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INVESTIGATION OF THE USE OF ARGENTATION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE ANALYSIS OF TRIGLYCERIDES

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SUMMARY

A basic method for the separation of triglyceride mixtures including the positional isomers 2-unsaturated-1,3-disaturated (SUS) and 1-unsaturated-2,3-disaturated (SSU) by argentation high-performance liquid chromatography is described. Temperature and silver loading have been investigated for their effect on the triglyceride capacity factors (k') and the resolution (R_s) between SUS and SSU. Quantitation of trisaturated (SSS), SUS and SSU in fat mixtures using an internal standard is also described.

INTRODUCTION

In 1938 Winstein and Lucas[1] showed that the complexation reaction between silver ions and alkenes was reversible and that equilibrium was attained very rapidly. The potential of this interaction for the separation of unsaturated compounds was realised when Dutton *et al.*[2] separated methyl oleate and methyl elaidate using a counter-current system. Subsequently, silver nitrate was incorporated into silicic acid adsorbents. De Vries[3,4] used column chromatography to separate fatty acid esters and triglycerides according to their number and geometry of double bonds, whilst Morris[5] resolved isomers and vinylogues of non-oxygenated and oxygenated fatty acid esters by thin-layer chromatography (TLC). Barrett *et al.*[6] also resolved glyceride mixtures by TLC. Much work has since been done using argentation chromatography to qualitatively and quantitatively analyse lipids, and some fine separations, even of positionally isomeric unsaturated species, have been achieved by TLC.

Argentation high-performance liquid chromatography (HPLC) has been used for the separation of a wide range of compounds including prostaglandins[8,9] insect sex attractants[10,11] and drugs[12,13] although its use within the lipid area is very limited[14].

A reproducible method for the separation of isomeric triglycerides has been developed by Dallas and Padley[15] using argentation TLC. Walker and Hammond[16] use a simpler and more quantitative modification of this procedure, incorporating phloxin (BDH, Poole, Great Britain) into the layer and scanning the plates with a Zeiss model PMQ3 densitometer in fluorescence mode. However, these procedures suffer from two main problems. (i) The error in quantitation is high when a tri-

glyceride component is less than 10% of total triglyceride and (ii) the linearity range is small (1–40 μg) and the approximate fat composition must be known.

This work concerns the investigation of argentation HPLC as a more rapid and quantitative approach to the analysis of unsaturated triglycerides.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a Waters Assoc. M6000 solvent delivery system and R401 differential refractometer (Waters Assoc., Hertford, Great Britain). Injection was achieved using a Specac 5000 p.s.i., 6-port injection valve (Spectroscopic Accessories, Sidcup, Great Britain) fitted with a 30-μl loop. The analytical column was 25 × 0.40 cm I.D. glass lined steel tubing (Scientific Glass Engineering, London, Great Britain) packed with 5 μm Partisil (Whatman, Maidstone, Great Britain) impregnated with silver nitrate. Semi-preparative work was carried out using 20 × 0.75 cm I.D. seamless stainless-steel tubing (H.S.C.P., Bourne End, Great Britain). Quantitation of the lipid components was carried out on the basis of peak area against an internal standard using an Infotronics CRS-208 integrator (Shannon Airport, Ireland). The column was maintained at a constant temperature using a jacket of 1/16 in. I.D. copper tubing through which water was circulated. The water temperature was maintained at a given level by balancing a Shandon (London, Great Britain) water heater/circulator and a Grant Instruments (Cambridge, Great Britain) refrigeration unit. Since benzene was used as the mobile phase all equipment was stored and used in a well ventilated fume cupboard.

