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# SEPARATION OF MOLECULAR SPECIES OF TRIACYLGLYCEROLS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH A SILVER ION COLUMN

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## SUMMARY

Molecular species representative of the wide range of triacylglycerols occurring in nature, ranging from relatively saturated fats such as that from sheep adipose tissue through polyunsaturated seed oils, including sunflower and linseed oils, to an oligounsaturated fish oil, have been resolved by high-performance liquid chromatography in the silver ion mode. The stationary phase consisted of an ion-exchange medium, which was a silica gel matrix with bonded sulphonic acid moieties, loaded with silver ions. The mobile phase for the more saturated fractions was a gradient of acetone into 1,2-dichloroethane–dichloromethane, then acetonitrile was introduced to elute polyunsaturated fractions. A mass detector was employed to monitor separations. Fractions were collected via a stream-splitter for identification and quantification by gas chromatography as methyl esters. Excellent resolution was obtained on the silver ion column with no contamination of fractions with silver ions, while the column was stable and retained its activity in prolonged use.

## INTRODUCTION

Silver ion chromatography has long been valued by lipid analysts, because it permits separation of distinct molecular fractions, differing solely in the degree of unsaturation[1,2]. The technique has been used largely in conjunction with thin-layer chromatography (TLC), with silver nitrate being incorporated into the silica gel layer. While this has given excellent results, there are a number of disadvantages in practice. For example, relatively large amounts of expensive silver nitrate are required, the separated components are not always easily visualised, autoxidation can occur on the TLC plate and some silver ions are generally eluted together with the fractions. There have been many attempts to adapt the technique to high-performance liquid chromatography (HPLC), but until relatively recently these had met with limited success only[3]. On the other hand, a stable ion-exchange column loaded with silver ions has been developed that has proved of value in some applications[4]. The procedures are rapid, reproducible and give clean fractions, uncontaminated by silver ions. HPLC with this column has recently been used to simplify complex mixtures of fatty acids of

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natural origin for subsequent identification by gas chromatography (GC)–mass spectrometry[5].

One of the more important applications of silver ion chromatography has been for the separation of molecular species of intact lipids, especially the triacylglycerols of the commercially important fats and oils[6]. By utilising aprotic mobile phases, it has been demonstrated that it is possible similarly to fractionate relatively simple natural fats, such as palm oil, by HPLC with the silver-loaded ion-exchange medium[4]. It is shown here that the method can be extended to a much wider range of natural triacylglycerols, including some that are highly unsaturated.

## EXPERIMENTAL

### *Samples and reagents*

The vegetable oils, *i.e.* sunflower, maize (corn), safflower, linseed and evening primrose, were obtained from local shops. A sample of subcutaneous fat was obtained from a sheep of the institute flock and one of rat parametrical adipose tissue was from a female Wistar rat; lipids were extracted with chloroform–methanol (2:1, v/v). The fish oil was a commercial sample of South African origin, probably from anchovies and pilchards, and was donated by Dr. R. J. Henderson of the NERC Unit of Aquatic Biochemistry in the University of Stirling. With each of the samples, triacylglycerols were purified, prior to HPLC analysis, by elution from a short column of Florisil™, with hexane–diethyl ether (4:1) as the mobile phase. All solvents were Analar or HPLC grades and were supplied by FSA Scientific (Loughborough, U.K.).

### *High-performance liquid chromatography*

The HPLC equipment and the silver ion column were as described previously[4]. In brief, a Spectra-Physics Model 8700 solvent delivery system was used (Spectra-Physics, St. Albans, U.K.), together with an ACS Model 750/14 mass detector [Applied Chromatography Systems, (ACS), Macclesfield, U.K.]. When required, a stream-splitter (approximately 10:1) was inserted between the column and the detector. A column (250 × 4.6 mm I.D.) of Nucleosil™ 5SA (kindly donated by ACS) was flushed with 1% aqueous ammonium nitrate solution at a flow-rate of 0.5 ml/min for 1 h, then with distilled water at 1 ml/min for 1 h. Silver nitrate (0.2 g) in water (1 ml) was injected onto the column via the Rheodyne valve in 50-$\mu$l aliquots at 1-min intervals; silver began to elute from the column after about 10 min. 20 min after the last injection, the column was washed with methanol for 1 h, then with 1,2-dichloroethane–dichloromethane (1:1, v/v) for a further hour.

