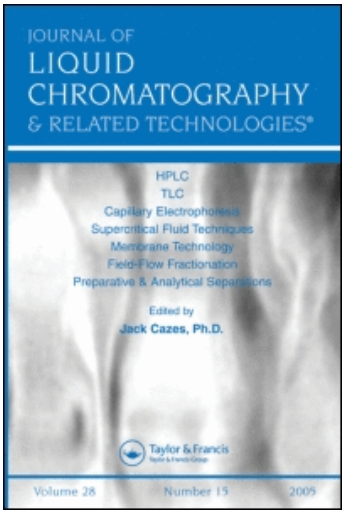

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SILVER ION HPLC OF p-METHOXYPHENACYL DERIVATIVES OF UNSATURATED FATTY ACIDS. II. CHAIN LENGTH VS. DOUBLE BOND POSITION

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ABSTRACT

The role of the chain-length and double bond position for the separation of positionally isomeric unsaturated fatty acid p-methoxyphenacyl esters by silver ion HPLC was evaluated. A clear effect of chain-length on retention was found, with k" decreasing when the number of carbon atoms in the chain increased. Thus, the resolution was affected both by the chain length and double bond position with the double bond position having the major impact. This allowed for the resolution of complex mixtures of fatty acids that differ in chain length and position of the double bonds by a single chromatographic run. Examples of such separation on model mixtures are presented.

INTRODUCTION

When discussing silver ion chromatography, it is always stressed that the separation of lipids is based on the number, configuration, and occasionally, the position of double bonds in the carbon chain; i.e. the chain-length was not considered as a factor in the resolution of components. However, a clear separation of fatty acid methyl esters with the same number of double bonds, but differing in chain length were reported with silver ion thin-layer chromatography (Ag-TLC). For example, separated were 20:4 and 18:4 (migrating in this

order);[1] 9,12-18:2 from 11,14-20:2 and 9,12,15-18:3 from 11,14,17-20:3;[2] 9-18:1 from 9-22:1;[3] 9,12-18:2 from 11,14-20:2 and 13,16-22:2, 6,9,12-18:3 from 8,11,14-20:3, 9,12,15-18:3 from 11,14,17-20:3 and 13,16,19-20:3.[4]

When introducing the stable silver ion column for silver ion high-performance liquid chromatography (Ag-HPLC), Christie also reported on the resolution of saturated fatty acids according to their chain-length and of the partial resolution of 20:4 from 18:4 (eluting in this order).[5] Recently, Adlof demonstrated an almost base-line resolution of 2:0, 4:0, 6:0, 10:0, and 16:0 methyl esters on dual ChromSpher Lipids columns with 1% acetonitrile in hexane and ascribed this phenomenon to normal phase effects occurring in Ag-HPLC.[6]

In our studies on the resolution of positionally isomeric fatty acids converted into p-methoxyphenacyl derivatives, we also observed the same effect: fatty acids with the same unsaturation but differing in the position of double bond(s) and the chain-length could be easily resolved in some cases. The question that arose was which of these two factors played the major role in resolution, and whether a longer-chain fatty acid always eluted ahead of those with shorter chains. Experiments were carried out with different series of positionally isomeric fatty acids, combining compounds with the same or different position of the first double bond (according to the traditional notation of position, i.e. from the carboxylic end). Since, in a series of papers we demonstrated the beneficial effect of the aromatic ester moiety on the resolution of positional isomeric fatty acids,[7-9] Ag-HPLC was employed, here, after conversion of the acids into p-methoxyphenacyl derivatives. The results of these experiments are presented in the present paper.

EXPERIMENTAL

Materials

Dichloromethane, acetonitrile, methanol, and iso-propanol were HPLC/UV-grade and were used without further purification. All other solvents were analytical grade. Hexane, when used as a mobile phase component, was left for 24 h over potassium hydroxide and then distilled. The isomeric fatty acids and the derivatizing reagents were purchased from Sigma-Aldrich (Poole, UK).

