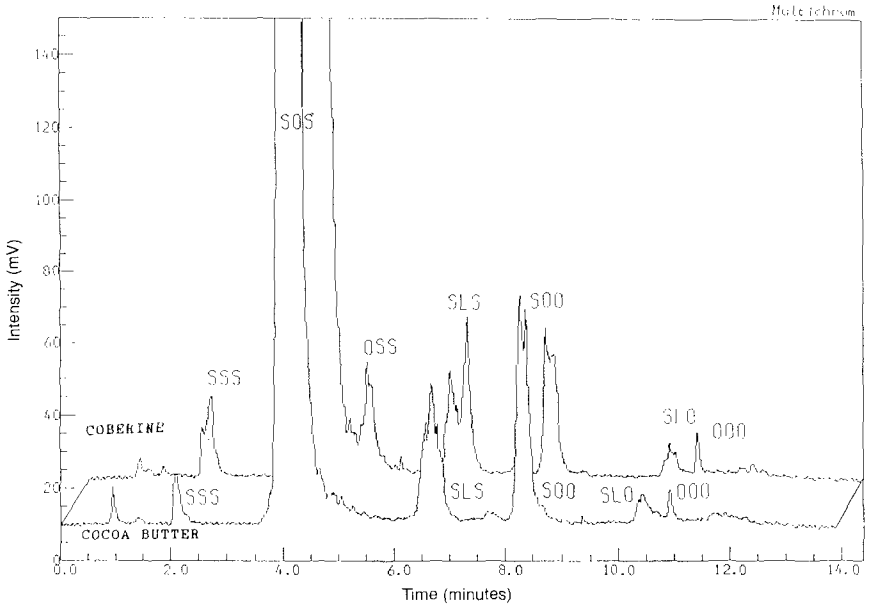

FIG. 2. Silver-complexation separation of TAG from cocoa butter and Coberine (comparative isometric plot). For conditions see text.

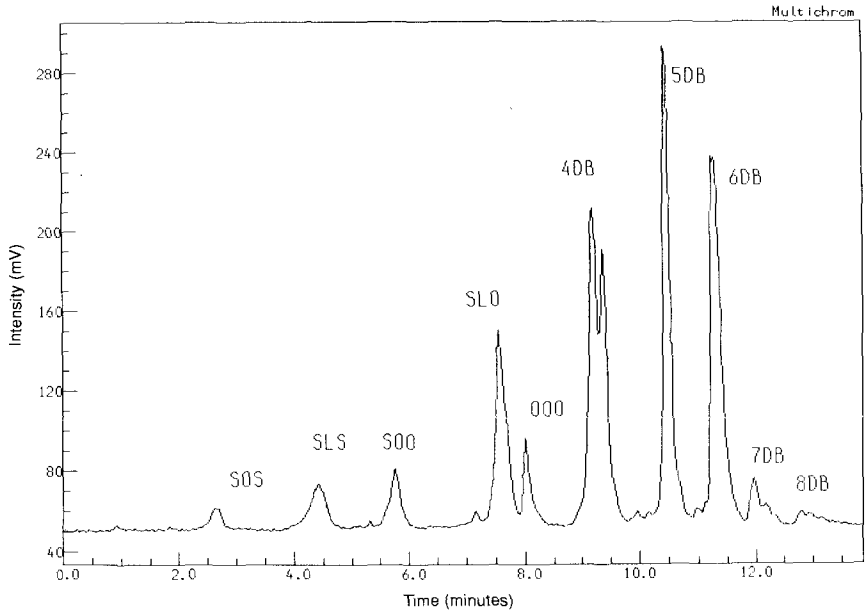

FIG. 3. Soybean oil chromatographed with Solvent B = toluene/ethyl acetate (3:1, v/v).

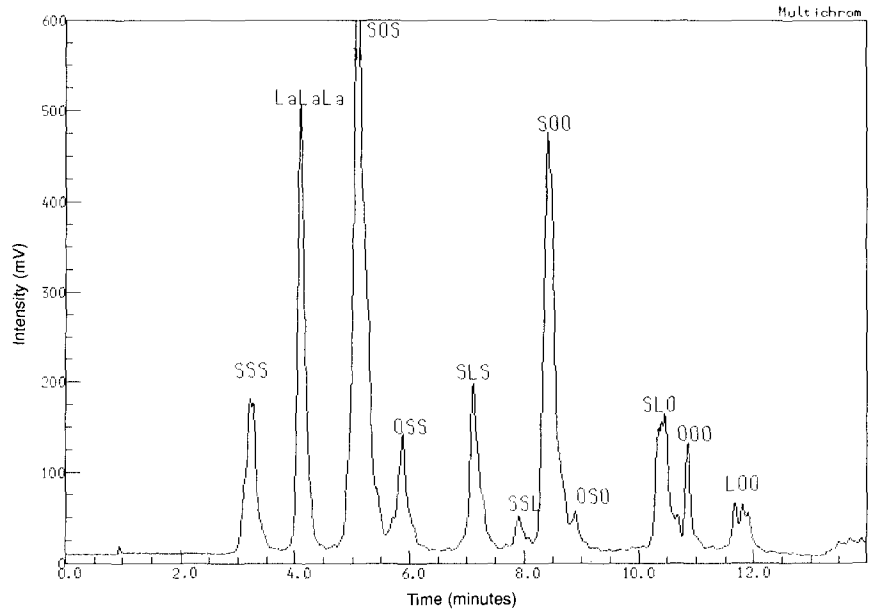

FIG. 4. Showing the resolution of trilaurin added to a sample of palm oil as an internal standard. For details see text.

