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SEPARATION OF *cis* AND *trans* ISOMERS OF UNSATURATED FATTY ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE SILVER ION MODE

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SUMMARY

High-performance liquid chromatography in the silver ion mode has been adapted for the analysis of positional and geometrical isomers of fatty acids, and especially for the determination of *trans* unsaturation in fats and oils. The stationary phase consisted of an ion-exchange medium, which was a silica gel matrix with bonded sulphonic acid moieties, loaded with silver ions. Fatty acids were converted into phenacyl esters so that UV detection at 242 nm could be used. The mobile phase for the separation of *trans*- and *cis*-monoenoic isomers was 1,2-dichloroethane–dichloromethane (1:1, v/v), and 0.5% of acetonitrile was added in order to elute geometrical isomers of linoleic and linolenic acids. Gradient elution was employed for mixtures containing a full range of components. Provided that the column temperature was kept constant, the retention times were reproducible over long periods of time, and the response of the detector was related in a rectilinear manner to the molar proportion of each fatty acid, irrespective of the structure. Monoenoic isomers from commercially hydrogenated soybean oil were identified by gas chromatography–mass spectrometry of picolinyl ester derivatives. The method was applied to natural fats containing *trans* double bonds, *i.e.*, sheep adipose tissues from three sites, to commercial margarines and cooking fats, and to soybean oil, rapeseed oil and a fish oil at various times during hydrogenation.

INTRODUCTION

A number of methods have been devised for the separation and/or determination of *cis* and *trans* isomers of unsaturated fatty acids (reviewed elsewhere[1]), but none of these is entirely satisfactory. Infrared spectrometry has been much used for this purpose[2], but gas chromatography (GC) on long packed columns or capillary columns of fused silica with highly polar liquid stationary phases now appears to be generally preferred. The effectiveness of the latter procedure has been widely debated[3–19], however, as it is not always easy to distinguish between peaks representing *cis*- and *trans*-monoenes.

Silver ion chromatography is also a useful technique for separating geometrical

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isomers of fatty acids (as the methyl ester derivatives) for subsequent analysis by GC, but the thin-layer chromatographic (TLC) procedures used hitherto have limited resolution, and are costly and messy[20]. On the other hand, a stable ion-exchange column loaded with silver ions has been developed for high-performance liquid chromatography (HPLC)[21] that has proved of value in the simplification of complex mixtures of fatty acids of natural origin for subsequent identification by GC–mass spectrometry (MS)[22–24] and for separating molecular species of triacylglycerols[25]. The procedures are rapid, reproducible and give clean fractions, uncontaminated by silver ions. Applications of this column to the isolation and determination of fatty acids containing *trans* double bonds in samples of natural and industrial origin are described in this paper.

EXPERIMENTAL

Materials and reagents

Lipid standards and reagents were supplied by Sigma (Poole, U.K.). All solvents were of AnalaR or HPLC grade and were supplied by FSA Scientific Apparatus (Loughborough, U.K.). Margarines and cooking fats were commercial brands and were purchased in a local supermarket. Adipose tissue samples were obtained from a freshly killed sheep of the Institute flock. Partially hydrogenated oils were donated by Dr. G. Thaxton of Van den Berghs & Jurgens (Purfleet, U.K.). Mixtures of the geometrical isomers of linoleic and linolenic acids were prepared from the parent compounds by nitric oxide-catalysed isomerization[26]. The reaction time was varied by trial and error to optimize the yield of products.

High-performance liquid chromatography

The HPLC equipment and the silver ion column were as described previously[22]. In brief, a Spectra-Physics (St. Albans, U.K.) Model 8700 solvent-delivery system was used, together with a Pye Unicam (Cambridge, U.K.) PU 4025 UV detector operated at 242 nm. A column (250 × 4.6 mm I.D.) of Nucleosil 5SA (kindly donated by Applied Chromatography Systems, Macclesfield, U.K.) was flushed with 1% 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 on to the column via the Rheodyne valve in 50-μl aliquots at 1-min intervals; silver began to elute from the column after about 10 min, and 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 1 h.