Liquid chromatography

Preparation and packing of silver nitrate impregnated silica. The percentage loading of the phase is calculated on a weight to weight basis, thus a 10% silver nitrate loading represents 1 g silver nitrate in 9 g dry silica. Dissolve 1 g silver nitrate in 50 ml methanol in a 150-ml round-bottomed flask covered with foil to protect it from light. Dry 9 g of silica overnight at 110° and then slurry in 50 ml of acetonitrile. Transfer slurry to the round bottom flask, shake for 1 min and then evaporate to dryness under reduced pressure at a temperature less than 30°. The phase is now ready for use and must be protected from light at all times.

The column was packed using the previously reported slurry packing procedure[17]. (a) Analytical: slurry 3.6 g of phase in 50 ml of carbon tetrachloride. Compress the phase to a compact bed using hexane at 6000 p.s.i. for 20 min. (b) Semi-preparative: use 9.5 g of phase and pack as above.

The effect of temperature on the analysis. The effect of temperature on (i) the capacity factors (k') of the lipids and (ii) the resolution of 2-unsaturated-1,3-disaturated (SUS) and 1-unsaturated-2,3-disaturated (SSU) was determined at temperatures from 6.5 to 21.5°.

The effect of silver nitrate loading on the analysis. Stationary phase containing between 2.5 and 15% silver nitrate was investigated for its effect on (i) the capacity factors of the lipids and (ii) the resolution of SUS and SSU.

Quantitation. A number of compounds were tested for their suitability as an internal standard. Having found one acceptable, a range of fourteen fat samples was

analysed by HPLC by melting approximately 1 g of fat into a 10-ml volumetric flask, adding approximately 200 mg of internal standard and making to volume with benzene. A 30-μl volume of this solution was injected on to the analytical column.

RESULTS AND DISCUSSION

Separation of trisaturated (SSS), SUS, SSU, 2-diunsaturated-1,3-disaturated (SLS), 2,3-unsaturated-1-saturated (SUU) and triunsaturated (UUU) triglycerides was obtained using benzene as a mobile phase although the resolution between SUS and SSU was poor at room temperature (Fig. 1). Initial attempts to use toluene as an alternative, less toxic solvent yielded poor resolution and peak shape (Fig. 2) although present studies with toluene and a different batch of silica have shown it to be equally suitable. This apparent contradiction is probably due to inter-batch variations in the silica and is currently under investigation. Using benzene, elution order of the peaks was as expected with the most highly unsaturated compound eluting last. The more sterically hindered isomer SUS was less strongly retained than, and eluted prior to, SSU. For similar reasons SLS eluted prior to SUU. UUU, containing three double bonds, was the most strongly retained.

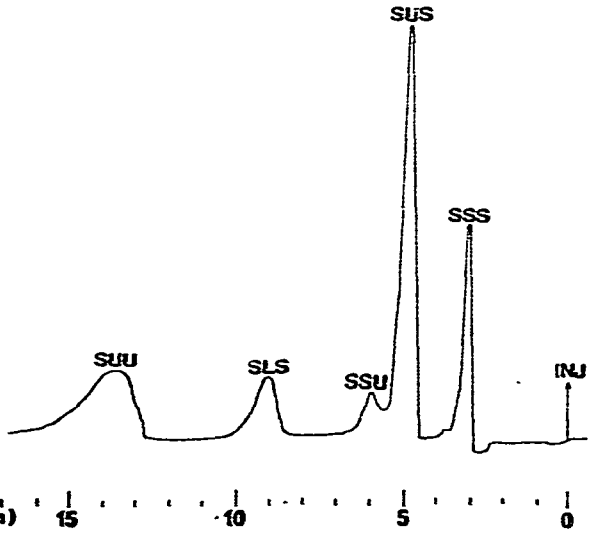

Fig. 1. Separation of triglycerides using a benzene mobile phase. Column: 25 × 0.4 cm I.D. glass lined steel. Packing: Partisil 5 with a 10% $AgNO_3$ loading. Flow-rate: 1 ml/min. Detector: refractive index. Sensitivity: S × 8. Injection volume: 30 μl. Temperature: ambient.

