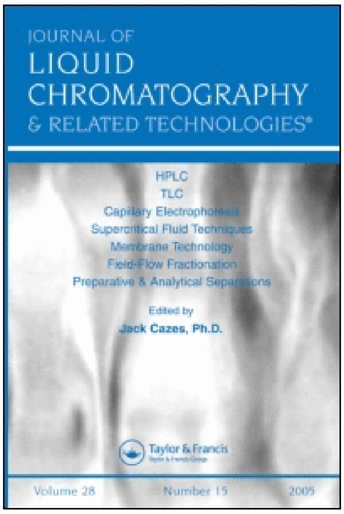

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# SILVER ION HPLC OF p-METHOXYPHENACYL DERIVATIVES OF UNSATURATED FATTY ACIDS. I. MOBILE PHASE EFFECTS

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## ABSTRACT

The effect of the mobile phase composition on the retention and resolution of positionally isomeric octadecamonoenoic (18:1), eicosamonoenoic (20:1), octadecatrienoic (18:3), and eicosatrienoic (20:3) fatty acids in silver ion high performance liquid chromatography was studied on model fatty acid mixtures. Prior to chromatography, the fatty acids were converted into p-methoxyphenacyl derivatives. Hexane-based and dichloromethane-based mobile phases that contained acetonitrile, methanol, and iso-propanol as modifiers were examined. All three modifiers ensured reliable resolution of the respective isomeric fatty acids and are of practical interest. In contrast to acetonitrile and methanol, iso-propanol was found to reverse the elution order of difficult to resolve components that gave adjacent chromatographic peaks. This finding indicates the active role of mobile phase in the chromatographic process.

## INTRODUCTION

In previous papers on silver ion high-performance liquid chromatography (Ag-HPLC) of positionally isomeric octadecenoates[1,2] and conjugated octadecadienoates,[3] we demonstrated that the conversion of fatty acids into p-methoxyphenacyl derivatives provided the best resolution of these isomers achieved so far on a single silver loaded column. Mobile phases used were either hexane- or dichloromethane-based with acetonitrile and occasionally with iso-propanol or methanol as modifiers. It has been observed in these studies that changes in the mobile phase composition not only substantially affected the retention and resolution (which could be expected),[2] but changed the elution order of some components.[2,3]

A more systematic study on this phenomenon was carried out in the present work. Naturally occurring positional isomers of octadecenoic, eicosenoic, octadecatrienoic, and eicosatrienoic fatty acids were employed as reference compounds. Mobile phases based on hexane or dichloromethane were studied, as previously;[2] acetonitrile, methanol, and iso-propanol were employed as modifiers.

## 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.[4] Briefly, 2 mg free fatty acid was reacted with an 0.5 mL solution (10 mg/mL in acetone) of α-bromo-p-methoxyacetophenone and with an 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 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 Pasteur pipettes, and the derivatives were eluted with diethyl ether. 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 comprised 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 plates by spectrodensitometry, using Shimadzu CS-930 densitometer (Shimadzu Corporation, Kyoto, Japan). Mixtures of hexane, dichloromethane, and acetonitrile, methanol, or iso-propanol 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.

Retention factors, k' and resolution Rs 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 repeatable injections of benzene. In some cases, the relative retention (k") was determined using *cis,cis* 11,14-20:2 fatty acid as a reference compound

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

## RESULTS AND DISCUSSION

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 changed while keeping constant the proportion of the modifier, and in the second, the hexane-dichloromethane ratio was kept constant and the volume part of the modifier was gradually changed. In the dichloromethane-based phases, the proportion of the modifier was changed within appropriate range. Acetonitrile was tested since it is traditionally used in Ag-HPLC of fatty acids.[1-3,6,7] Methanol has been also employed as the modifier in Ag-HPLC of fatty acids as phenacyl esters. Separated were species with 0 to 6 double bonds,[6] as well as, the configurational isomers of linolenic acid.[8] In a previous paper, we used iso-propanol as modifier for the resolution of conjugated linoleic acid (CLA) isomers and reported about changes in the elution order of *cis,trans/trans cis* CLA.[3]