The three solvent reservoirs contained the following: (A) 1,2-dichloroethane–dichloromethane (1:1, v/v); (B) acetone; (C) acetone–acetonitrile (9:1, v/v). For the separation of sheep subcutaneous fat, a linear gradient of A to B was generated over 40 min at a flow-rate of 0.75 ml/min. With rat adipose tissue and linoleic acid-rich seed oils, gradients of A to 50% A–50% B over 15 min, then to 50% B–50% C over a further 25 min, and held at this for 5 min, were employed. For linolenic acid rich seed oils, C was changed to acetone–acetonitrile (4:1, v/v) and the flow-rate was increased to 1 ml/min; gradients of A to 50% A–50% B over 10 min, then to 70% B–30% C over 20 more min, and finally to 100% C over a further 30 min, were utilised. The last elution scheme was also used with the fish oil except that the final solvent mixture was

maintained for an additional 10 min. Samples (0.25–0.8 mg) were applied to the column in dichloroethane solution (5–10 $\mu$l). Fractions were collected manually via the stream splitter and methyl nonadecanoate was added to each as an internal standard.

*Gas chromatography*

The methyl ester derivatives of the fatty acids from each fraction were prepared by sodium methoxide-catalyzed transesterification[7]. A Carlo Erba Model 4130 capillary gas chromatograph (Carlo Erba, Crawley, U.K.), fitted with split/splitless injection, was equipped with a capillary column (25 m × 0.22 mm I.D.) of fused silica coated with Carbowax 20M (Chrompak, London, U.K.). It was temperature-programmed from 165°C (held at this for 3 min) at 4 to 195°C, and held at this point for a further 20 min. Hydrogen was the carrier gas. Components were quantified by electronic integration.

## RESULTS

The utility of a gradient of acetone into 1,2-dichloroethane–dichloromethane for the separation of simple natural triacylglycerols was demonstrated earlier[4]. With aprotic solvents of this kind, there is no danger of transesterification of solutes being catalysed by residual free sulphonic acid groups in the stationary phase, as has been observed by others in related circumstances[8]. However, this basic elution scheme is only of value with triacylglycerols containing relatively low amounts of linoleic acid. The separation of one such sample is illustrated in Fig. 1, *i.e.* of triacylglycerols from sheep subcutaneous adipose tissue. In this instance, one additional complication is the presence of fatty acids with *trans* double bonds or with conjugated diene systems (largely 9-*cis*-11-*trans*-octadecadienoic acid[9]). The more abundant fractions such as SSS, SSM and SMM (see Table I for abbreviations) are clearly apparent and are particularly well resolved. In addition, there are a number of other peaks and some of these were found to contain *trans*-monoenes and the conjugated diene following

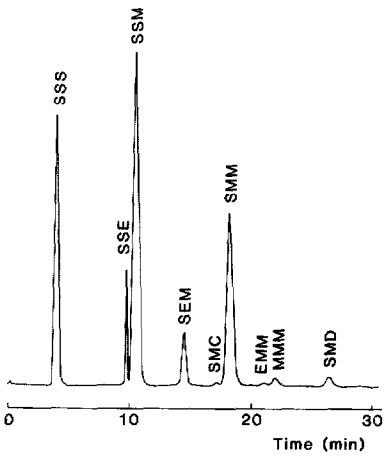


Fig. 1. Separation of triacylglycerols from sheep subcutaneous adipose tissue by HPLC with a silver ion column and mass detection. See the Experimental section for practical details, and Table I for abbreviations.

TABLE I

FATTY ACID COMPOSITION (MOL% OF THE TOTAL) OF THE TRIACYLGLYCEROLS OF SHEEP SUBCUTANEOUS ADIPOSE TISSUE AND OF FRACTIONS OF THIS OBTAINED BY SILVER ION HPLC

Abbreviations: S = saturated; M = monoenoic (*cis*-); E = elaidic (*trans*-monoenoic); C = conjugated dienoic; D = dienoic; T = trienoic fatty acids; br-i = isomethyl branched; ai = ante-isomethyl branched.