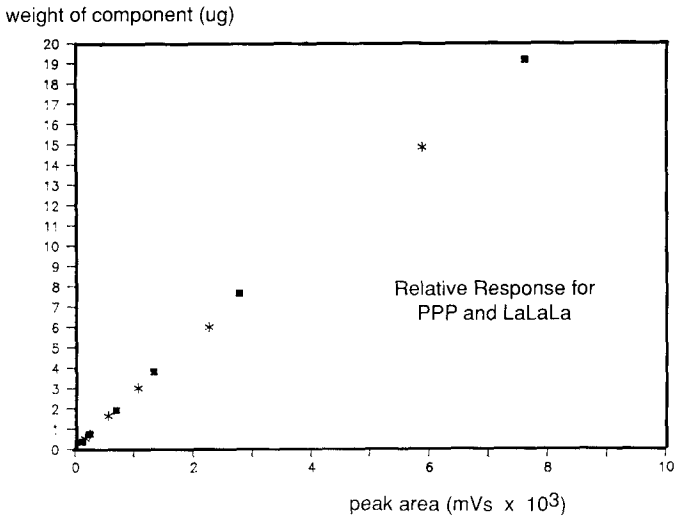

FIG. 5. Linearity of response for PPP and LaLaLa (Tracor 945 detector).

TABLE 2

Reproducibility Data for Palm Oil (Data is area%, n=7)

	TAG						*a*
	SSS	SOS	OSS	SLS	SOO	SOL+OOO	>3db
Mean	8.7	30.8	7.3	9.4	25.9	13.8	4.0
S.D.	0.1	0.3	0.1	0.2	0.3	0.2	0.1

[a]a>3DB = sum of TAG with more than three double bonds.

TABLE 3

Data for Randomized Palm Oil (% Area)

	TAG										*a*
	SSS	SOS	OSS	SLS	SSL	SOO	OSO	SLO	OOO	SSLe	>3db
Calc. (%w/w)	14.0	9.9	19.8	3.0	6.1	14.0	7.1	12.6	5.0	0.2	8.2
Found	14.0	10.8	20.4	2.8	6.1	14.1	7.8	10.7	6.0	—	7.2

[a]a>3DB = sum of TAG with more than three double bonds.

TABLE 4

Data for Pure Synthetic TAG (n=4)

	PPP	POP	OPP	PLP	POO	OOO
Wt% composition	6.3	33.0	4.7	9.0	29.5	17.5
Determined area%						
Mean	6.3	33.5	4.7	8.8	29.1	17.6
S.D.	0.2	0.4	0.3	0.3	0.1	0.3

laboratory for high-temperature capillary gas chromatography of TAG (12).

Despite the advances made in high-resolution capillary gas chromatography and reversed-phase HPLC of TAG over the last few years, silver-complexation chromatography remains the only direct way to analyze such materials for both unsaturation and isomeric configuration. Our work has shown that silver nitrate-loaded columns can provide high-speed and high-resolution separations simultaneously, together with an acceptable practical working lifetime. In addition, the approach described can provide a high degree of reproducibility and quantitative accuracy for this type of chromatography.

ACKNOWLEDGMENT

G. Sassano, H. Brownlow and D. Phillips (Unilever Research, Colworth House) provided technical input; Dr. F. Padley actively encouraged this work over a number of years; Loders Croklaan b.v., The Netherlands, gave permission to publish this paper.

REFERENCES

1. De Vries, B., *Chem. Ind. (London)* 1049 (1962).
2. Barrett, C.B., M.S.J. Dallas and F.B. Padley, *J. Am. Oil Chem. Soc. 40*:580 (1963).
3. Dallas, M.S.J., and F.B. Padley, *Lebensm.-Wiss. u. Technol. 10*: 328 (1977).
4. Hammond, E.W., *Chemistry Ind. (London) 20*:710 (1981).
5. Smith, E.C., A.D. Jones and E.W. Hammond, *J. Chrom. 188*:205 (1980).
6. Aitzetmuller, K., *Prog. Lipid Res. 21*:171 (1982).
7. Hammond, E.W., and J.W. Irwin, *HPLC in Food Analysis*, edited by R. Macrae, Academic Press Ltd., Academic Press Ltd., 1988, pp. 96-132.
8. Takano, S., and Y. Kondoh, *J. Am. Oil Chem. Soc. 64*:380 (1987).
9. Christie, W.W., *J. High Res. Chrom. and Chrom. Comm. 10*:148 (1987).
10. Christie, W.W., *J. Chrom. 454*:273 (1988).
11. Litchfield, C., *Analysis of Triglycerides*, Academic Press, 1972, pp. 248-250.
12. Geeraert, E., and P. Sandra, *J. High. Res. Chrom. and Chrom. Comm. 8*:415 (1985).

[Received October 30, 1990; accepted March 1, 1991]