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Silver ion high-performance liquid chromatography of isomeric *cis*- and *trans*-octadecenoic acids
Effect of the ester moiety and mobile phase composition

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Abstract

The effects of the ester moiety and of the mobile phase composition on the resolution of *cis* and *trans* positional isomers of octadecenoates have been studied by silver ion HPLC with UV detection. The efficiency of the separation increases in the order phenethyl<phenacyl<*p*-methoxyphenacyl esters. Retention and resolution are substantially affected by small changes in the proportion of acetonitrile in the mobile phase. Dichloromethane influences retention but has a small effect on resolution. Clear resolution of *cis* and *trans* positional isomers of octadecenoic acid in partially hydrogenated sunflower oil has been achieved after conversion into *p*-methoxyphenacyl esters on a silver ion column by isocratic elution with a mobile phase of hexane–dichloromethane–acetonitrile (60:40:0.2, v/v) in only 22 min.

Keywords: Silver ion liquid chromatography; Geometrical isomers; Positional isomers; Derivatization, LC; Mobile phase composition; Octadecenoic acids; Fatty acids

1. Introduction

A simple procedure for preparation of a stable silver ion column for high-performance liquid chromatography (Ag-HPLC) [1] and the appearance later of a commercial column of this type (ChromSpher Lipids™ from Chrompack) has caused a rapid revival of this technique in lipid analysis [2,3]. It is accepted by many authors that silver ion chromatography has no rival for clear and unambiguous differentiation between lipid species differing in the number and, particularly, the configuration of double bonds [2,4–6]. Methods for separation and determination of *cis* and *trans* fatty acids in modified oils and fats are of importance in industrial processing, but also for human nutrition and metabolic studies. Efficient procedures based on Ag thin-layer chromatography (Ag-TLC) [7] and Ag-HPLC [8–10] are now available and can solve most of the problems of the separation and determination of geometrical isomers.

The modification of plant oils by partial hydrogenation presents a greater challenge for analysis. Series of isomers that differ in double bond position and geometry are formed. Thus, besides the separation of *cis* from *trans* isomers, resolution of the positional isomers is required in each group. The usual method chosen is gas chromatography (GC)

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[11] on long (50–100 m) highly polar capillary columns. Despite the high efficiency of these columns, overlapping of *cis* and *trans* isomers and formation of mixed peaks are observed when GC is applied as the only method [12]. Better and less ambiguous results are obtained when fatty acids are first fractionated into *cis* and *trans* groups by Ag-TLC [13] or Ag-HPLC [14] and then analysed by GC. Undoubtedly, the best solution is to perform the entire analysis by a single Ag-HPLC separation. The potential of this technique was first demonstrated by Christie and Breckenridge [8] on the resolution of the phenacyl esters of partially hydrogenated soybean oil. Recently, Adlof and co-workers described the resolution of positionally isomeric *cis* and *trans* octadecenoic fatty acids (as methyl esters) from a partially hydrogenated vegetable oil, and thus confirmed the high efficiency and great practical significance of Ag-HPLC in this analysis [15].

On examining the behaviour of methyl and phenacyl esters of positionally isomeric octadecenoic and octadecadienoic fatty acids on an Ag-HPLC column [16], we found that, as in Ag-TLC of methyl esters [17], retention depends on the position of the double bond in the chain. An unexpected finding was that the k' values were strongly affected by the nature of the ester moiety. Derivatization with unsaturated alcohols [18], with normal- and branched-chain alcohols and with substituted aromatic compounds has been also examined [19], and some of these provided promising results for practical purposes. Improved resolution of the three naturally occurring 18:1 isomers, petroselinic (*cis*-6-18:1), oleic (*cis*-9-18:1) and *cis*-vaccenic (*cis*-11-18:1) acids, as phenacyl derivatives (compared to methyl esters) was demonstrated, for example. A method for their determination in seed oils by Ag-TLC has been proposed recently [20].