| | *Total* | *SSS* | *SSE* | *SSM* | *SEM* | *SMC* | *SMM* | *SSD* | *EMM* | *MMM* | *SMD* | *Check* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 14:0 | 3.3 | 6.5 | 5.4 | 3.7 | 1.0 | 1.1 | 1.2 | 2.2 | | | 1.0 | 2.9 |
| 15:0 | 0.9 | 1.3 | 0.6 | 0.8 | 0.4 | 0.5 | 0.4 | 1.1 | | | 0.6 | 0.6 |
| 16:0 | 26.1 | 44.3 | 31.7 | 29.9 | 15.9 | 17.5 | 17.0 | 35.1 | | | 19.3 | 25.3 |
| 16-br-i | 0.6 | 0.9 | 0.5 | 0.7 | 0.3 | 0.2 | 0.4 | 0.7 | | | 0.7 | 0.6 |
| 16-br-ai | 0.9 | 1.5 | 1.1 | 1.1 | 0.5 | 0.4 | 0.6 | 0.9 | | | 0.8 | 0.9 |
| 16:1 | 2.3 | | 0.5 | 1.4 | 2.2 | 1.5 | 3.0 | | 13.4 | 3.5 | 1.5 | 2.0 |
| 17:0 | 1.7 | 3.1 | 2.1 | 2.1 | 1.2 | 1.1 | 1.1 | 2.5 | | | 1.1 | 1.7 |
| 17:1 | 0.5 | | | 0.5 | 0.5 | 0.6 | 1.2 | | 1.0 | 1.1 | 0.4 | 0.6 |
| 18:0 | 21.2 | 42.4 | 32.8 | 26.8 | 14.0 | 13.4 | 10.3 | 32.8 | | | 10.9 | 21.7 |
| 18:1($n-9$) | 34.9 | | 22.6 | 32.6 | 34.2 | 32.7 | 63.2 | | 68.0 | 90.3 | 31.9 | 38.2 |
| 18:1($n-7$) | 4.4 | | 2.8 | 0.4 | 28.2 | 1.7 | 1.6 | | 17.6 | 5.2 | 1.3 | 3.9 |
| 18:2 | 1.4 | | | | | 8.9 | | 24.8 | | | 30.5 | 1.0 |
| 18:3 | 0.6 | | | | | | | | | | | |
| 18:2conj. | 1.2 | | | | 1.6 | 20.3 | | | | | | 0.7 |
| % of the total | | 14.3 | 5.1 | 35.6 | 9.6 | 2.6 | 25.2 | 1.5 | 2.1 | 2.6 | 1.5 | |

transesterification and analysis by capillary GC and silver ion HPLC[10] as indicated. Thus, fractions containing one mole of *trans*-monoene with two of saturated fatty acids and one saturated, one *cis*- and one *trans*-monoenoic fatty acid are present. A similar result was obtained with bovine milk fat (not illustrated). No comparable separation appear to have been recorded by silver ion TLC.

Each of the fractions was collected via the stream-splitter and transesterified in the presence of an internal standard for identification and quantification by GC. The mass (or "light-scattering") detector itself can only be used for quantification purposes after extensive calibration, although it permits a wide range of solvents and gradients to be used and is exceedingly useful when developing methodology[3]. The results are shown in Table I. Each of the fractions contain broadly the spectrum and proportions of fatty acids expected, the deviations being greatest in minor fractions or those which are incompletely resolved. As many trace fatty acids were resolved by capillary GC and were omitted from the calculations as unidentifiable, the results are perhaps better than is immediately apparent. As a check on the recoveries, the fatty acid composition of the whole was computed from the relative proportions in each of the fractions; the agreement is acceptable for most of the components.

In order to elute triacylglycerols containing higher proportions of linoleic acid, it was necessary to introduce some acetonitrile into the mobile phase. As an example, the separation of the triacylglycerols of rat parametrical adipose tissue is illustrated in Fig. 2. Many more distinct fractions are seen than in the previous sample. Some of these have shoulders or appear to be double, and this may be because of partial resolution of positional isomers in the triacylglycerols or because of chainlength differences or variations in the positions of the double bonds in fatty acids. Thus the two components of the trimonoenoic fraction are 18:1–18:1–18:1 and 18:1–18:1–16:1 respectively. All of the early fractions are well resolved, but fractions containing linolenic acid tend to overlap with those containing two dienoic acids. The relative proportions of each can, however, be determined from the fatty acid compositions, which were obtained as before and are listed in Table II. Again each fraction contains the relative proportions