## Retention and Resolution of cis 6-, 9-, 11-18:1

Figure 1 presents the respective k' values plotted against the increasing proportions of dichloromethane (Figure 1A) and the modifier (Figure 1 B) for

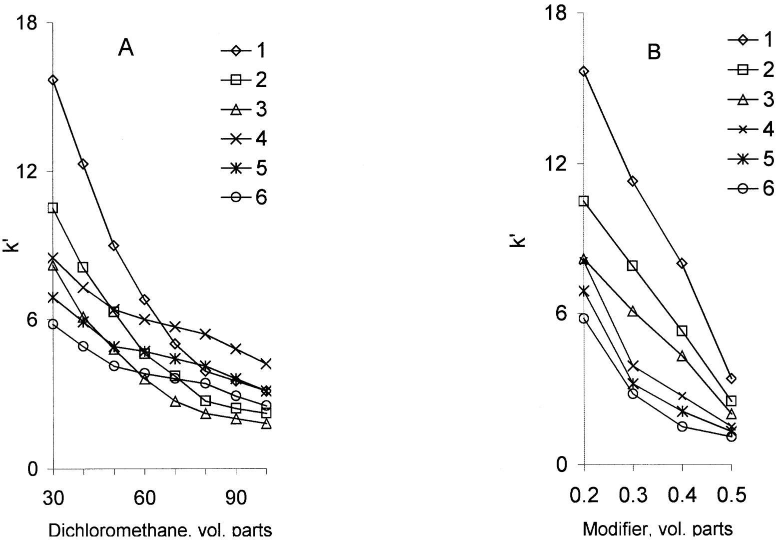

**Figure 1**. Effect on retention (k') of **A**, dichloromethane content in hexane-based mobile phases at 0.2 volume parts of modifier and **B**, modifier content in hexane-dichloromethane, 70:30 (v/v). 6-18:1 (lines **1** and **4**), 9-18:1 (lines **2** and **5**) and 11-18:1 (lines **3** and **6**); iso-propanol (lines **1-3**) and methanol (lines **4-6**) as modifiers. Nucleosil 100-5SA column laboratory loaded with silver ions; flow rate, 1.5 mL/min.

hexane-based mobile phases. Data for methanol and iso-propanol are presented only, since the effect of acetonitrile has been reported earlier.[2] In general, all three modifiers tested affected the retention in the same way. K' values rapidly decreased when the proportions of either dichloromethane or the modifier increased in the ranges from 30 to 100 and from 0.2 to 0.5, respectively. As is evident from the figure, the k' values were higher in iso-propanol than in methanol containing mobile phases. In contrast, while the general pattern was the same in dichloromethane-based phases, k' was higher in the presence of methanol (Figure 2, A-C). Irrespective of the mobile phase composition, the elution order remained the same: 11-, 9-, 6-18:1 (in order of increasing retention).

The resolution of these octadecamonoenoic isomers was not affected by the changes of the mobile phase composition, as is evident from Figure 3 A and Figure 3 B. The resolution remained complete (Rs >> 1) over the whole range of mobile phases tested. The best resolution was achieved with hexane-based phases and iso-propanol as modifier. The Rs values were higher than those found previously under identical conditions with acetonitrile.[2]