The subject of the present work was to examine the effect of the ester moiety on the resolution of *cis* and *trans* positionally isomeric octadecenoic fatty acids after conversion into phenethyl, phenacyl and *p*-methoxyphenacyl derivatives. Conditions were found for clear separation of *trans* from *cis* fatty acids and simultaneous resolution of positional isomers within each group. A single laboratory-prepared Ag-HPLC column was utilised with UV detection. The procedure was then applied to a sample of esters from partially hydrogenated sunflower oil.

2. Experimental

2.1. Materials

Dichloromethane and acetonitrile were HPLC-grade solvents and were used without further purification. All other solvents were analytical grade. Hexane, when used as mobile phase component for Ag-HPLC, was left for 24 h over potassium hydroxide and then distilled. The isomeric *cis*- and *trans*-octadecenoic fatty acids and the derivatizing reagents were from Sigma-Aldrich (Poole, UK). The partially hydrogenated sunflower oil sample was a gift of the late Professor A. Andreev, from the Institute of Catalysis at the Bulgarian Academy of Sciences.

2.2. Derivatization

Phenethyl derivatives were prepared by acid-catalysed esterification according to Liu and Hammond [21]. The sample (10 mg) was dissolved in hexane (0.5 ml) and 1 ml of 1% sulphuric acid in phenethyl alcohol was added. The mixture was heated for 1 h in boiling water. Aqueous sodium chloride (ca. 1%) was added and the derivatives were extracted twice with 1 ml of hexane. The hexane extracts were concentrated to ca. 0.5 ml (under nitrogen) and the derivatives were purified by TLC on silica gel G on laboratory-made glass plates (4×19 cm) with a mobile phase of light petroleum–acetone (100:10, v/v). The band was detected under UV light after spraying (the edges only, after careful covering the rest of the plate) with 2,7-dichlorofluorescein solution. The products were recovered from the silica gel band by elution with diethyl ether.

Phenacyl and *p*-methoxyphenacyl derivatives were produced exactly as described by Wood and Lee [22] from the free fatty acids, after the partially hydrogenated oil sample (2 mg) was hydrolysed with 0.1 *M* potassium hydroxide in 95% ethanol [4]. Several clean-up procedures were tested for the purification of the phenacyl and *p*-methoxyphenacyl esters: elution from Florisil mini column (Pasteur pipette) or

from a Florisil Isolute™ column (500 mg) (International Sorbent Technology, Mid Glamorgan, UK) with hexane–acetone (99:1) or hexane–diethyl ether (10:0.5, v/v); elution from a BondElut™ NH_2 column (Analytichem International, Cambridge, UK) or Isolute™ NH_2 column (International Sorbent Technology) with hexane–diethyl ether (9:1, v/v); TLC on silica gel with mobile phase light petroleum–acetone (100:10, v/v). The purity of the products was checked by TLC (Alufolio silica gel 60, Merck, Darmstadt, Germany) with a mobile phase of hexane–acetone (100:8, v/v).

2.3. Silver ion TLC

The three major fatty acid groups of the oil sample were determined by Ag-TLC as described by Chobanov et al. [7]. Saturated, total *trans* and total *cis* methyl octadecenoates (positional isomers not differentiated) were detected by charring and quantified by densitometry in the reflection zigzag mode at 450 nm and 1.2×1.2 mm beam-slit (Shimadzu CS-930 densitometer, Shimadzu DR-2 electronic integrator).

2.4. Silver ion HPLC

An ISCO (Lincoln, NE, USA) HPLC system equipped with model 2350 isocratic pump, Valco C6W injection valve with 10 μl sample loop and V4 UV/Vis detector was used. The column of Nucleosil 100-5SA (250×4.6 mm; Hichrom, Reading, UK) was converted to the silver ion form as described earlier [1]. The mobile phase hold-up time was determined by repeated injection of benzene. The mean value of six measurements was 2.26±0.01 min at 21°±2°C. Samples, ca. 2 μg of each standard fatty acid (approximately 25 μg in total) and 20 μg from the partially hydrogenated oil, were injected as solutions in hexane (10 μl). Mixtures of hexane–dichloromethane–acetonitrile were used as mobile phase at a flow-rate of 1.5 ml/min. The proportions depended on the nature of derivative and are given in the Section 3.

