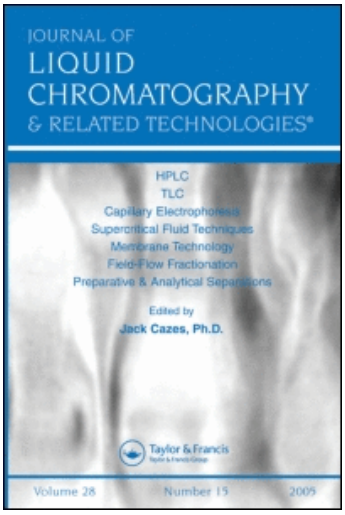

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SILVER ION HPLC OF p-METHOXYPHENACYL DERIVATIVES OF UNSATURATED FATTY ACIDS. III. MOBILE PHASE EFFECTS ON *TRANS* 6-, 9-, AND 11-18 : 1

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ABSTRACT

The effect of the mobile phase composition on the retention and resolution of *trans* positionally isomeric 6-, 9-, and 11-18 : 1 fatty acids was studied on a model mixture by silver ion high performance liquid chromatography (Ag-HPLC). Prior to chromatography, the fatty acids were converted into p-methoxyphenacyl derivatives. Hexane-based and dichloromethane-based mobile phases modified with either acetonitrile or methanol, or isopropanol were examined. The modifiers were found to have a different effect on the resolution, depending on the main solvent component in the mobile phase. In case of hexane being the main mobile phase solvent, best resolution was achieved when using isopropanol as a modifier. In dichloromethane-based mobile phases, best results were achieved with acetonitrile or

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methanol. The three *trans* isomers were completely resolved in practically all mobile phases tested, the elution order being 11-, 9-, 6-18 : 1, irrespective of the mobile phase composition.

INTRODUCTION

Octadecenoic fatty acids with *trans* double bonds have been linked to several effects adverse to health (1). In recent years, various methods have been developed for separation and quantitative determination of *trans* positionally isomeric fatty acids (2). In the most widely used analytical approach, gas-liquid chromatography of fatty acid methyl esters, despite the use of a sophisticated instrumentation, overlapping of peaks of positional isomers with *trans* and *cis* double bonds may occur (2). In order to increase the credibility of the result, silver ion chromatography (mostly thin-layer chromatography, TLC) is applied as a preliminary step (3). A series of papers on the retention and resolution of positionally isomeric *cis*- and *trans*-fatty acids by silver ion chromatography clearly showed the advantage of using aromatic and not the conventional methyl derivatives in the analysis. Best results were achieved, so far, after conversion of fatty acids in p-methoxyphenacyl derivatives (4–7). On the other hand, the resolution of isomeric fatty acids depends, also, on the mobile phase composition (5–8). In a previous paper, we demonstrated the mobile phase effects on retention and resolution of p-methoxyphenacyl derivatives of positionally isomeric fatty acids with *cis* double bonds (8). Here a systematic study was carried out on p-methoxyphenacyl derivatives of *trans* 6-, 9-, and 11-18 : 1, which are typical for dairy products and foods, containing partially hydrogenated oils (9). Mobile phases based on hexane or dichloromethane were studied and acetonitrile, methanol, and isopropanol were the modifiers.

EXPERIMENTAL

Materials

Dichloromethane, acetonitrile, methanol, and isopropanol were HPLC/UV-grade (Merck, Darmshtadt, Germany) 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).

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Derivatization

The p-methoxyphenacyl esters were prepared according to Wood and Lee (10). Briefly, 2 mg free fatty acid were reacted with 0.5 mL solution (10 mg/mL in acetone) of α-bromo-p-methoxyacetophenone and with a 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 silica gel G-TLC on laboratory made glass plates after single development, with a 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 fluorescent indicator (the edges of the plate only, after careful covering the rest of the plate). The bands were scrapped, transferred to small columns, and the derivatives were eluted with diethyl ether. The solvent was evaporated under nitrogen and the derivatives were redissolved in hexane to give 0.2 mg/mL solutions.

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 (250 × 4.6 mm; Hichrom, Reading, UK) was converted to the silver ion form as described by Christie (11). The injection volume was 10 μL (sample size of 1–2 μg of each derivative). p-Methoxyphenacyl esters were detected at 270 nm. The UV absorption maximum was determined on silica gel TLC plates by spectrodensitometry using Shimadzu CS-930 densitometer (Shimadzu Corporation, Kyoto, Japan). Mixtures of hexane, dichloromethane, and acetonitrile, or methanol or isopropanol were used as mobile phases at a flow-rate of 1.5 mL/min at 21 ± 2°C.