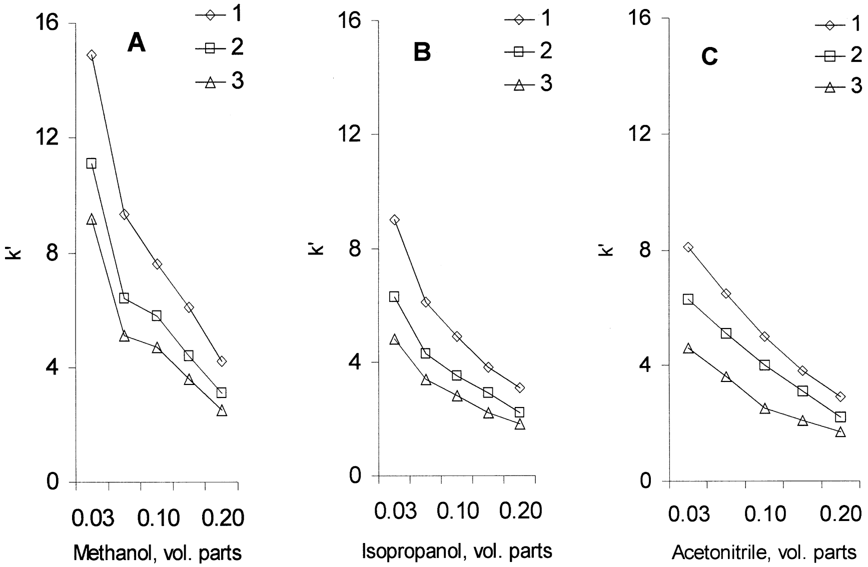

**Figure 2**. Effect of the modifier in dichloromethane-based mobile phases on the retention (k') of 6-18:1 (line **1**), 9-18:1 (line **2**) and 11-18:1 (line **3**). Other conditions as in Figure 1.

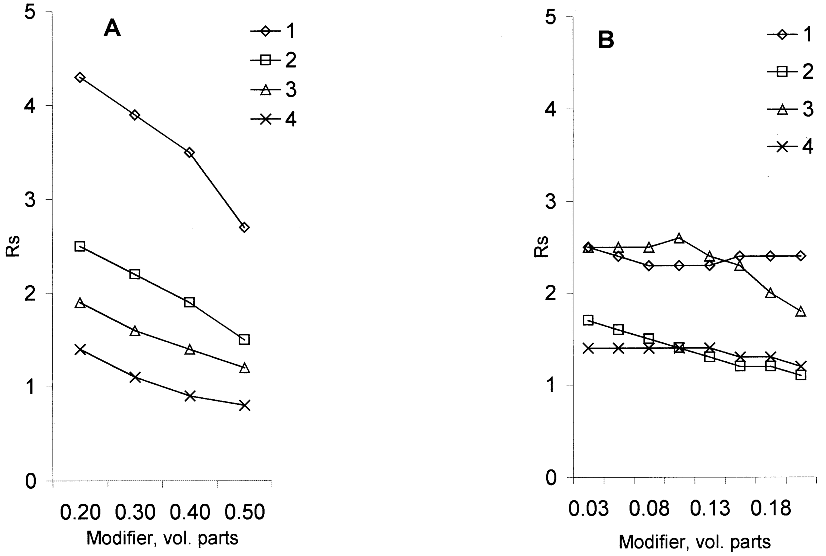

**Figure 3**. Effect of the iso-propanol (lines **1** and **2**) and methanol (lines **3** and **4**) as modifiers in hexane-dichloromethane, 70:30 (v/v) (**A**) and dichloromethane (**B**) mobile phase on the resolution (Rs) of 6-/9-18:1 (lines **1** and **3**) and 9-/11-18:1 (lines **2** and **4**). Other conditions as in Figure 1.

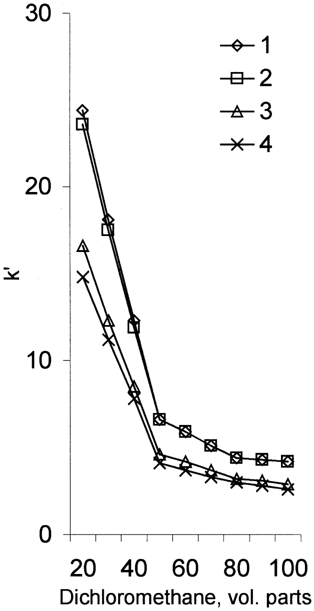

**Figure 4**. Effect of dichloromethane content in hexane-based mobile phases on the retention (k’) of 8-20:1 (line **1**), 5-20:1 (line **2**), 11-20:1 (line **3**) and 13-20:1 (line **4**) with methanol as modifier. Other conditions as in Figure 1.