Retention factors ($k' = t_r - t_0/t_0$) and resolution ($R_s = (t_{r2} - t_{r1})/\frac{1}{2}(W_1 + W_2)$) were determined as the mean of three parallel measurements, with relative standard error not exceeding 4.0%. t_r, t_{r1}, t_{r2} are the retention times of the respective isomeric species, t_0 is the column hold-up time (see above); W_1 and W_2 are the widths (on the time axis) of the respective peaks at the base-line.

Data were collected and integrated using ISCO Chemresearch version 2.3 software.

3. Results and discussion

3.1. Notes on the procedure

The phenacyl esters of fatty acids were proposed first for use in reversed-phase HPLC since they improved detection greatly and allowed for direct quantification by UV detectors [23,24]. Derivatization according to Wood and Lee [22] was rapid and easy to perform with an almost 100% yield. Careful purification was, however, necessary. Most of the impurities eluted with the solvent front, but a noise peak overlapped with the peak of the saturated fatty acids and other noise peaks appeared in the chromatogram. Several cleaning procedures were tested (see Section 2) and TLC on silica was found to produce the cleanest products. Light petroleum–acetone (100:10, v/v) or hexane–diethyl ether (80:20, v/v) [21] were suitable mobile phases. The derivatives formed well defined bands, with R_F values of 0.7 for phenethyl, of 0.4 for phenacyl and of 0.3 for *p*-methoxyphenacyl derivatives.

245 nm was a practical working wavelength for phenacyl and *p*-methoxyphenacyl derivatives, close to that used by others [8,10,22]. A wavelength of 220 nm was recommended for detection of phenethyl derivatives [21] but could not be used in the present work because dichloromethane has a cut-off at 230 nm. Phenethyl derivatives were detected at 254 nm, therefore, and sensitivity of detection was less than the optimum.

3.2. Mobile phase composition

In our previous Ag-HPLC studies on aromatic derivatives with an evaporative light-scattering detector, a baseline resolution of 6-, 9- and 11-18:1 fatty acids was achieved with a mobile phase of a 1:1 mixture of dichloromethane–dichloroethane with

0.025 volume parts of acetonitrile [16,19]. Adlof et al. [15], on the other hand, employed hexane with 0.08% acetonitrile for the Ag-HPLC resolution of positionally isomeric *cis* and *trans* methyl esters with UV detection at 206 nm. Therefore, a question arose whether hexane-based mobile phases are suitable as well for the resolution of positional isomeric 18:1 fatty acids, after conversion to aromatic esters. Hexane-based mobile phases and UV detection have been examined in this work. Preliminary experiments revealed, however, that: (i) hexane–acetonitrile phases were not suitable since concentrations greater than 1.5% acetonitrile, suggested as a solubility limit by Adlof [9], were required to elute the aromatic derivatives from the column with little or no resolution of positional isomers; (ii) dichloromethane is a suitable third component providing the required solvent strength and, as mediator, ensuring good solubility of acetonitrile in hexane;(iii) the presence of acetonitrile, equal or greater than 0.1 volume parts (depending on the ester moiety), was essential for the resolution of positional isomers in hexane-based phases. Thus, mixtures of hexane, dichloromethane and acetonitrile have been tested for the resolution of the aromatic derivatives with isocratic elution.

The effect of acetonitrile on the retention (k') and the resolution (R_s) (on the example of *cis*- and *trans*-6-18:1 and 9-18:1 *p*-methoxyphenacyl derivatives) is demonstrated in Fig. 1A and Fig. 1B, respectively. k' and R_s decreased rapidly when the content of acetonitrile increased from 0.1 to 0.5 volume parts [at a constant hexane–dichloromethane ratio (70:30)]; that is, small changes in the acetonitrile content caused substantial effects on retention and resolution. The *trans* isomers were affected to a much greater extent than *cis* isomers.