Retention factors, k′, and resolution, R_S, were determined as a mean of three parallel measurements, with relative standard error not exceeding 4% rel. The column hold-up time was determined by repeated injections of benzene.

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

RESULTS AND DISCUSSION

In a previous paper, (6) we demonstrated that the resolution of positionally isomeric octadecenoic fatty acids with *cis* double bonds improved in the following order of esters: 2-naphthylmethyl < 9-anthrylmethyl < 2-naphthacyl

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≈ p-methoxyphenacyl esters. Improved resolution in the same order of derivatives was established, also, for the investigated octadecenoic positional isomers with *trans* double bonds (Figure 1). For that reason, we chose p-methoxyphenacyl esters as the most appropriate derivatives for our chromatographic investigations on *trans* 6-, 9-, and 11-18 : 1.

Two types of mobile phases were examined throughout this work: hexane-dichloromethane-modifier (denoted below as hexane-based) and dichloromethane-modifier (denoted as dichloromethane-based). Two sets of experiments were carried out with hexane-based phases. In the first, the proportion of dichloromethane was increased while keeping constant the proportion of the modifier, and in the second, the hexane-dichloromethane ratio was kept constant (70 : 30, v/v) and the volume part of the modifier was gradually increased. In the

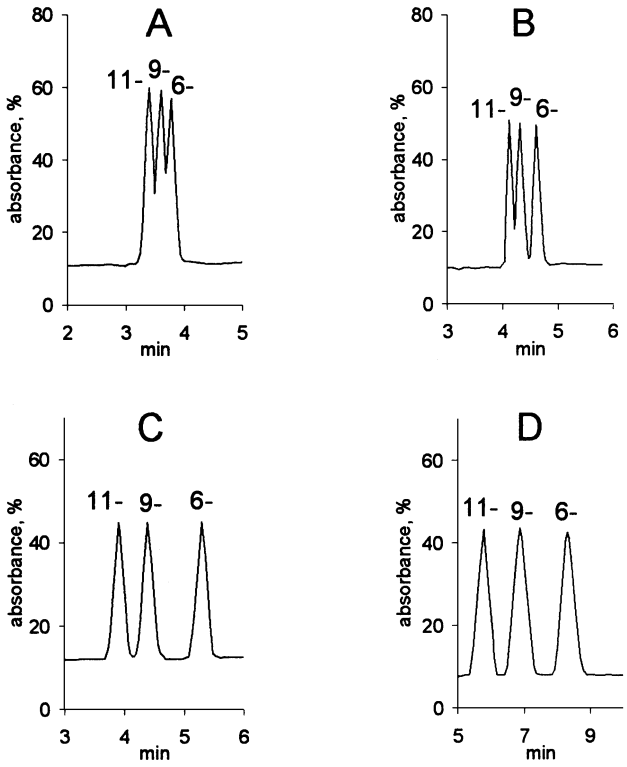

Figure 1. Ag-HPLC separation of trans 6-, 9-, and 11-18 : 1 as: (**A**) 2-naphthylmethyl esters; (**B**) 9-anthrylmethyl esters; (**C**) 2-naphthacyl esters; (**D**) p-methoxyphenacyl esters. Silver loaded Nucleosil 100-5SA column with mobile phase dichloromethane-acetonitrile, 100 : 0.025 (v/v) at a flow rate of 1.5 mL/min.

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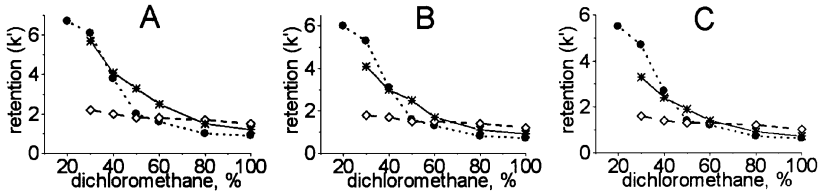

Figure 2. Retention factors (k′) in Ag-HPLC of (**A**) *trans* 6-18 : 1; (**B**) *trans* 9-18 : 1; (**C**) *trans* 11-18 : 1 as p-methoxyphenacyl esters. Mobile phase hexane-dichloromethane, x + y = 100 and 0.2 volume parts modifier acetonitrile (●), methanol (◇) or isopropanol (∗). Other conditions as in Figure 1.

dichloromethane-based phases, the proportion of the modifier was changed within appropriate range. Acetonitrile, methanol, and isopropanol were the modifiers, as in our previous study on isomers, with *cis* double bonds (8).