For most of the analytical work, the column temperature was maintained at 38°C in a thermostated oven. 1,2-Dichloroethane–dichloromethane (1:1, v/v) (mixture A) at a flow-rate of 1.5 ml/min was the mobile phase for the separation of isomeric monoenes, and the same solvent with the addition of 0.5% acetonitrile (mixture B) at a flow-rate of 0.75 ml/min was employed for isomeric dienes and trienes. With samples containing a wide range of components, such as hydrogenated fats, the column was eluted with solvent A for 13 min, then changed in one step to A–B (75:25) with a gradient to 100% B over 20 min.

Derivatization

Lipids were hydrolysed with 1 *M* potassium hydroxide in 90% ethanol at room temperature overnight. After acidification and extraction, the free fatty acids were converted into the phenacyl derivatives as described by Wood and Lee[27]. Prior to HPLC analysis, phenacyl esters were purified by elution from a Bond Elut NH_2 column with hexane–diethyl ether (9:1, v/v). The methyl ester derivatives of the fatty acids were prepared from lipid samples by sodium methoxide-catalysed trans-esterification[28]. Picolinyl ester derivatives were prepared as described elsewhere[29]; in brief, the mixed anhydride of each fatty acid with trifluoroacetic acid was reacted with 3-(hydroxymethyl)pyridine (10-fold molar excess) in the presence of 4-dimethyl-aminopyridine (1.2 molar proportion) as catalyst.

Gas chromatography and gas chromatography–mass spectrometry

For analytical purposes, a Carlo Erba (Crawley, U.K.) Model 4130 capillary gas chromatograph, 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.). The temperature was programmed from 165°C (held for 3 min) at 4°C/min to 195°C (held for 20 min). Hydrogen was the carrier gas. Components were quantified by electronic integration. The derivatives were submitted to GC–MS as described elsewhere[30] (except that the upper temperature of the column was 10°C lower), *i.e.*, a fused-silica capillary column (25 m × 0.2 mm I.D.), coated with a cross-linked (5% phenylmethyl) silicone (Hewlett-Packard, Wokingham, U.K.), with helium as carrier gas, was programmed from 60 to 220°C at 50°C/min then to 250°C at 1°C/min. The column outlet was connected directly to the ion source of a Hewlett-Packard 5970 mass-selective detector, operated at an ionization energy of 70 eV.

RESULTS AND DISCUSSION

Many different derivatives of fatty acids have been employed for the separation of fatty acids by HPLC[31], and strongly UV-absorbing esters such as the phenacyl derivatives have the advantage that they can be detected with some sensitivity by their absorbance at 242 nm. In addition, the detector response is to the molar proportion rather than the weight proportion of the compound. Phenacyl derivatives were therefore used here.

Preliminary experience with the silver ion column indicated that excellent results were obtainable with chlorinated solvents as the mobile phase[21,25]. Dichloromethane gave the best results but was too volatile alone, so 1,2-dichloroethane–dichloro-methane (1:1, v/v) was employed initially. This gave baseline separations of the phenacyl derivatives of 9-*trans*-, 11-*trans*-, 9-*cis*- and 11-*cis*-octadecenoic acids. Standard mixtures containing these components were used to check the linearity of the detector response. Initially, this was found to be variable, as were the elution times of the compounds. As the strength of complex formation between the silver ions and double bonds is known to be affected appreciably by temperature[32], the column was placed in a thermostated oven. It seems probable that better results would be obtained if this were maintained at a sub-ambient temperature, but 38°C was the lowest stable temperature possible with the equipment available. When the column temperature was

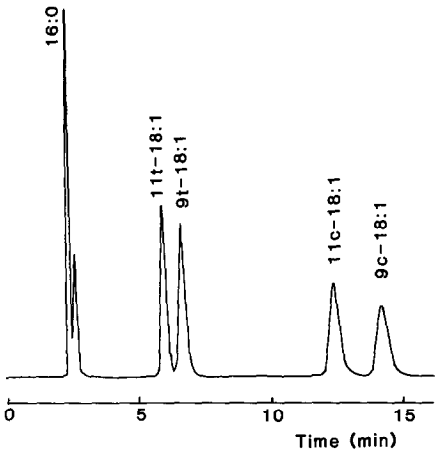

Fig. 1. Separation of the phenacyl derivatives of 16:0, 11*t*-18:1, 9*t*-18:1, 11*c*-18:1 and 9*c*-18:1 by HPLC in the silver ion mode. The column temperature was 38°C and the mobile phase was 1,2-dichloroethane–dichloromethane (1:1, v/v) at a flow-rate of 1.5 ml/min with detection at 242 nm.