The effect of temperature on the analysis

The effect of column temperature on the resolution between SUS and SSU is shown in Fig. 3. As might be anticipated, resolution was found to increase rapidly for a steady reduction in temperature. Similarly a graph of capacity factor against temperature also shows an increase in k' at lower temperature (Fig. 4). All further work was carried out at 6.8° in order to maximise the separation of SUS and SSU (benzene freezes at 6°).

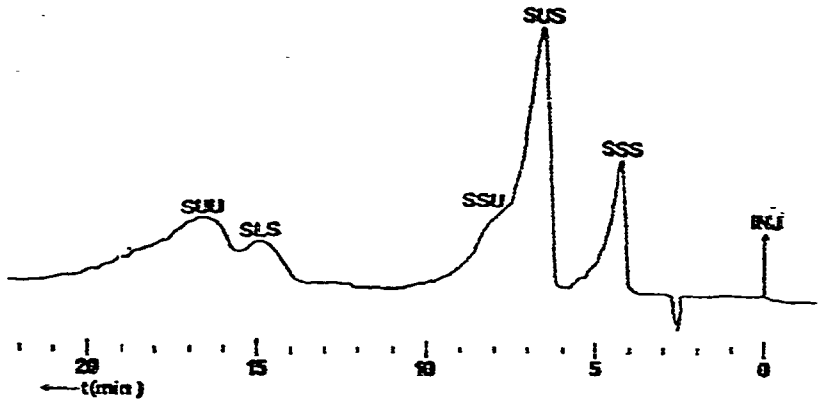

Fig. 2. Separation of triglycerides using a toluene mobile phase. Conditions as in Fig. 1.

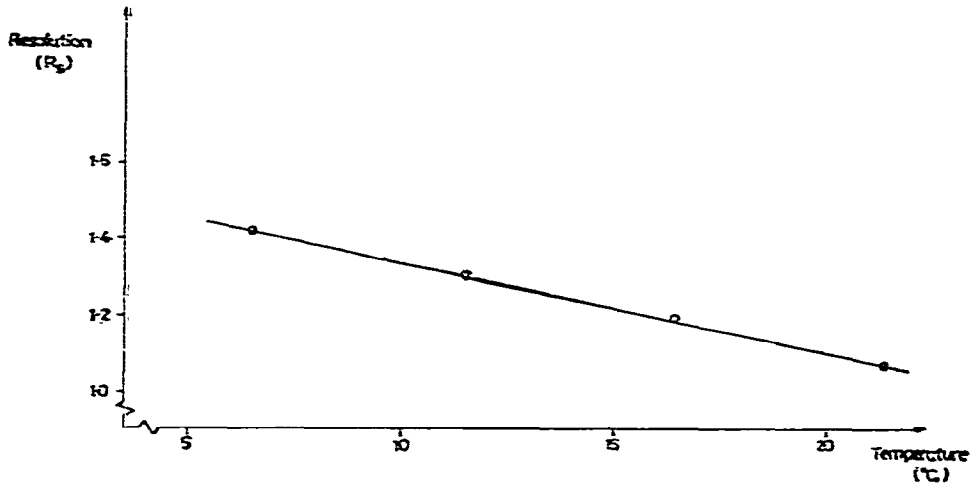

Fig. 3. The effect of column temperature on the resolution (R_s) of SUS and SSU.

The effect of silver nitrate loading on the analysis

The effect of silver nitrate loading on the capacity factors for the lipids is shown in Fig. 5. Surprisingly k' increased for decreasing silver nitrate loading but this trend was reversed below a 5% loading for SUU and SLS. This would indicate a strong contribution to lipid capacity factors from the silica surface in addition to the silver ion–double bond complex. This is supported by the fact that those lipids containing a double bond were characterised by pronounced peak tailing, indicative of a mixed retention mechanism. Fig. 6 shows the effect of silver nitrate loading on the resolution between the positional isomers SUS and SSU. A small but steady increase in resolution was observed when the loading of $AgNO_3$ was reduced from 15 to 5% but below this level the resulution decreased markedly. This would indicate that a 5% $AgNO_3$ loading represents the optimum for separation of these two isomers. In practice 5% loading leads to an unacceptably long analysis time for the complete triglyceride mixture. A 10% loading, therefore, represents a convenient compromise between speed of analysis and good resolution.