Derivatization

The p-methoxyphenacyl esters were prepared according to Wood and Lee.[10] Briefly, 2 mg free fatty acid was reacted with 0.5 mL solution (10 mg/mL in acetone) of α-bromo-p-methoxyacetophenone and with 0.5 mL solution (10 mg/mL in acetone) of triethylamine for 15 min in a boiling water bath. Acetic

acid (70 µL) was added, and the sample was heated for an additional 5 min. The derivatives were purified by TLC on silica gel G hand-made glass plates after single development with mobile phase of hexane-acetone in proportion 100:12 (v/v) (R_f=0.35). The esters were detected under UV light after spraying with a fluorescent indicator (the edges of the plate only, after carefully covering the rest of the plate). The bands were scrapped, transferred to Pasteur pipettes, and the derivatives were eluted with diethyl ether. The diethyl ether was evaporated under nitrogen and the derivatives were redissolved in hexane to give 0.2 mg/mL solutions. Each ester gave a single peak in Ag-HPLC. Working solutions were prepared, composed of isomers with the same chain-length and the same unsaturation, by mixing equal volumes of the respective solutions. The solvent was evaporated under nitrogen and the residue was redissolved in hexane to give 0.2 mg/mL final solution.

Silver Ion HPLC (Ag-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, Nucleosil 100-5SA (250x4.6 mm; Hichrom, Reading, UK) was converted to the silver ion form as described by Christie.[5] The injection volume was 10 µL (sample size of 1-2 µg of each derivative). p-Methoxyphenacyl esters were detected at 270 nm. The respective absorption maxima were determined on silica gel TLC plate by spectrodensitometry using Shimadzu CS-930 densitometer (Shimadzu Corporation, Kyoto, Japan). Mixtures of hexane, dichloromethane, and acetonitrile, methanol or isopropanol, as a modifier (as specified in Results & Discussion) were used as mobile phases at a flow-rate of 1.5 mL/min at 21 ± 2°C.

Relative retention factors, k" and resolution Rs were determined as a mean of three parallel measurements, with relative standard error not exceeding 4% rel.; p-methoxyphenacyl *trans*-6-18:1 was used as the internal standard. The column hold-up time was determined by repeatable injection of benzene.

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

RESULTS AND DISCUSSION

The work of Wilson and Sargent[4] gives many examples of successfully separating fatty acids with the same number of double bonds but different chain-length by using Ag-TLC. At a closer look, it was evident that the compounds were grouped according to the same position of the last double bond (when counted from the carboxylic end), i.e., compared were fatty acids of the series (n-3) and (n-6). They differed and, in some cases, the difference was substan-

tial, in the position of the first double bond. On the other hand, when studying the chromatographic behavior of octadecenoic positional isomers, a hypothesis was offered which considered the position of the double bond in the chain.[8] The elution order observed by Ag-HPLC[7] resembled, closely, the famous sinusoidal migration order of octadecenoates in Ag-TLC.[11] To explain the retention order, the formation of a three-center complex between a double bond in favorable position, a fragment of the ester moiety, and a silver ion was suggested.[8] In general, the suggestion predicts that fatty acids with a first double bond in positions 5, 6, 7, and 8 should be held stronger than those with a double bond in position 9, and beyond. Although this was postulated for monoenoic fatty acids, we observed the same rule was valid for some isomeric pairs of p-methoxyphenacyl, all *cis* octadecatrienoates and eicosatrienoates in hexane-dichloromethane-acetonitrile or dichloromethane-acetonitrile mobile phases where, obviously, elution order was determined by the position of the first double bond in the chain.[12]

In Figure 1, the k" values of a series of fatty acids with different chain lengths and the same position of the first double bond were plotted against the number of double bonds. Many isomers were included, except 18:2 and 20:2, which were not commercially available. All k" values were recalculated for a mobile phase of dichloromethane-acetonitrile, 100:0.025 (v/v) which was the best for separation of 18:1 isomers. A clear tendency of decreasing retention with increasing number of carbon atoms in the chain was observed. The impact of this fact on the chromatographic resolution is demonstrated by Table 1 and Figures 2 and 3.