Hexane-Based Mobile Phases

Figures 2 and 3 present the k′ values plotted against the increasing proportions of dichloromethane and the modifier, respectively. In general, k′ values decreased when the proportions of dichloromethane were increased from 30- to 100- and those of the modifier—in the range 0.2 to 0.5 volume parts. Irrespective of the mobile phase composition, the elution order of *trans* isomers remained the same: 11-, 9-, 6-18 : 1 (in order of increasing retention).

As is evident from Figure 2, in mobile phases containing up to 40% dichloromethane, k′ decreased depending on the modifier on the: acetonitrile > isopropanol > methanol. This order reversed to methanol > isopropanol >

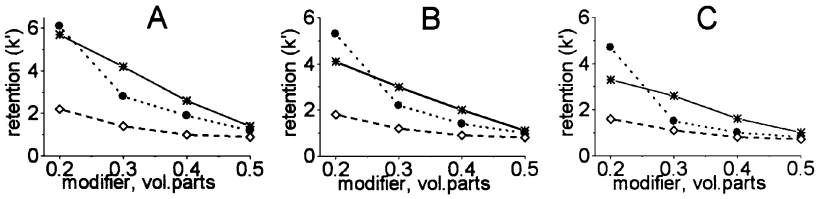

Figure 3. Retention factors (k′) in Ag-HPLC of (**A**) *trans* 6-18 : 1; (**B**) *trans* 9-18 : 1; (**C**) *trans* 11-18 : 1 as p-methoxyphenacyl esters. Mobile phase hexane-dichloromethane, 70 : 30 (v/v) and modifier acetonitrile: (●), methanol (◇) or isopropanol (∗). Other conditions as in Figure 1.

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acetonitrile, when dichloromethane proportions in the mobile phase was higher than 80%.

When the modifier proportion in the mobile phase increased from 0.2 to 0.5 volume parts, the retention of *trans* 6-, 9-, and 11-18 : 1 rapidly decreased (Figure 3). In mobile phases containing up to 0.3 volume parts modifier, the respective k′ values of *trans* isomers decreased in the order: acetonitrile > isopropanol > methanol. Over this limit the order was changed to isopropanol > acetonitrile > methanol.

The effect on resolution (R_S) of *trans* 6-, 9-, and 11-18 : 1 is presented in Figures 4 and 5 where the R_S values are plotted against the increasing proportions of dichloromethane and the modifier in the mobile phase, respectively. In general, acetonitrile and methanol had very similar effect on the resolution of the positional isomers tested. The R_S values of 6-/9-18 : 1 were higher than that of 9-/11-18 : 1 in the respective mobile phases.

Increasing proportion of dichloromethane in hexane-based mobile phases affected the resolution of *trans* 6-/9-18 : 1 and 9-/11-18 : 1 in a different way (Figure 4 A and B, respectively). It is evident that the resolution of 6-/9-18 : 1 slightly improved with either acetonitrile or methanol as modifiers and worsened in the presence of isopropanol (Figure 4 A). The resolution of 9-/11-18 : 1 was practically unaffected by the increasing proportion of dichloromethane in mobile phases in the presence of acetonitrile or methanol, and slightly decreased when isopropanol was the modifier (Figure 4 B). In these mobile phases, the three *trans* isomers were completely resolved.

Figure 5 presents the effect of the modifier proportion in hexane-dichloromethane, 70 : 30 (v/v) mobile phases. It is evident, that the resolution of *trans* 6-/9-18 : 1 and 9-/11-18 : 1 gradually decreased when the proportion of the modifier was increased in the range 0.2 to 0.5 volume parts. The

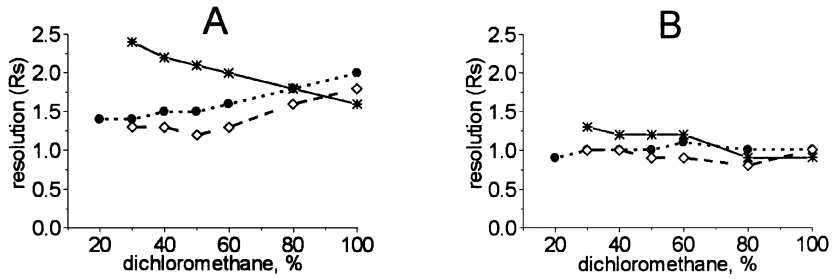

Figure 4. Resolution (R_S) in Ag-HPLC of (**A**) *trans* 6-/9-18 : 1 and (**B**) *trans* 9-/11-18 : 1 as p-methoxyphenacyl esters. Mobile phase hexane-dichloromethane, $x + y = 100$ and 0.2 volume parts modifier acetonitrile (●), methanol (◇) or isopropanol (∗). Other conditions as in Figure 1.