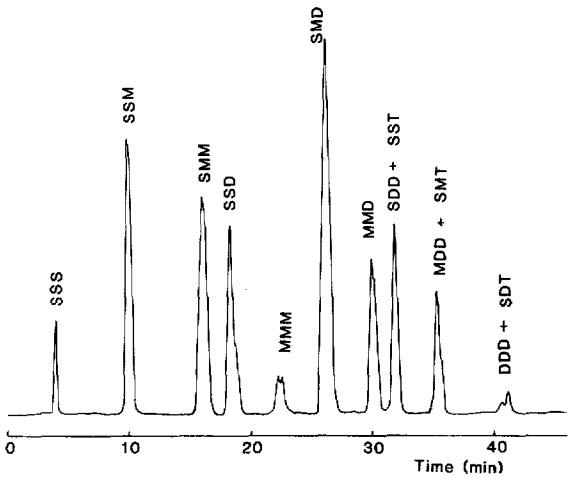


Fig. 2. Separation of triacylglycerols from rat parametrial adipose tissue by HPLC with a silver ion column and mass detection. See the Experimental section for practical details, and Table I for abbreviations.

TABLE II

FATTY ACID COMPOSITIONS (MOL% OF THE TOTAL) OF THE TRIACYLGLYCEROLS OF RAT PARAMETRIAL ADIPOSE TISSUE AND OF FRACTIONS OF THIS OBTAINED BY SILVER ION HPLC

Abbreviations: see Table I.

| | *Total* | *SSS* | *SSM* | *SMM* | *SSD* | *MMM* | *SMD* | *MMD* | *SDD + SST* | *MDD + SMT* | *DDD + SDT* | *Check* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 14:0 | 2.3 | 2.3 | 1.4 | 0.9 | 1.8 | | 1.0 | | 1.2 | 0.3 | 0.9 | 1.0 |
| 15:0 | 0.5 | 0.9 | 0.6 | 0.4 | 0.7 | | 0.3 | | 0.5 | 0.2 | 0.4 | 0.4 |
| 16:0 | 32.6 | 84.8 | 55.9 | 28.6 | 54.7 | | 27.4 | | 28.9 | 6.7 | 18.2 | 32.3 |
| 16:1 | 7.6 | | 5.7 | 12.3 | | 17.2 | 5.9 | 12.0 | | 5.9 | | 6.1 |
| 17:0 | 0.3 | 0.6 | 0.5 | 0.2 | 0.4 | | 0.3 | | 0.3 | | | 0.3 |
| 18:0 | 3.3 | 11.4 | 6.8 | 3.2 | 6.3 | | 2.9 | | 3.0 | 1.0 | 2.0 | 3.7 |
| 18:1($n-9$) | 22.7 | | 26.8 | 48.9 | | 72.0 | 23.6 | 44.3 | | 21.0 | | 24.4 |
| 18:1($n-7$) | 3.2 | | 2.3 | 5.6 | | 10.7 | 3.7 | 8.1 | | 4.2 | | 3.4 |
| 18:2 | 26.1 | | | | 36.1 | | 34.9 | 35.6 | 64.1 | 54.8 | 65.7 | 27.3 |
| 18:3 | 1.4 | | | | | | | | 2.0 | 6.2 | 12.8 | 1.0 |
| % of the total | | 3.8 | 14.3 | 15.5 | 12.5 | 3.2 | 22.1 | 8.9 | 9.5 | 7.5 | 2.7 | |

of the various types of fatty acid expected, and the check on the recovery by computation of the overall composition from those of the fractions is excellent.

A more challenging separation perhaps is that of the linoleic acid-rich seed oils used for human consumption, and the separation of sunflower seed oil, in which the same gradient elution scheme as for the last sample was used, is illustrated in Fig. 3. Again, well-shaped peaks are obtained and excellent resolution of all the main fractions is achieved with species containing linoleic acid being predominant. The quantitative data on the fatty acid compositions and the proportions of the various molecular species is listed in Table III. As before, the check on the overall composition indicates that the recovery is essentially complete. Very similar results were obtained with maize (corn) and safflower oils (not illustrated) except that the relative heights of the main peaks varied somewhat. In some commercial samples of these vegetable oils, there appeared to be some isomerised linoleic acid, and additional small peaks containing these components eluted in front of the corresponding main peaks on silver ion chromatography.