Table 1 demonstrates the role of the double bond position and of chain-length on the resolution of several selected series of fatty acids. Thus, the pair 9-16:1/9-18:1 was partially resolved over the whole range of hexane- and dichloromethane-based mobile phases tested, as was the series of 13-18:1, 13-20:1 and 13-22:1 (in order of decreasing k"). Under identical experimental conditions, the pair 9-16:1/6-18:1 and the series 6-18:1, 11-20:1 and 13-22:1 (k" decreased in this order) were fully resolved. As evident from Table 1, the number of carbon atoms in the chain affected the resolution to a certain extent but the position of the double bond had the leading role. Thus, fatty acids with the different carbon chain but the same position of the double bond are only partially resolved, the longer chain fatty acid being eluted first. Complete resolution was achieved when both the chain-length and position of the double bond favored the resolution. In Table 1, it is also shown that a small but statistically significant difference, was determined for the Rs values of eicosenoates and docosaenoates, depending on the mobile phase composition: Rs in hexane-dichloromethane-acetonitrile was higher than in dichloromethane-acetonitrile.

Figures 2 and 3 demonstrate the practical output of the above observation. Ag-HPLC separations of mixtures of octadecenoates and eicosenoates (Figure

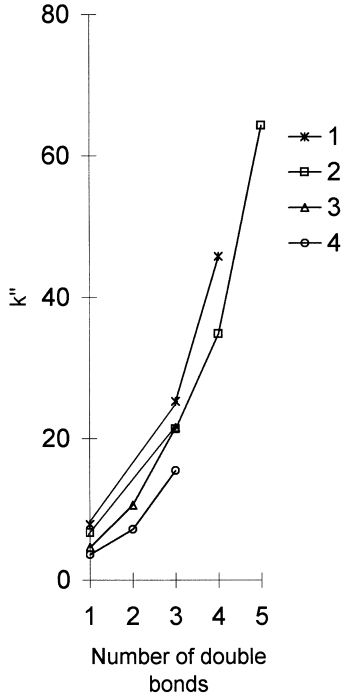

Figure 1. Relative retention, **k"**, vs. number of double bonds, **n**, for p-methoxyphenacyl esters of unsaturated fatty acids. **1**, 6-18:n; **2**, 5-20:n; **3**, 11-20:n; **4**, 13-22:n. k" was recalculated for a mobile phase of dichloromethane:acetonitrile, 100:0.025 (by volume), internal standard was *trans* 6-18:1.

2), and of octadecatrienoates and eicosatrienoates (Figure 3) are shown. All effects discussed above are clearly demonstrated by the resolution presented in Figure 2. Figure 3 demonstrates that a difference in chain length allowed differentiation between triunsaturated positional isomers, despite poorer resolution. Isocratic elution was applied in both cases, which indicated that much better results could be expected by using a gradient.

Thus, two factors seem to govern the resolution of unsaturated fatty acids: the number and position of the double bond and the number of carbon atoms. When and if these factors act in the same direction, a complete resolution of the components can be achieved. This explained the interesting separation achieved by Wilson and Sargent by Ag-TLC:[4] separated were fatty acids of the same unsaturation but of different chain lengths, and of favorable difference in