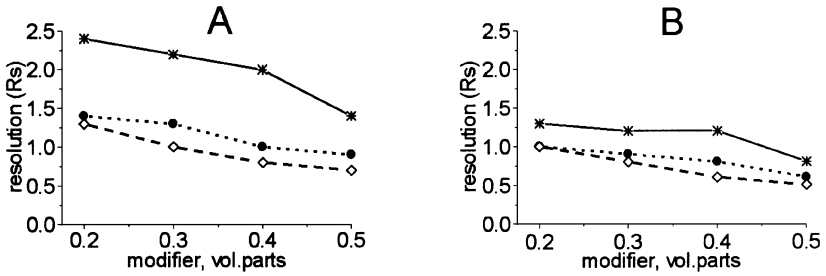

Figure 5. Resolution (R_S) in Ag-HPLC of (**A**) *trans* 6-/9-18 : 1 and (**B**) *trans* 9-/11-18 : 1 as p-methoxyphenacyl esters. Mobile phase hexane-dichloromethane, 70 : 30 (v/v) and modifier acetonitrile (●), methanol (◇) or isopropanol (∗). Other conditions as in Figure 1.

corresponding R_s values decreased in the following order of modifiers: isopropanol > acetonitrile > methanol. The three *trans* isomers were completely resolved in mobile phases containing isopropanol.

Dichloromethane-Based Mobile Phases

Figure 6 presents the k′ values of *trans* 6-, 9-, and 11-18 : 1 plotted against the increasing proportion of the modifier in the mobile phase. Expectedly, the retention of isomers decreased under these chromatographic conditions.

It is evident from Figure 6, that acetonitrile and isopropanol, as modifiers, had very similar effects on the retention of the three *trans* isomers. The corresponding k′ values decreased in the following order of modifiers: methanol > isopropanol ≥ acetonitrile. Irrespective of the mobile phase composition, the elution order of *trans* isomers remained the same: 11-, 9-, 6- 18 : 1 (in order of increasing retention).

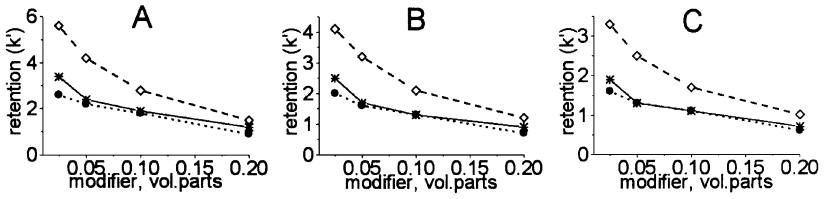

Figure 6. Retention factors (k′) in Ag-HPLC of (**A**) *trans* 6-18 : 1; (**B**) *trans* 9-18 : 1; (**C**) *trans* 11-18 : 1 as p-methoxyphenacyl esters. Mobile phase dichloromethane and modifier acetonitrile (●), methanol (◇) or isopropanol (∗). Other conditions as in Figure 1.

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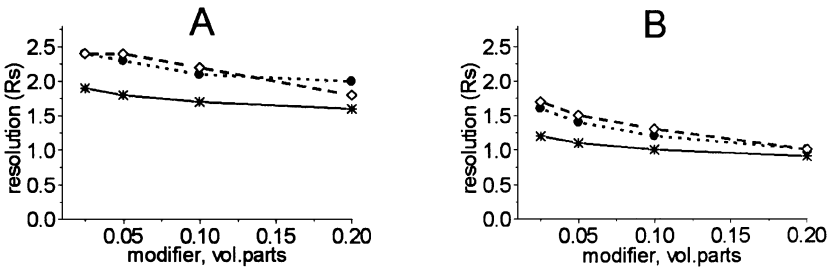

Figure 7. Resolution (R_S) in Ag-HPLC of (**A**) *trans* 6-/9-18 : 1 and (**B**) *trans* 9-/11-18 : 1 as p-methoxyphenacyl esters. Mobile phase dichloromethane and modifier acetonitrile (●), methanol (◇) or isopropanol (∗). Other conditions as in Figure 1.