## Retention and Resolution of 5-, 8-, 11- and 13-20:1

The general pattern of the plots of k’ against the mobile phase composition were the same as above. A few examples are given here, therefore, to illustrate the results. A rapid drop of k’ values was observed when the dichloromethane proportion was increased in hexane-based phases as is demonstrated in Figure 4, for the example of methanol as modifier at 0.2 volume parts. At the same content in the mobile phase, the graphs for acetonitrile and iso-propanol had practically the same pattern. The results revealed that: (i) the strength of the mobile phases increased in the order methanol, iso-propanol, acetonitrile; (ii) k’ decreased more rapidly with the increasing dichloromethane content in methanol containing phases; (iii) k’ of 5- and 8-20:1 were the same over almost the whole interval of methanol-containing phases while promising differences in these values were measured when using iso-propanol or acetonitrile.

Expectedly, k’ decreased rapidly when the proportion of the modifier in hexane-based mobile phase was increased in the range from 0.2 to 0.6 volume parts (Figure 5, iso-propanol as modifier). The graphs with methanol and ace-

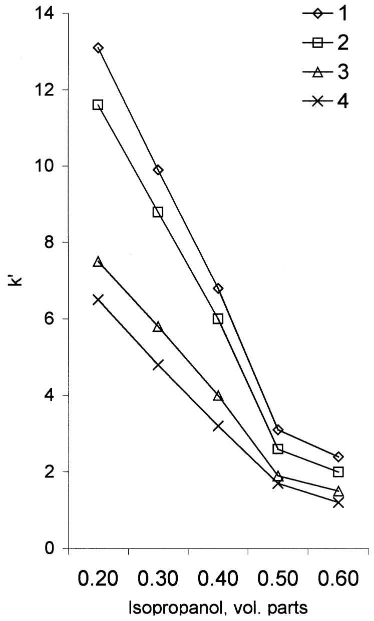

**Figure 5**. Effect of iso-propanol content in hexane-dichloromethane, 70:30 (v/v) on the retention (k') of 5-20:1 (line **1**), 8-20:1 (line **2**), 11-20:1 (line **3**) and 13-20:1 (line **4**). Other conditions as in Figure 1.

tonitrile had the same pattern. The strength of the phases increased in the order: iso-propanol<methanol≈acetonitrile.

In dichloromethane-based phases, the plots of k' against the increasing proportion of the modifier (from 0.025 to 0.2 volume parts) presented the same general pattern (Figure 6). The solvent strength of the mobile phases increased in the same order as above.

The k' values revealed that the order of elution was, in general, 13-, 11-, 5-, 8-20:1 (by increasing retention) with formation of two pairs: 11-/13-20:1 and 5-/8-20:1, which, were very clearly resolved from each other. It was evident, also, that while no problems should be expected with the resolution of 11- and 13-20:1, that of 5- and 8-20:1 could be problematic. This was confirmed in the practice and is illustrated here with the plots of Rs against the mobile phase composition (Figure 7 A-C). Most important observation was that when by changing the modifier, changes in the elution order were observed. Thus, 5-20:1 eluted ahead of 8-20:1 in hexane-dichloromethane-acetonitrile, 70:30:0.2 (v/v/v) (Figure 8 A), while the reversed order was found in hexane-

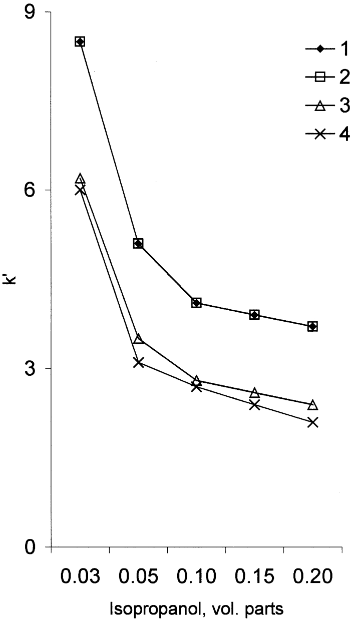

**Figure 6**. Effect of the iso-propanol content in dichloromethane on the retention of 8-20:1 (line **1**), 5-20:1 (line **2**), 11-20:1 (line **3**) and 13-20:1 (line **4**). Other conditions as in Figure 1.

dichloromethane-iso-propanol, 70:30:0.5 (v/v/v) (Figure 8 B). As observed previously with CLA isomers,[3] effected were the difficult to resolve 5- and 8-20:1, which gave adjacent peaks in Ag-HPLC.