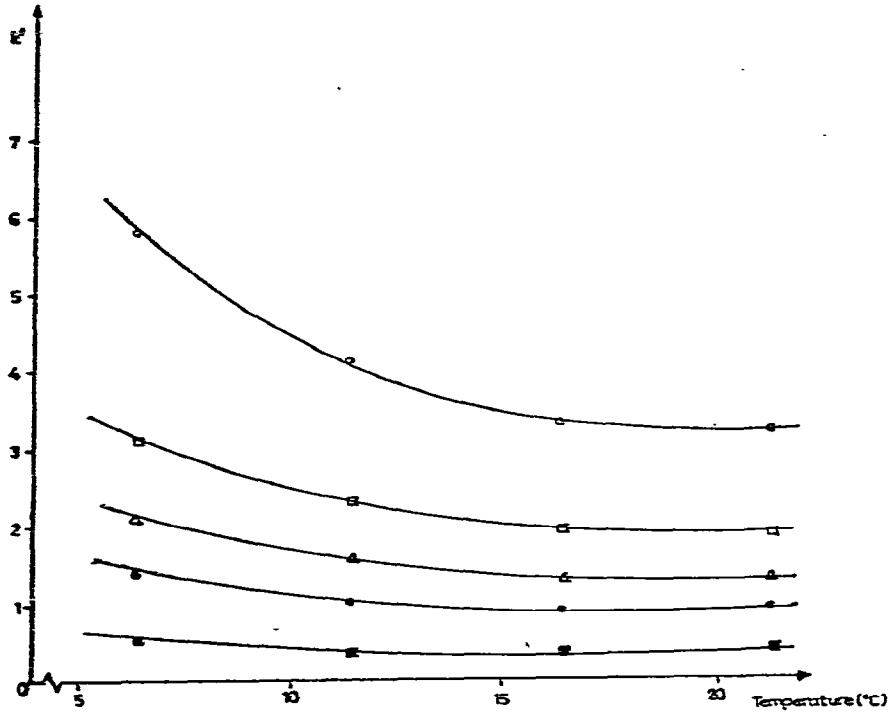

Fig. 4. The effect of column temperature on triglyceride capacity factors (k'). Key: ■, SSS; ●, SUS; △, SSU; □, SLS; ○, SUU.