The resolution of the three *trans* isomers under the same conditions is presented in Figure 7. As seen, the R_S values slightly decreased with increasing proportions of the modifier in the mobile phase. The modifier effect decreased in the order: methanol $\approx$ acetonitrile > isopropanol. In all mobile phases tested, the three *trans* isomers were completely resolved.

The hexane-based and dichloromethane-based mobile phases, which ensured the highest R_s values of *trans* 6-, 9- and 11-18 : 1, are summarized in Table 1. The results confirm the assumption (7,8,12), that acetonitrile is not unique as a modifier for the separation of fatty acid positional isomers as p-methoxyphenacyl esters. In hexane-based mobile phases, isopropanol should be the preferred for complete resolution of *trans* 6-, 9-, and 11-18 : 1 (Table 1). In contrast to some eicosamonoenoic, (8) eicosatienoic, (8) and conjugated octadecadienoic positional isomers, (7) and exactly as with *cis* 6-, 9-, and 11-18 : 1, (5,8) the three *trans* octadecenoic isomers were eluted in the same order (11-, 9-, 6-18 : 1, according to the increasing retention) irrespective of the mobile

Table 1. Hexane-Based and Dichloromethane-Based Mobile Phases for the Most Complete Ag-HPLC Resolution (R_S) of *trans* 6-, 9- and 11-18 : 1 as p-Methoxyphenacyl Esters

	6-/9-18 : 1	9-/11-18 : 1
Mobile Phase	Hex-DCM-iPrOH[a], 70 : 30 : 0.2	Hex-DCM-iPrOH, 70 : 30 : 0.2
R_S	2.4	1.3
Mobile Phase	DCM-AcN/MeOH[b], 100 : 0.025	DCM-AcN/MeOH, 100 : 0.025
R_S	2.4	1.6

[a] Hex = hexane; DCM = dichloromethane; iPrOH = isopropanol.
[b] AcN/MeOH = acetonitrile or methanol.

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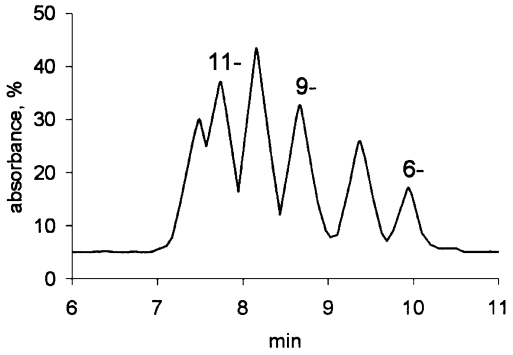

Figure 8. Ag-HPLC separation of *trans* positionally isomeric 18 : 1 fatty acids in partially hydrogenated sunflower oil after conversion into p-methoxyphenacyl esters. Silver loaded Nucleosil 100-5SA column with mobile phase hexane-dichloromethane-acetonitrile, 60 : 40 : 0.2 (v/v/v) at a flow rate of 1.5 mL/min.

phase modifier (isopropanol, acetonitrile or methanol). On the other hand, in dichloromethane-based mobile phases, methanol ensured the same complete separation as acetonitrile (Table 1) and could be used as a mobile phase modifier for resolution of *trans* 6-, 9-, and 11-18 : 1 as p-methoxyphenacyl esters. In all mobile phases tested, the *trans* isomers 6-/9-18 : 1 were better resolved than were 9-/11-18 : 1. A respective chromatogram for dichloromethane-based mobile phase, with acetonitrile as a modifier, can be seen in Figure 1 D.

Summarizing, it is evident, that both hexane-based and dichloromethane-based mobile phases modified with either acetonitrile or methanol or isopropanol provided reasonably good resolution of positionally isomeric *trans* 6-, 9-, and 11-18 : 1 fatty acids as p-methoxyphenacyl esters. The data presented here would be helpful for rapidly establishing the optimal mobile phase composition for a given *trans* fatty acid sample. For example, Ag-HPLC separation of a series of *trans* positionally isomeric 18 : 1 fatty acids in partially hydrogenated sunflower oil could be achieved in only 11 min after conversion into p-methoxyphenacyl esters (Figure 8). The mobile phase that provided sufficiently good resolution of the isomers was hexane-dichloromethane-acetonitrile, 60 : 40 : 0.2 (v/v/v).

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