## Retention and Resolution of 18:3 and 20:3 Positional Isomers

Many of the data discussed above, were valid for the two pairs of 18:3 (6,9,12- and 9,12,15-) and 20:3 (5,8,11- and 11,14,17-) p-methoxyphenacyl esters. Certainly, these derivatives were retained much stronger than monoenes and dienes and mobile phases with much higher content of a modifier were required for relevant elution. Also, the ranges of solvent proportions of hexane- and dichloromethane-based phases that provided reliable selectivity of resolution were quite narrow. Under identical experimental conditions, the retention of eicosatrienoic fatty acids was weaker than that of octadecatrienoic fatty acids. This is seen from the example given in Table 1. While the measured differences in k' between the isomers could be expected,[9] it was interesting to monitor the resolution. In earlier papers, a hypothesis of formation of a three-cen-

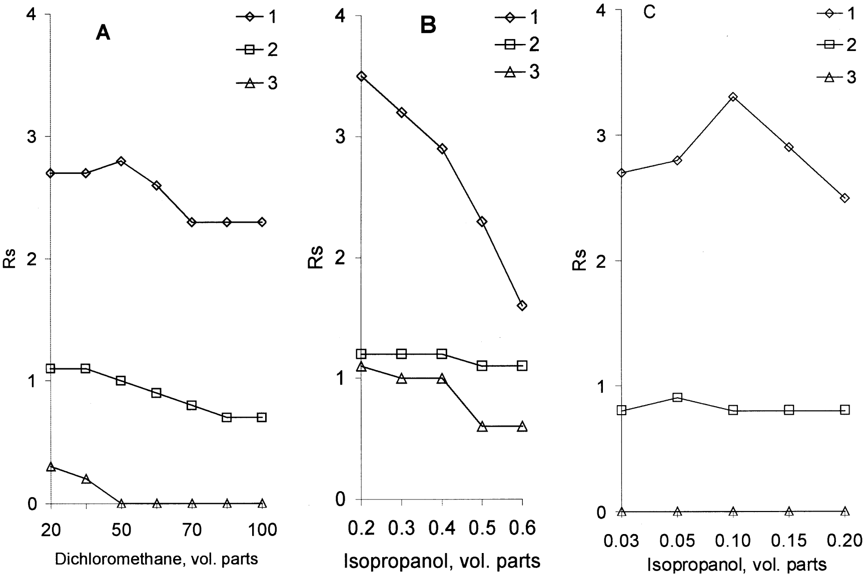

**Figure 7**. Effects of mobile phase composition on the resolution (Rs) of 20:1 positional isomers. Variation of dichloromethane content in hexane-dichloromethane-methanol, 100-X:X:0.2 (v/v/v) mobile phases(**A**); variation of iso-propanol content in hexane-dichloromethane-iso-propanol, 70:30:X (v/v/v) (**B**). 11-/8-20:1 (line **1**), 13-/11-20:1 (line **2**), 5-/8-20:1 (line **3**). Other conditions as in Figure 1.

ter complex was suggested on the basis of the retention and resolution defined by the position of the single double bond;[1,9,10] the three centers being: a suitably positioned double bond, a fragment of the ester moiety, and a silver ion. It was not clear whether the higher number of double bonds would enhance, or suppress the resolution. Also, it was not clear whether the elution order of 18:1 isomers as defined earlier[2,9] would hold. Obviously, the 18:3 isomers were suitable example to examine the retention and resolution in this aspect since the first double bonds were in positions 6- and 9-, respectively, exactly as in the 18:1 analogues. The result was, that in the best resolving mobile phase, dichloromethane-acetonitrile, 100:0.7 (v/v) a Rs value of 0.9 was determined, while a value of 3.4 was found for the pair of 6-/9-18:1 in the corresponding most selective mobile phase of dichloromethane-acetonitrile 100:0.025.[9] Evidently, while the increased number of double bonds led to a substantial increase in retention, resolution decreased with a value of about the same magnitude. The elution order, however, was exactly the same as with 18:1 fatty acids, i.e. 9,12,15- 8:3 eluted ahead the 6, 9, 12-18:3 (Figure 9 A). This fact