The effect of dichloromethane content has been examined, by changing the hexane–dichloromethane ratio from 80:20 to 0:100 (v/v) while keeping the acetonitrile content constant at 0.2 volume parts. The increase of dichloromethane proportion resulted in a rapid decrease in retention, the minimum being at a hexane–dichloromethane ratio 40:60 (v/v). k' values remained almost constant during further increase of dichloromethane content to hexane–dichloromethane 0:100 (v/v) (Fig. 2A). *Cis* isomers were more strongly affected. Despite the decrease in k' values,

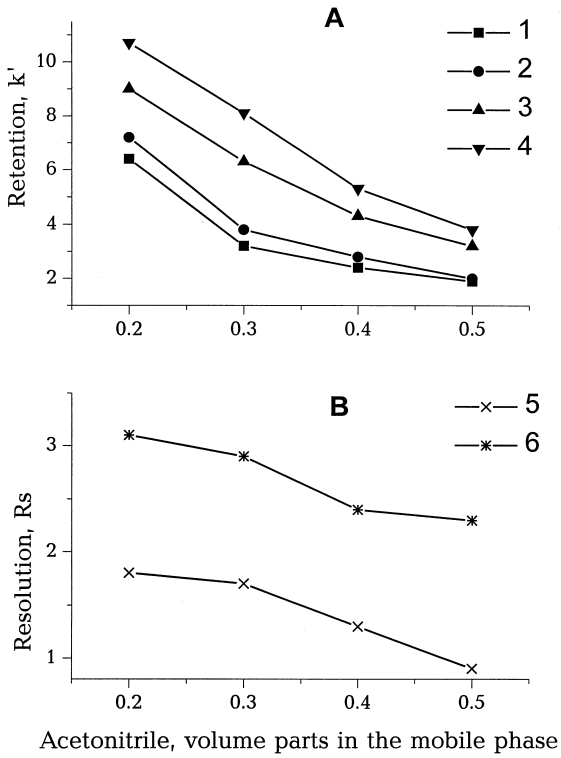

Fig. 1. Effect of acetonitrile content in the mobile phase on the retention, k', (A) and on the resolution, R_s, (B) of: 1,*trans*-9-18:1; 2, *trans*-6-18:1; 3, *cis*-9-18:1; 4, *cis*-6-18:1; 5, *trans* 6-18:1/9-18:1; 6, *cis* 6-18:1/9-18:1 fatty acid *p*-methoxyphenacyl esters at hexane–dichloromethane (70:30, v/v) and flow-rate 1.5 ml/min.

resolution remained excellent throughout the whole range of dichloromethane contents in the mobile phase (R_s values are close to or greater than 1.5) – Fig. 2B. However, R_s of *cis* isomers gradually increased with the dichloromethane content in the mobile phase (hexane–dichloromethane ratios from 80:20 to 0:100), while R_s of *trans* isomers remained constant up to hexane–dichloromethane 40:60 and slightly increased when dichloromethane in the ratio reached 100% (note that acetonitrile proportion was kept constant at 0.2 volume parts). These results reveal that dichloromethane eventually has a more complex effect on the resolution, and not only to provide a suitable solvent strength. Further work may be necessary to determine the exact mechanism.