By increasing the proportion of acetonitrile in the final stages of elution to a higher level, it proved possible to obtain good separations of molecular species of seed oils containing trienoic acids. Fig. 4 illustrates the separation of linseed oil, which contains a high proportion of 18:3 ($n-3$), and the compositional data are listed in Table IV. In this instance also, a large number of fractions, up to and including trilinolenin, are resolved. Many of these comprise single molecular fractions, and only those species containing a single linolenoyl residue tend to overlap with those having two linoleoyl residues. In spite of the high degree of unsaturation, the compositions of each fraction contain close to the expected proportions of the fatty acids, while the check involving summation of the composition of the various fractions confirms that quantitative recoveries are obtained. With evening primrose oil, which contains 8% of $\gamma$-linolenic acid [18:3($n-6$)], fractions up to DDT were eluted but some small changes in the order of elution were observed in comparison to linseed oil that were presumably

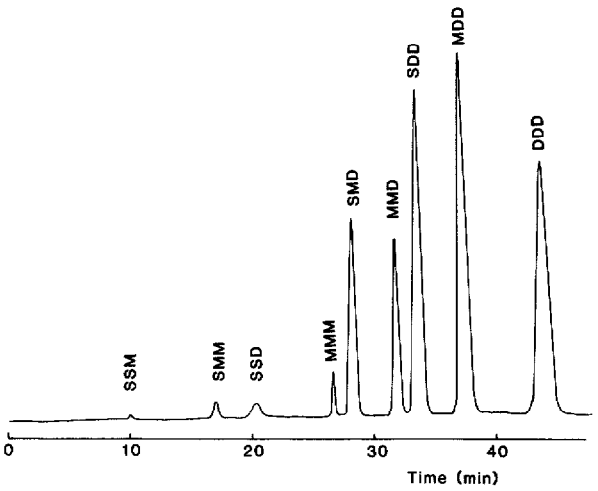


Fig. 3. Separation of triacylglycerols from sunflower seed oil by HPLC with a silver ion column and mass detection. See the Experimental section for practical details, and Table I for abbreviations.

TABLE III

FATTY ACID COMPOSITIONS (MOL% OF THE TOTAL) OF THE TRIACYLGLYCEROLS OF SUNFLOWER SEED OIL AND OF FRACTIONS OF THIS OBTAINED BY SILVER ION HPLC

Abbreviations: see Table I.

| | *Total* | *SSM* | *SMM* | *SSD* | *MMM* | *SMD* | *MMD* | *SDD* | *MDD* | *DDD* | *Check* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 16:0 | 7.3 | 37.2 | 17.5 | 36.9 | | 19.6 | | 17.4 | | | 6.7 |
| 18:0 | 4.9 | 27.7 | 12.8 | 27.5 | | 11.7 | | 14.4 | | | 5.0 |
| 18:1 | 21.9 | 35.1 | 69.6 | | 100.0 | 34.2 | 61.9 | | 31.9 | | 23.4 |
| 18:2 | 65.9 | | | 35.5 | | 34.5 | 38.1 | 68.2 | 68.1 | 100.0 | 64.9 |
| % of the total | | 0.6 | 2.3 | 2.8 | 3.9 | 10.2 | 9.3 | 17.7 | 26.8 | 26.6 | |

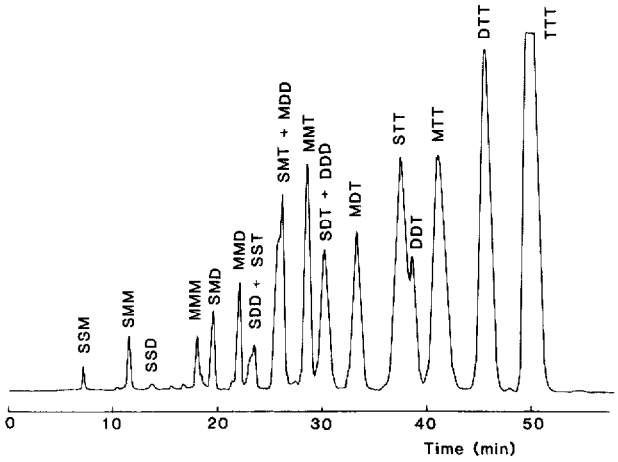


Fig. 4. Separation of triacylglycerols from linseed oil by HPLC with a silver ion column and mass detection. See the Experimental section for practical details, and Table I for abbreviations.

due to the different position of the double bonds in the trienoic component (separation not illustrated). Thus, SST and MMD elute together, SDT elutes just ahead of DDD, while MDT elutes just after DDD, *i.e.* fractions containing 18:3($n-6$) tend to elute before those containing 18:3($n-3$).