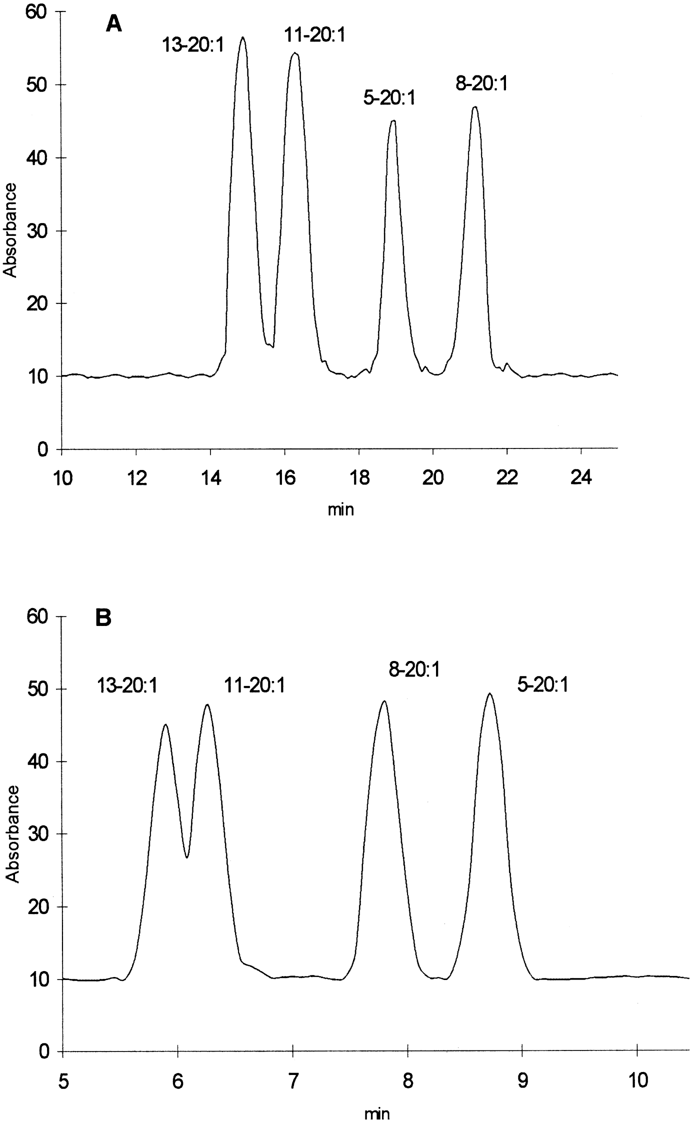

**Figure 8**. Separation of 20:1 isomers in Ag-HPLC: **A**, with mobile phase hexane-dichloromethane-acetonitrile, 70:30:0.2 (v/v/v); **B**, with mobile phase hexane-dichloromethane-iso-propanol, 70:30:0.5 (v/v/v). Other conditions as in Figure 1.

### Table 1

### Retention Factors, k′, of *cis* Trienoic p-Methoxyphenacyl Esters in Ag-HPLC with Mobile Phase Dichloromethane-Acetonitrile*

| **Fatty Acid** | **k′$^a$** |
|---|---|
| 11, 14, 17-20:3 | 2.7 |
| 5, 8, 11-20:3 | 3.1 |
| 9, 12, 15-18:3 | 3.4 |
| 6, 9, 12-18:3 | 3.7 |

---

* 100:0.4, v/v. [a] $k' = k'_{FA}/k'_{11,14\text{-}20:2}$

eventually supports the hypothesis about the formation of a three center complex and its role in the resolution of positionally isomeric fatty acids in Ag-HPLC.

The same mobile phase also ensured the best resolution of the two 20:3 isomers (Figure 9 B). The Rs value was 1.1 and the elution order resembled that of the respective 18:1 isomers.[9] The better resolution of the 20:3 isomers could be eventually ascribed to the difference between the position of the respective first double bonds: 5,8,11- and 11,14,17-20:3. According to earlier results,[9] this difference in double bond positions should ensure a large difference in retention and, hence, good resolution.