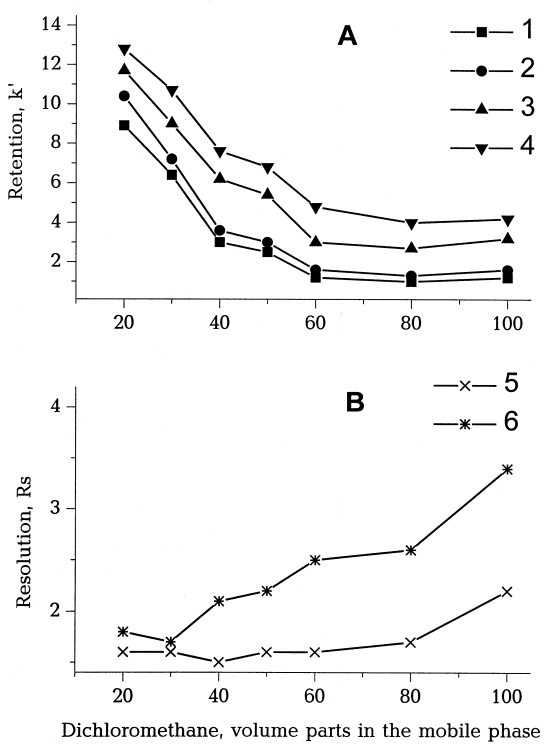

Fig. 2. Effect of dichloromethane content in the mobile phase on the retention, k', (A) and on the resolution, R_s, (B) of: 1, *trans*-9-18:1; 2, *trans*-6-18:1; 3, *cis*-9-18:1; 4, *cis*- 6-18:1; 5, *trans* 6-18:1/9-18:1; 6, *cis* 6-18:1/9-18:1 fatty acid *p*-methoxyphenacyl esters at hexane–dichloromethane, $v+v=100$ and 0.2 vol. parts acetonitrile; flow-rate 1.5 ml/min.

Mobile phases with lower than 10 volume parts of dichloromethane were not strong enough to elute the unsaturated *p*-methoxyphenacyl esters from the column. Values of 10.5 and 36.2 for k' of the 16:0 derivative were measured with hexane–dichloromethane–acetonitrile (90:10:0.2 and 99:1:0.2, v/v) with run durations of 120 min.

The same effects were valid in general for the other derivatives. Because of the lower overall polarity, lower dichloromethane contents were required to elute these derivatives from the column for comparable run times.

The distinctive effect of acetonitrile on the resolution was in agreement with previous observations and conclusions. It further supports the suggestion that acetonitrile participates in specific interactions with the silver ions in the column [15,19] and/or in π–π interactions with the double bond, as has been assumed in reversed–phase HPLC [25].

3.3. Effect of the ester moiety

Figs. 3–5 demonstrate the resolution achieved at the most effective composition of the mobile phase for each derivative. Experiments were carried out on standard mixtures (data not shown) and on a sample of fatty acids from partially hydrogenated sunflower oil. The sample contained saturated (29%), *trans*–octadecenoic (46%) and *cis*-octadecenoic (25%) fatty acids as determined by Ag-TLC/densitometry. Some of the positional isomers were identified unambiguously by spiking the sample with the appropriate pure standards.

It is clear from these results that the resolution improved in the order phenethyl<phenacyl<*p*-methoxyphenacyl esters. With hexane-based mobile phases, conditions for resolution of positional isomers as phenethyl esters were not found. *Trans*- and *cis*-phenethyl derivatives tended to overlap giving a single, broad peak. Partial resolution of only part of the late eluting *cis* isomers was observed (Fig. 3A). However, this may be useful in some circumstances, for example for simple fractionation of fatty acids into groups according to unsaturation, as seen from Fig. 3B. The mobile phase employed was hexane–dichloromethane–acetonitrile (85:15:0.3, v/v), and the duration of the isocratic run was only 8 min. A semi-quantitative estimation gives 30% for saturated; 50% for the *trans*- and 20% for the *cis*-octadecenoates (data from a single run), which was sufficiently close to that determined by Ag-TLC (see above in this subsection). More complex samples (containing dienes and trienes, for example) could be fractionated by modifying the proposed chromatographic conditions (i.e. by step-wise or linear gradient elution). An advantage of using phenethyl esters for preliminary fractionation (compared to methyl esters) is that further resolution of positional isomers is possible by GC under conventional experimental conditions as shown by Liu and Hammond [21].