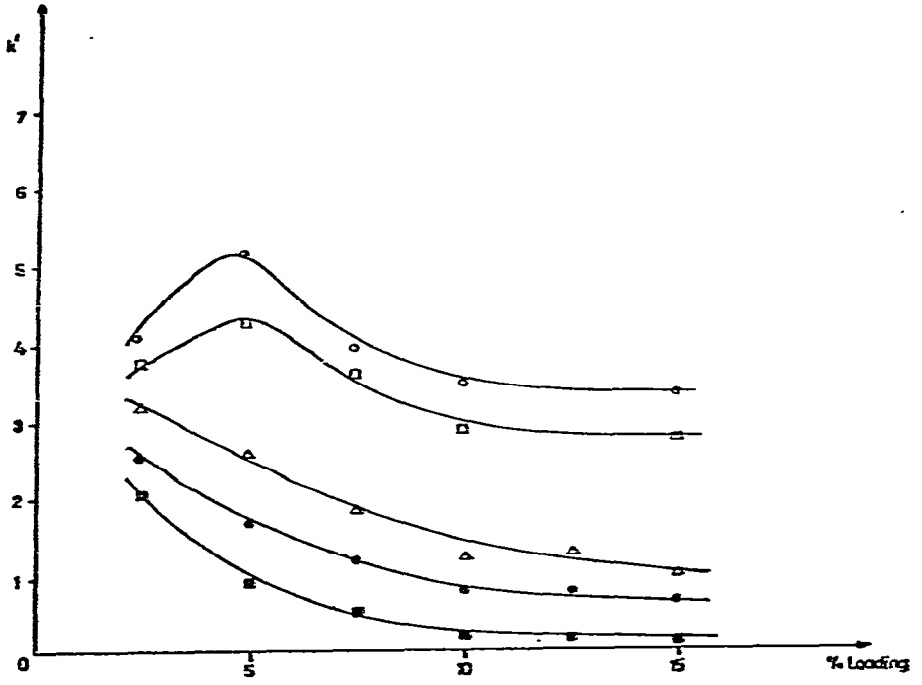

Fig. 5. The effect of silver nitrate loading on triglyceride capacity factors (k'). Key as in Fig. 4.

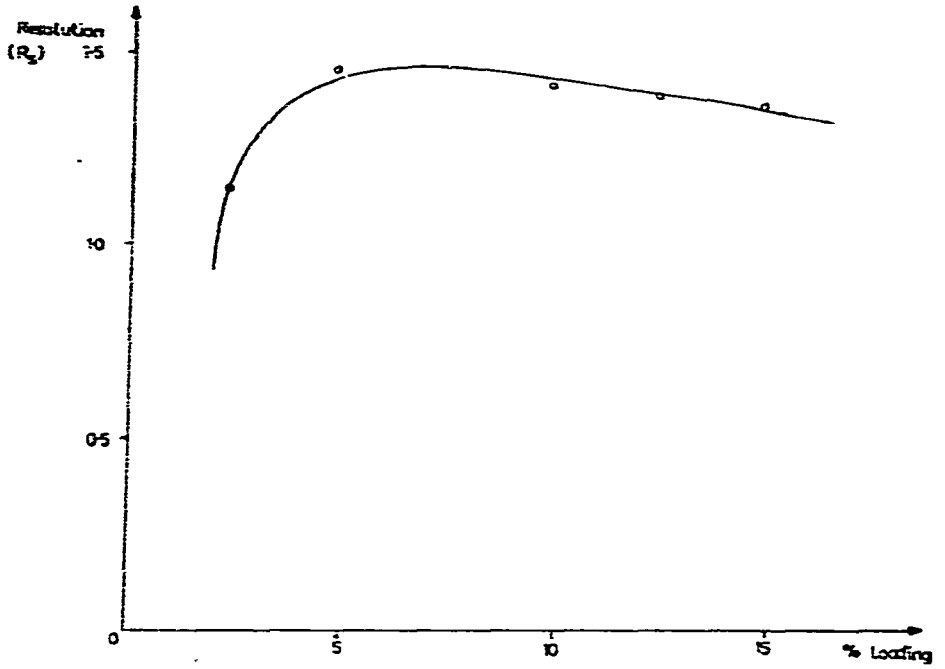

Fig. 6. The effect of silver nitrate loading on the resolution (R_s) of SUS and SSU.

Quantitation

Methyl stearolate, methyl linoleate and methyl oleate were tested for their suitability as internal standards but had to be discarded due to the fact that they eluted too close to a lipid component. Methyl santalbate (the methyl ester of octadec-