When using iso-propanol as a modifier, however, the elution order of both pairs of trienoic fatty acids reversed (Figure 10 A and B).

Summarizing the results of the present work, it was clear that: (i) acetonitrile was not unique as a mobile phase modifier for the separation of positionally isomeric fatty acids as p-methoxyphenacyl derivatives; methanol and iso-propanol ensured the same fine separations; (ii) depending on the sample composition and the type of the solvent system chosen as a mobile phase, either of these three modifiers provided reliable resolution.

In confirmation of earlier result,[3] iso-propanol affected the elution order of difficult to resolve isomers, which gave adjacent peaks in Ag-HPLC by reversing the elution order obtained with acetonitrile. There is no plausible explanation of this phenomenon so far. Interaction of the mobile phase, both with the fatty acid molecule or/and with the alkyl-benzenesulfonic acid moieties that bear the silver ions, may take place. Also, the fact that iso-propanol containing phases affected the elution order of closely related isomers, eventually indicated that fatty acid

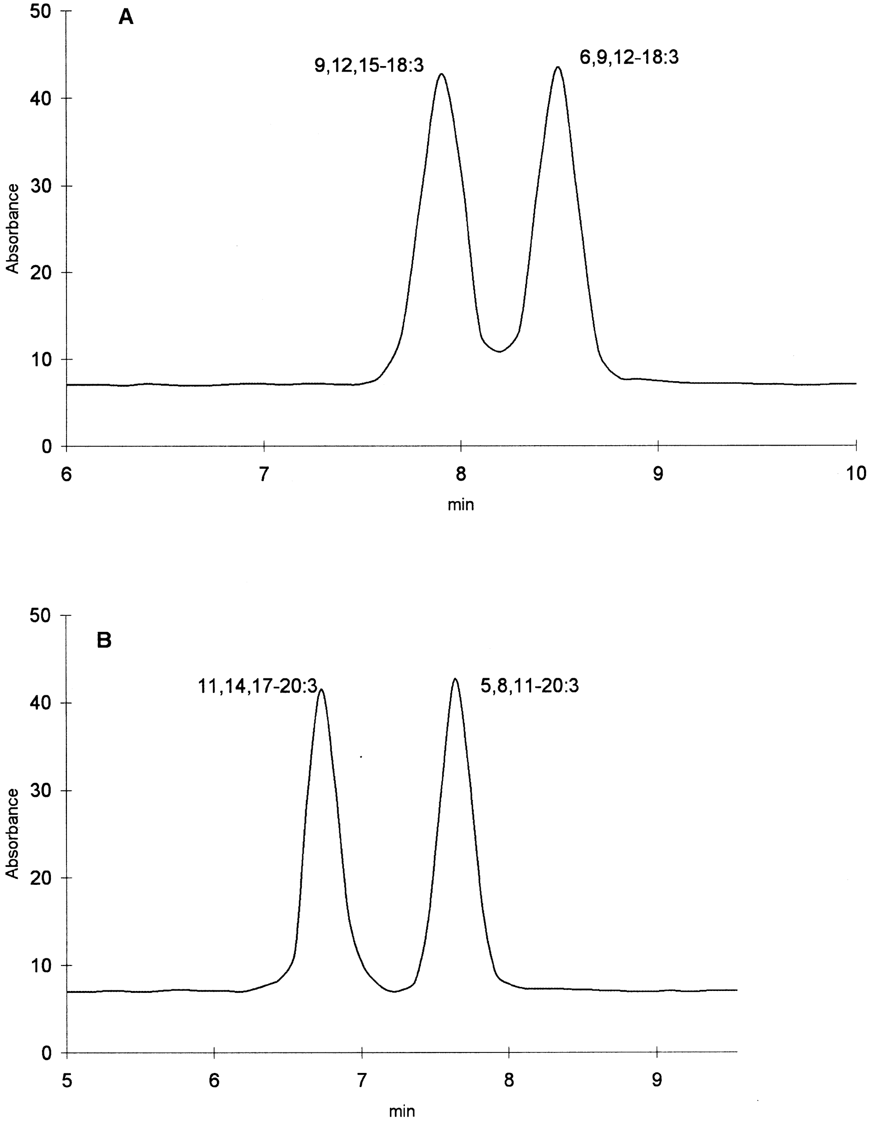

**Figure 9**. Separation of 18:3 (**A**) and 20:3 (**B**) isomers in Ag-HPLC with mobile phase dichloromethane-acetonitrile 100:0.7 (v/v). Other conditions as in Figure 1.