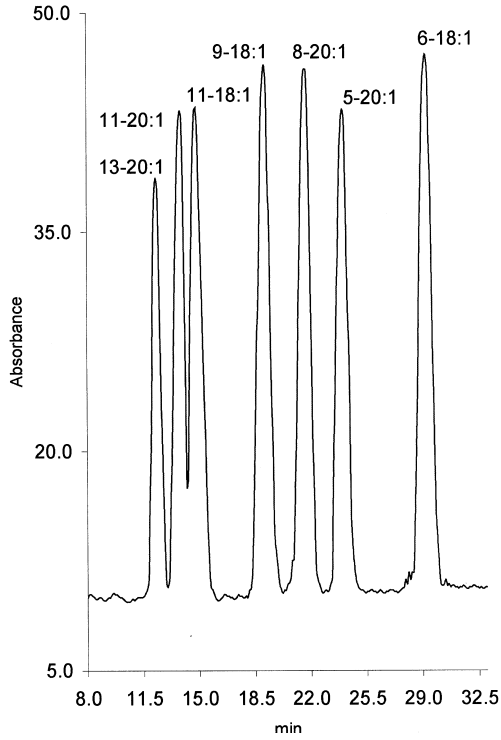

Figure 2. Separation of a mixture of positionally isomeric octadecenoic and eicosaenoic fatty acid p-methoxyphenacyl esters by Ag-HPLC. Conditions: Nucleosil 100-5SA column laboratory loaded with silver ions; mobile phase, hexane-dichloromethane-isopropanol, 60:40:0.2 (by volume); flow rate, 1.5 mL/min.

the position of the first double bonds. If and when the two factors act in the opposite direction, partial or no resolution of fatty acids was to be expected.

A third element of the system which should also be taken into account is the mobile phase composition. So far, there were some indications that longer chain fatty acids were eventually better resolved in hexane-based mobile phases.

Effects of chain-length were observed in the early years of silver ion chromatography of unsaturated molecules when gas-liquid chromatography was the techniques employed. They were ascribed to the formation of less stable silver ion complexes with elongation of the carbon chain.[13] At this stage, it is not possible to estimate whether this fact, or the "normal phase" effects suggested by Adlof[6] were taking place.

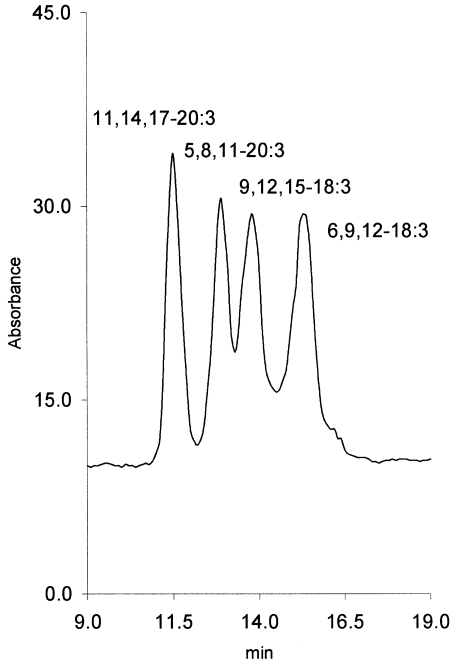

Figure 3. Separation of a mixture of positionally isomeric octadecatrienoic and eicosatrienoic fatty acid p-methoxyphenacyl esters by Ag-HPLC. Mobile phase, dichloromethane-acetonitrile, 100:0.4 (by volume); other conditions as in Figure 2.

Table 1

Resolution, Rs, of Some Monounsaturated Fatty Acids of Different Chain-Length

Fatty Acids	Mobile Phase[a] Hex:DCM:AcN[b,c]	DCM:AcN
9-18:1/9-16:1	0.5[b]	0.5
9-16:1/6-18:1	1.2[b]	2.1
13-22:1/11-20:1	1.6[b]	1.3
11-20:1/6-18:1	3.9[b]	5.8
13-22:1/13-20:1	0.7[c]	0.5
13-20:1/13-18:1	0.5[c]	0.7

[a] Hex, hexane, DCM, dichloromethane, AcN, acetonitrile. [b] Solvent ratio: 70:30:0.2. [c] Solvent ratio: 80:20:0.2.

From a practical point of view, these results and the findings of Christie[5] and Adlof,[6] show that the Ag-HPLC possesses a very strong resolution power. Of potential practical interest is that many complex mixtures which include positionally isomeric fatty acids of different chain length (as in marine organisms, for example) may be successfully resolved by Ag-HPLC.

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