The ultimate test of the methodology is whether it will give acceptable separations of molecular fractions from a fish oil that contains substantial amounts of polyunsaturated fatty acids including 20:5($n-3$) and 22:6($n-3$). In the sample of triacylglycerols from a commercial fish oil of South African origin used here, these comprised 18.3 and 7.4% respectively of the total fatty acids. With an elution scheme

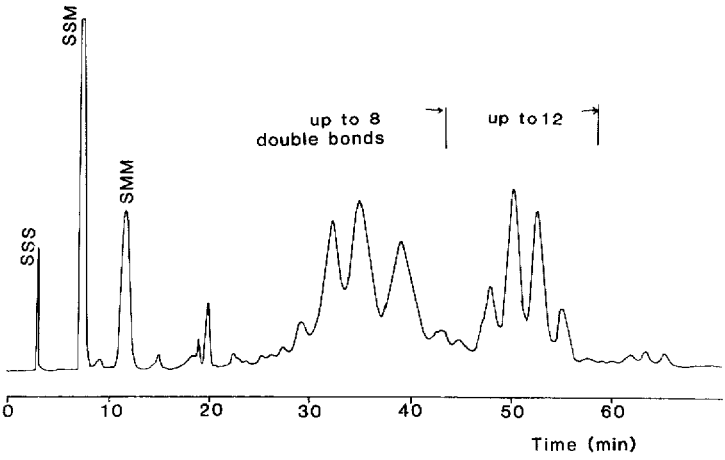


Fig. 5. Separation of triacylglycerols from a commercial fish oil of South African origin by HPLC with a silver ion column and mass detection. See the Experimental section for practical details, and Table I for abbreviations.

TABLE IV

FATTY ACID COMPOSITIONS (MOL% OF THE TOTAL) OF THE TRIACYLGLYCEROLS OF LINSEED OIL AND OF FRACTIONS OF THIS OBTAINED BY SILVER ION HPLC

Abbreviations: see Table I.

| | *Total* | *SSM* | *SMM* | *SSD* | *MMM* | *SMD* | *MMD* | *SST + SDD* |
|---|---|---|---|---|---|---|---|---|
| 16:0 | 6.3 | 32.4 | 14.0 | 28.5 | | 15.9 | | 28.5 |
| 18:0 | 3.2 | 32.7 | 20.6 | 32.8 | | 12.0 | | 16.5 |
| 18:1 | 17.0 | 34.8 | 65.5 | | 100.0 | 36.7 | 64.8 | |
| 18:2 | 15.8 | | | 38.7 | | 35.4 | 35.2 | 40.4 |
| 18:3 | 57.7 | | | | | | | 14.6 |
| % of the total | | 0.3 | 0.8 | 0.6 | 1.3 | 1.4 | 2.1 | 1.9 |

similar to that for linseed oil but extended somewhat, the separation shown in Fig. 5 was obtained. The resolution is far from perfect, but some considerable simplification is obviously possible. Because of the complexity, a few representative fractions only were analysed to determine the nature of the separations, but it is evident that species with twelve and more double bonds in total are eluted. Unfortunately, it appeared that in spite of a careful purification some autoxidised or polymerised material remained that contributed to peak broadening. As many positional isomers of unsaturated fatty acids, differing in chain length, are also present, some peak broadening is probably inevitable. The potential of the procedure for this type of separation is evident, nonetheless, and the work will be repeated when a better defined and fresher sample of fish triacylglycerols is available.