The isocratic separation of the same sample after

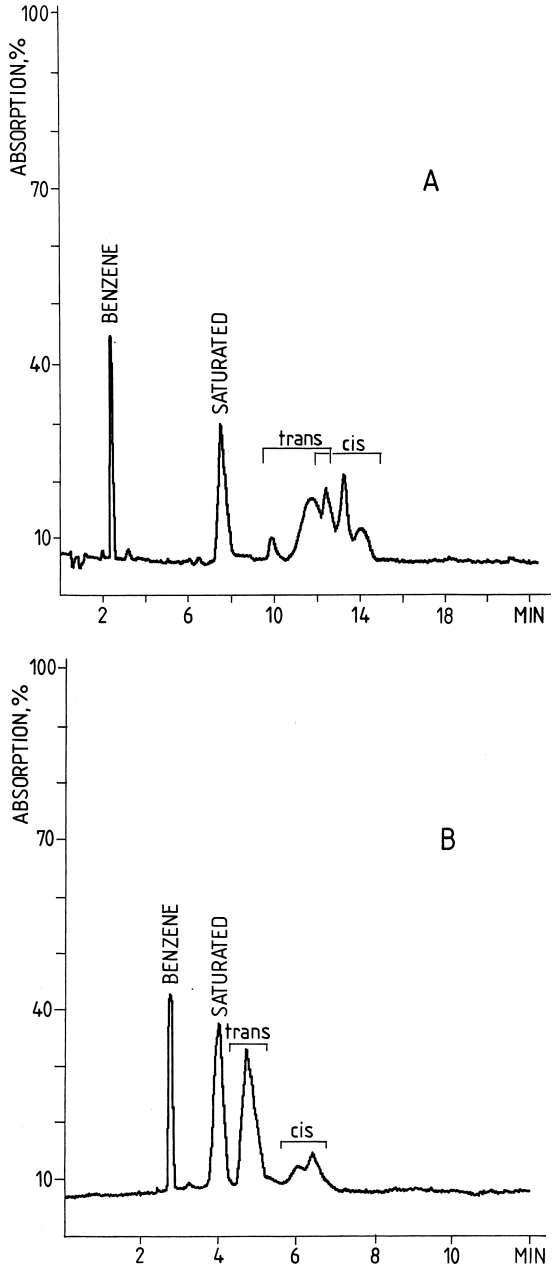

Fig. 3. Separation of isomeric fatty acids in partially hydrogenated sunflower oil after conversion into phenethyl esters by isocratic Ag-HPLC and detection at 254 nm; flow-rate 1.5 ml/min. A, mobile phase hexane–dichloromethane–acetonitrile (98:2:0.1, v/v); B, mobile phase hexane–dichloromethane–acetonitrile (85:15:0.3, v/v).

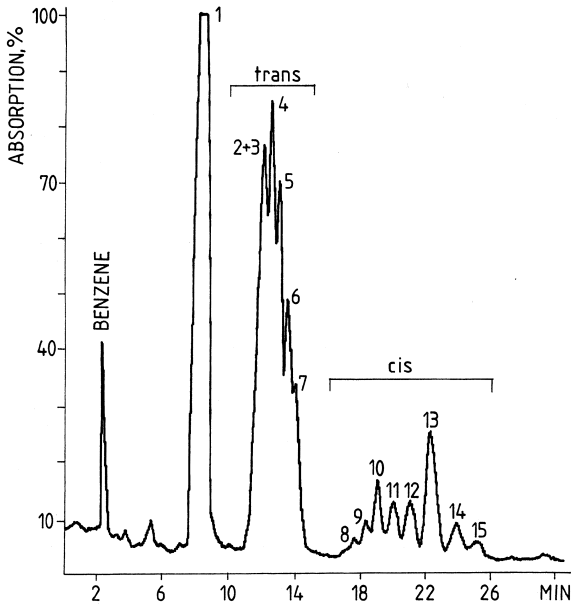

Fig. 4. Separation of isomeric fatty acids in partially hydrogenated sunflower oil after conversion into phenacyl esters by isocratic Ag-HPLC and detection at 245 nm; mobile phase hexane–dichloromethane–acetonitrile (90:10:0.2, v/v); flow-rate 1.5 ml/min. 1, saturated; 2/3 unknown/*trans*-11-18:1; 4, unknown; 5, *trans*-9-18:1; 6, unknown; 7, *trans*-6-18:1; 8, *cis*-13-18:1; 9, *cis*-12-18:1; 10, *cis*-15-18:1; 11, *cis*-11-18:1; 12, unknown; 13, *cis*-9-18:1; 14, unknown; 15, *cis*-6- and *cis*-7-18:1 fatty acids.