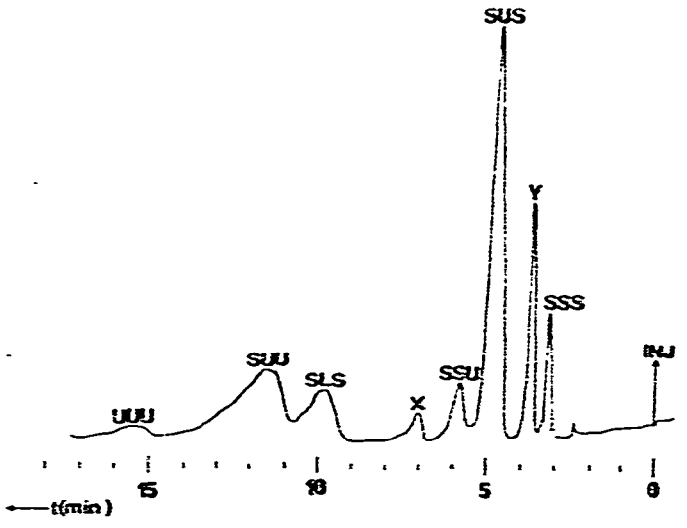

Fig. 7. Separation of triglycerides at 6.8° using a benzene mobile phase. Conditions as in Fig. 1. Y = Methyl santalbate.

9-yne-11-*trans*-enoic acid obtained from the oil of *Santalum album* where santalbic acid comprises some 80% of total fatty acids) was found to elute between SSS and SUS without interfering and showed good linearity based on peak area over a wide range of concentration. The response of the three lipid components SSS, SUS and SSU was determined over the same concentration range and compared with the internal standard. Fig. 7 shows a typical chromatogram obtained under the optimum conditions of temperature and phase loading on which quantitation was carried out. Again good linearity was obtained and a constant response factor of 1.66 relative to the internal standard was shown to exist for all three components. (The refractive indices of all three components are virtually identical.) SSS is not soluble above 5% in benzene and hence solutions containing high SSS concentrations had to be diluted to come into the appropriate concentration range. For all quantitation work, therefore, approximately 1 g of fat mixture was dissolved in 10 ml of benzene. The concentration renge of the internal standard and the three triglycerides SSS, SUS and SSU for which quantitation is valid is therefore:

Triglyceride	*Concentration in benzene solution (%)*
Internal standard (methyl santalbate)	0–10 (in practice 2% is generally used)
SSS	0– 5
SUS	0–10
SSU	0–10

A range of fourteen different fat mixtures were separated and quantitated in duplicate to determine the reproducibility of the method. For each triglyceride type (SSS, SUS, SSU) coefficients of variation were calculated for the concentration ranges 0–10%, 10–30% and > 30%. The results are presented in Table I.

TABLE I

REPRODUCIBILITY OF QUANTITATION FOR SSS, SUS AND SSU IN FAT MIXTURES

Triglyceride	*Concentration range (%)*	*Number of values in range*	*Average CV (%)*
SSS	0–10	6	8.1
	10–30	6	5.5
	>30	2	1.7
SUS	0–10	1	10.6
	10–30	2	2.5
	>30	11	3.4
SSU	0–10	9	7.7

Semi-preparative work

Using a 7.5 mm I.D. column and a 500-μl loop it was possible to apply and separate up to 200 mg of fat mixture. Since the triglycerides SOS, (2-oleo-1,3-distearin), POS (1-palmito-2-oleo-3-monostearin) and POP (2-oleo-1,3-dipalmitin) elute together as an SUS fraction under the HPLC conditions, this technique was therefore used to isolate this fraction for further analysis by gas–liquid chromatography (GLC) on the basis of chain length.

CONCLUSIONS

Using argentation chromatography with a benzene mobile phase at 6.8° it is possible to separate the triglyceride types SSS, SSU, SUS, SLS, SUU and UUU in triglyceride mixtures although peak shape progressively deteriorates as double bond content increases. The optimum support loading for the separation of the positional isomers SUS and SSU is 5%. Using this method SSS, SUS and SSU fractions in fat mixtures can be quantitated using an internal standard, *e.g.* methyl santalbate. The method can be used in a semi-preparative mode using a 7.5 mm I.D. column and injecting up to 200 mg of fat. This allows milligram quantities of lipid fractions to be prepared for further analysis by GLC.

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