kept constant in this way, the response of the detector to all fatty acid derivatives was found to be the same and constant, and relative to mass was rectilinear and passed through the origin (0–200 μg), while the elution times were constant over long periods of use of the column. The nature of the separation obtained with the standard mixture is illustrated in Fig. 1. Excellent resolution is achieved and the peak shapes are close to symmetrical. The results of a comparison of the composition of this standard mixture with that determined by the HPLC procedure (isocratic elution) are listed in Table I. It can be seen that the agreement is indeed excellent and that the standard deviations are small.

Large numbers of positional and geometrical isomers of monoenoic fatty acids are produced during the hydrogenation of vegetable oils and an application of the separation procedure to partially hydrogenated soybean oil is illustrated in Fig. 2.

TABLE I

COMPARISON BETWEEN THE ACTUAL COMPOSITIONS (%) OF STANDARD MIXTURES (DETERMINED GRAVIMETRICALLY) AND THOSE DETERMINED BY ISOCRATIC AND GRADIENT HPLC PROCEDURES (MEAN ± STANDARD ERROR OF FOUR DETERMINATIONS)

c = cis; *t* = trans.

Isocratic elution scheme			*Gradient elution scheme*		
Fatty acid	*Actual*	*Found*	*Fatty acid*	*Actual*	*Found*
16:0	35.13	34.79 ± 0.16	16:0	7.26	6.97 ± 0.04
11*t*-18:1	9.91	9.71 ± 0.12	*trans*-Monoenes	13.04	12.91 ± 0.03
9*t*-18:1	22.52	22.18 ± 0.32	*cis*-Monoenes	13.04	12.21 ± 0.03
11*c*-18:1	9.91	10.04 ± 0.50	Dienes	33.15	35.16 ± 0.15
9*c*-18:1	22.52	23.29 ± 0.25	Trienes	33.52	32.74 ± 0.15

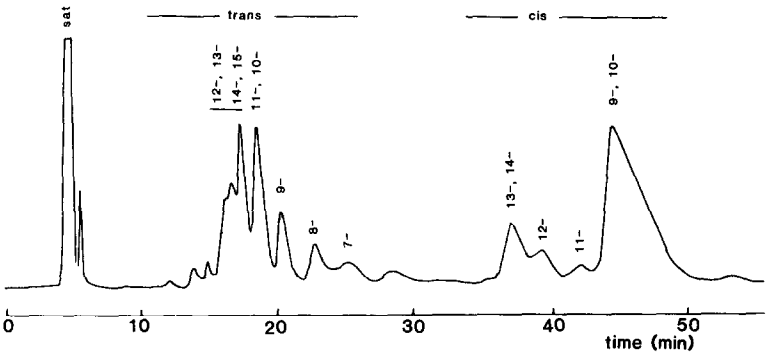

Fig. 2. Separation of the phenacyl derivatives of the saturated and monoenoic fatty acids from hydrogenated soybean oil by HPLC in the silver ion mode. Experimental condition as in the legend to Fig. 1. Abbreviation: sat = saturated fatty acids.

Relatively few standards are available for identification purposes, so peaks were collected as they eluted and were converted into the picolinyl ester derivatives for examination by GC–MS[30,33]. With such a complex mixture, some overlap is inevitable, but most of the main products were identified and are labelled in Fig. 2. A systematic study of the migration of isomeric octadecenoates on thin layers of silicagel impregnated with silver nitrate indicated a sinusoidal relationship between double bond position and complex formation with the silver ions, with double bonds in positions 5–7 being retained most strongly and those in positions 11–13 being affected less strongly than adjacent isomers[34]. A broadly similar phenomenon appears to be seen with the silver ion HPLC column in this work. Whereas a superficially similar chromatographic trace was obtained with an HPLC column containing silica gel impregnated with silver nitrate in work reported from another laboratory, the order of elution of the individual components was reportedly different (isomers were not identified by unequivocal means, however)[35].