conversion into phenacyl derivatives is shown in Fig. 4. Partial resolution of five *trans*- and clear differentiation of eight *cis*-isomer peaks was obtained.

Much better resolution, for a shorter analysis time (22 min), was achieved under the same isocratic conditions after converting the fatty acids to *p*-methoxyphenacyl derivatives (Fig. 5). Three of the six peaks of the *trans* isomers were identified as *trans*-11-18:1, *trans*-9-18:1 and *trans* 6-18:1 (in the order of increased retention), although it is not clear whether these are single components. Only two, of the eight *cis* isomers were not identified. The 13-, 12-, 15- and 11-18:1 (ordered according to the increasing k′ value) are difficult to resolve and to identify. 12- and 15-18:1 only partially separated with phases of lower dichloromethane content (up to 50 volume parts). These species formed a mixed peak in dichloromethane-rich mobile phases which, depending on the exact mobile phase composition,

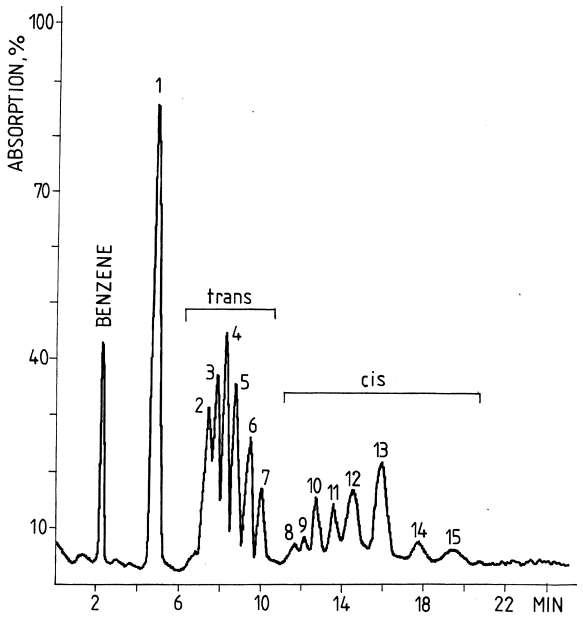

Fig. 5. Separation of isomeric fatty acids in partially hydrogenated sunflower oil after conversion into *p*-methoxyphenacyl esters by isocratic Ag-HPLC and detection at 245 nm; mobile phase hexane–dichloromethane–acetonitrile (60:40:0.2, v/v); flow-rate 1.5 ml/min. 1, saturated; 2 unknown; 3 *trans*-11-18:1; 4, unknown; 5, *trans*-9-18:1; 6, unknown; 7, *trans*-6-18:1; 8, *cis*-13-18:1; 9, *cis*-12-18:1; 10, *cis*-15-18:1; 11, *cis*-11-18:1; 12, unknown; 13, *cis*-9-18:1; 14, unknown; 15, *cis*-6- and *cis*-7-18:1 fatty acids.

was not well resolved either from 13-18:1 or from 11-18:1.

Unfortunately, *cis*-6-18:1 and *cis*-7-18:1, both as phenacyl and *p*-methoxyphenacyl esters eluted as a single peak, under most of the conditions examined, since the difference of about 0.1 k' units in favour of 6-18:1 is not sufficiently large. Partial resolution was observed only with the standard fatty acid mixture (equal concentration of these species in the sample) with a mobile phase of hexane-dichloromethane–acetonitrile (80:20:0.2, v/v).