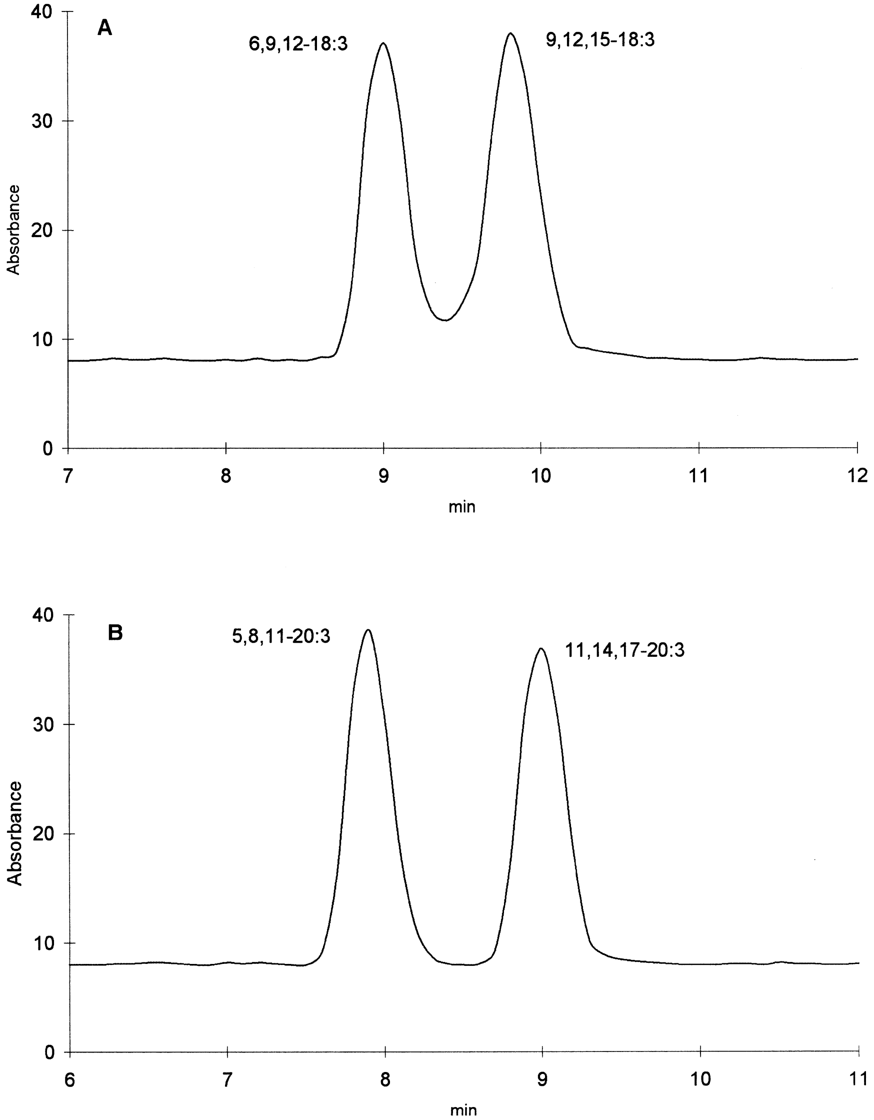

**Figure 10**. Separation of 18:3 (**A**) and 20:3 (**B**) isomers in Ag-HPLC with mobile phase dichloromethane-iso-propanol, 100:3 (v/v). Other conditions as in Figure 1.

molecules were twisted in such a way that double bonds in different positions were favored in the formation of three center complexes with silver ions.

## ACKNOWLEDGMENTS

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