In some samples, fatty acids with a conjugated diene system (predominantly the 9-*cis*,11-*trans* isomer) tended to co-elute with the last of the *trans*-monoenes. A similar phenomenon has been noted with some silver ion TLC systems[36].

Similarly, geometrical isomers of linoleic and linolenic acids, the most abundant polyenoic fatty acids in seed oils, are not available commercially, but mixtures can be prepared from the parent compounds by nitric oxide-catalysed isomerization. After conversion to the phenacyl derivatives, the various components eluted slowly with chlorinated solvent alone as the mobile phase, but addition of only 0.5% of acetonitrile speeded up the separation appreciably. The separation achieved with the isomeric dienes derived from linoleic acid is shown in Fig. 3A. Three peaks are apparent, the last of which is the natural 9-*cis*,12-*cis* isomer; the first must be 9-*trans*,12-*trans*-octadecadienoate, and the second peak is presumably a mixture of the 9-*cis*,12-*trans* and 9-*trans*,12-*cis* compounds. The separations are better than those reported from HPLC in the reversed-phase mode[27]. With the geometrical isomers of linolenic acid and the same isocratic HPLC system, six peaks emerge, the first of which is presumably the all-*trans* isomer and the last the all-*cis* isomer (Fig. 3B). Eight isomers should be formed in the reaction. A substantial amount of chemical degradative work would be

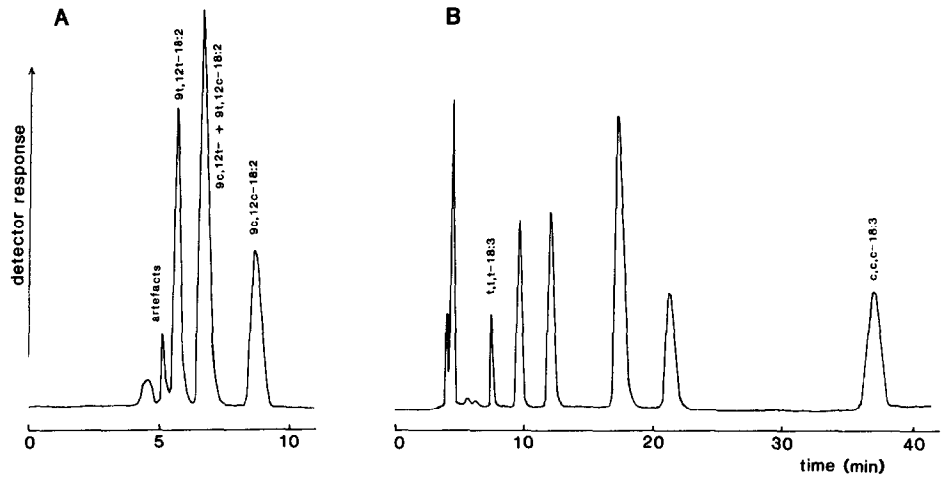

Fig. 3. Separation of the phenacyl derivatives of the geometrical isomers of (A) linoleic and (B) linolenic acids by HPLC in the silver ion mode. The column temperature was 38°C and the mobile phase was 1,2-dichloroethane–dichloromethane–acetonitrile (49.75:49.75:0.5, v/v/v) at a flow-rate of 0.75 ml/min with detection at 242 nm. Note the change of scale on the time axis.

necessary to identify the remaining isomers. In this instance, the resolution was better than that attained by capillary GC[37]. The potential of the system for the isolation of related isomers, from insect pheromones for example, is obvious.

Although all-*trans*-octadecatrienoate tended to elute with the *cis*-dienes, this was not a problem in practice with samples of hydrogenated fats containing dienoic and trienoic fatty acids. A gradient elution scheme was adapted from the isocratic mobile phases used for monoenes and polyenes for the separation of such mixtures. As discussed above, the response of the detector to different fatty acids is constant, as only the phenacyl group affects this. Provided that the temperature of the column was kept constant, the elution times were reliable. The accuracy and reproducibility were determined by analysing a standard mixture of palmitic, oleic, linoleic and linolenic acids that had been isomerized with nitric oxide as the catalyst. The results (listed in Table I) show excellent agreement between the theoretical and actual value with small standard errors. When the system was applied to real samples, a spurious but constant peak was obtained in blank runs and tended to coincide with the main diene peak. This was eliminated mathematically in calculating the results.