The observed effect of the ester moieties agrees with our suggestions [3,19] for the participation of the ester group in the interaction with the silver ions. The ester group offers a second reaction site, probably the carbonyl oxygen of the phenacyl and *p*-methoxyphenacyl moieties, favouring the resolution of positionally isomeric monoenes. Phenethyl derivatives that do not offer such a site have no positive effect, but are efficient substitutes for methyl esters for rapid determination of the *trans* fatty acid content in a sample.

As UV detection provides the advantage of a direct quantification of the resolved species [8], we consider that resolution of positionally isomeric *cis* and *trans* fatty acids as *p*-methoxyphenacyl esters has great potential in the analysis of fatty acids in partially hydrogenated plant oils.

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References

[1] W.W. Christie, J. High Resolut. Chromatogr. Chromatogr. Commun. 10 (1987) 148.
[2] B. Nikolova-Damyanova, in: W.W. Christie (Ed.), Advances in Lipid Methodology–1, The Oily Press, Ayr, 1992, pp. 181.
[3] G. Dobson, W.W. Christie, B. Nikolova-Damyanova, J. Chromatogr. B 671 (1996) 197.
[4] W.W. Christie, Lipid Analysis, Pergamon Press, Oxford, 1982.
[5] W.W. Christie, High-performance Liquid Chromatography and Lipids—A Practical Guide, Pergamon Press, Oxford, 1987.
[6] C. Litchfield, Analysis of Triglycerides, Academic Press, New York, 1972.
[7] D. Chobanov, R. Tarandjiiska, B. Nikolova-Damyanova, J. Planar Chromatogr. 5 (1992) 157.
[8] W.W. Christie, G.H.McG. Breckenridge, J. Chromatogr. 469 (1989) 261.
[9] R.O. Adlof, J. Chromatogr. A 659 (1994) 95.
[10] P. Juaneda, J-L. Sebedio, W.W. Christie, J. High Resolut. Chromatogr. 17 (1994) 321.
[11] W.W. Christie, Gas Chromatography and Lipids—A Practical Guide, The Oily Press, Ayr, 1989, pp. 100–101.
[12] Z.Y. Chen, G. Pelletier, R. Hollywood, W.M.N. Ratnayake, Lipids 30 (1995) 15.
[13] J. Molkentin, D. Precht, Chromatographia 41 (1995) 267.

[14] T.G. Toschi, P. Capella, C. Holt, W.W. Christie, J. Sci. Food Agric. 61 (1993) 261.
[15] R.O. Adlof, L.C. Copes, E.A. Emken, J. Am. Oil Chem. Soc. 72 (1995) 571.
[16] B. Nikolova-Damyanova, W.W. Christie, B. Herslof, J. Chromatogr. 609 (1992) 133.
[17] F.D. Gunstone, I.A. Ismail, M.S.F. Lie Ken Jie, Chem. Phys. Lipids 1 (1967) 376.
[18] B. Nikolova-Damyanova, W.W. Christie, B. Herslof, J. Chromatogr. A 693 (1995) 235.
[19] B. Nikolova-Damyanova, W.W. Christie, B. Herslof, J. Chromatogr. A 749 (1996) 47.
[20] B. Nikolova-Damyanova, Sv. Momchilova, W.W. Christie, Phytochem. Anal. 7 (1996) 136.
[21] L. Liu, E.G. Hammond, J. Am. Oil Chem. Soc. 72 (1995) 749.
[22] R. Wood, T. Lee, J. Chromatogr. 254 (1983) 237.
[23] B.D. Durst, M. Milano, E.J. Kikta Jr., S.A. Connelly, E. Grushka, Anal. Chem. 47 (1975) 1797.
[24] R.F. Borch, Anal. Chem. 47 (1975) 2437.
[25] B. Nikolova-Damyanova, in: W.W. Christie (Ed.), Advances in Lipid Methodology—4, The Oily Press, Dundee, 1997, pp. 193–251.