## DISCUSSION

The results described above illustrate that HPLC in the silver ion mode can replace analogous TLC procedures for the separation of the full range of triacylglycerols likely to be encountered in natural samples. Excellent resolution is obtained in general, and indeed the time for each fractionation can be reduced appreciably by increasing the flow-rate of the mobile phase or the rate of change of the gradient. The mass detector could certainly be used for quantification purposes if need be following careful calibration, but better results would probably be obtained with a detector operating on the transport-flame ionisation principle. However, the lengthy procedure of transesterification in the presence of an internal standard for GC determination, that is employed here, has the merit of giving detailed information on the composition of each fraction. In contrast to fractions obtained by silver ion TLC, the solvent eluting from the silver ion HPLC column contains no silver as an impurity so no clean-up step is required prior to transesterification. Most of the natural samples analysed here have been analysed previously by silver ion TLC (as reviewed by Litchfield[6]), but never with the convenience and clarity obtained here.

A column of this type has been in continuous use in the author's laboratory for miscellaneous separations with no detectable loss of resolution for more than one year. Periodically, there is a build up in the operating pressure, that appears to be due to the

| *SMT + MDD* | *MMT* | *SDT + DDD* | *MDT* | *STT + DDT* | *MTT* | *DTT* | *TTT* | *Check* |
|---|---|---|---|---|---|---|---|---|
| 13.9 | | 14.9 | | 11.0 | | | | 4.5 |
| 9.6 | | 9.1 | | 8.6 | | | | 3.3 |
| 32.6 | 65.1 | | 33.1 | | 31.8 | | | 15.9 |
| 17.7 | | 48.3 | 33.2 | 21.3 | | 31.3 | | 15.7 |
| 26.2 | 34.9 | 27.8 | 33.7 | 59.1 | 68.2 | 68.7 | 100.0 | 60.7 |
| 6.6 | 6.2 | 5.0 | 6.3 | 15.1 | 12.1 | 14.4 | 25.9 | |

presence of material tightly bound to the stationary phase, but this can be cleared with relative ease by elution with methanol–acetonitrile (1:1, v/v) into which a solution of a few milligrams of silver nitrate in acetonitrile is injected.

The order of elution of triacylglycerol species is comparable but not identical to that obtained by TLC, but this may be due in part to variation in the mobile phases used by analysts. However, the only important difference is that those fractions containing one linoleoyl residue are sometimes separated from those containing two dienoic fatty acids in the TLC procedures[11,12]. With the HPLC elution schemes described here, the order of migration is SSS > SSM > SMM > SSD > MMM > SMD > MMD > SDD = SST > SMT = MDD > MMT > SDT = DDD > MDT > STT ⩾ DDT > MTT > DTT > TTT, where T is 18:3($n-3$). When the positional isomer of linolenic acid, *i.e.* 18:3($n-6$), is present, the species containing this component are retained less strongly. Internal double bonds in polyunsaturated fatty acids almost certainly interact less strongly with silver ions than do outer ones, otherwise fractions with twelve or more double bonds from fish oils would not have eluted in a reasonable time. It should, therefore, be possible to analyse triacylglycerols from any natural source by this means.

In recent years many excellent separations of triacylglycerol species have been obtained by HPLC in the reversed-phase mode[3]. Separations are then attained both according to the combined chainlengths of the fatty acyl residues, and the total number of double bonds, each one reducing the retention time of the species by the equivalent of about two methylene groups. It is then rarely easy to identify components of complex mixtures. Fewer fractions are obtained when HPLC in the silver ion mode is employed, but as the separation is on the basis of a single molecular property, it is a rather simpler problem to identify components. In the analysis of confectionary fats especially, it can be important to determine the proportions of the trisaturated and disaturated–monounsaturated species by a rapid method as these govern the physical properties. The technique described here should permit analyses of this kind in a manner suited to routine quality control. On the other hand, it may be of particular value to consider silver ion chromatography as a complementary technique to reversed-phase procedures rather than as a rival. Many more molecular species can be isolated by subjecting fractions isolated by silver ion HPLC to reversed-phase

chromatography than would be possible using either technique on its own.

Although the elution schemes described here are for triacylglycerols only, they could almost certainly be adapted with little difficulty to the fractionation of other simple lipids, such as cholesterol esters or wax esters, and to the analysis of diacylglycerol derivatives prepared by the phospholipase C-catalysed hydrolysis of phospholipids. A priori, there would appear to be no reason why intact glycosphingolipids or phospholipids should not be resolved in a similar manner after further modifications to the mobile phase.

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