The system can be used to determine the relative proportions of different geometrical isomers in oils and fats of natural or commercial origin. Some examples are listed in Table II. The adipose tissue from ruminant animals contains fatty acids with *trans* double bonds that are formed during biohydrogenation in the rumen. The data obtained here are higher than the mean values recorded elsewhere[38], but results from individual sheep can vary appreciably. As a check, samples were also analysed by capillary GC on a column that did not differentiate between geometrical isomers, and the results were mutually consistent.

Several commercial margarines and cooking fats that were examined using the procedure were found to contain between 4.5 and 20.6% of *trans*-monoenes. In these

TABLE II

FATTY ACID COMPOSITIONS (MOL%) OF SOME OILS AND FATS CONTAINING *trans*-UNSATURATION

Source	*Fatty acids*				
	Saturated	*trans-Monoenes*	*cis-Monoenes*	*Dienes*	*Trienes*
Sheep adipose tissues:					
Intestinal	60.2	8.2	27.4	3.4	0.8
Subcutaneous	52.5	7.3	36.1	4.1	
Perirenal	61.1	7.2	24.9	6.8	
Margarines:					
A	43.6	17.2	22.8	13.4	3.0
B	45.8	20.6	17.5	12.7	3.4
C	44.8	6.2	39.5	8.3	1.3
D	43.5	4.5	41.9	8.6	1.5
Cooking fats:					
E	42.4	18.8	17.8	20.2	0.7
F	21.3	11.1	44.9	16.2	6.5
Hydrogenated soybean oil:					
Start	13.3	2.0	23.7	53.1	7.8
Mid-point	16.7	25.9	38.1	17.4	1.9
End	28.9	51.0	17.5	2.6	
Hydrogenated rapeseed oil:					
Start	10.0	1.5	54.1	26.2	8.3
Mid-point	9.5	4.9	51.5	23.4	10.7
End	13.8	12.8	56.5	12.7	4.2
Hydrogenated fish oil:					
Start	44.5	4.0	38.6	12.9	
Mid-point	44.3	10.0	32.2	14.6	
End	39.5	16.3	18.6	25.6	

samples, the *trans*-monoenes are formed as by-products of the catalytic hydrogenation process used to alter the physical properties of native vegetable or fish oils. To demonstrate the efficacy of the HPLC methodology, it was applied to some oils taken at various stages of a commercial hydrogenation process. In each instance, the content of *trans*-monoenoic fatty acids is low just after the start of hydrogenation and rises rapidly as the reaction progresses, although the absolute degree of hydrogenation varied among the samples. The soybean oil received much more processing than did the rapeseed oil, which has already a high content of monoenoic fatty acids. The proportion of *trans* fatty acids in the former rises quickly until it is half of the total, while the content of di- and trienoic acids in the soybean oils decreases at the same rate over the period, as expected. With the hydrogenated fish oil, the analytical results indicate only part of the process as the tetra- to hexaenoic components are not eluted by the HPLC system used here. However, there is a rise in the content of *trans*-monoenes as the reaction progresses together with increases in the proportion of dienoic components presumably, formed by partial hydrogenation of the poly-unsaturated fatty acids. Hydrogenated fish oils are notoriously difficult to analyse

because of the wide range of chain lengths and positional isomers in the fatty acid constituents. It should be possible to extend the gradient elution system to obtain a more comprehensive analysis with such samples. Again, a broad check on the data by GC gave consistent results.

In continued use over 18 months, the column lost some of its resolving power. This could be restored in part by washing the column with polar solvents such as methanol–acetonitrile mixtures and by injecting fresh silver nitrate on to the column at 1–2 monthly intervals. It was eventually necessary to replace the column.

HPLC in the silver ion mode, with a column of the type described here, therefore has the capacity to provide an alternative means to those in use elsewhere for the separation and analysis of fatty acids with *trans* unsaturation in fats and oils. It may also be of value for the isolation of positional and geometrical isomers for structural or other studies.

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