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Separation of isomeric compounds by reversed-phase high-performance liquid chromatography using Ag^+ complexation

Application to *cis-tram* fatty acid methyl esters and retinoic acid photoisomers

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ABSTRACT

The improvement of the chromatographic efficiency using Ag^+ complexing reversed-phase liquid chromatography for the analysis of geometric isomers was studied. The influence of the nature and concentration of silver salts on r-complex formation was investigated with two types of sample, fatty acid methyl esters and *trans*-retinoic acid photoisomers. First, the separations of *cis–trans*-linoleic acids and *cis–trans*-oleic acids were confirmed. Second, the π-complexation was shown to occur with more complex chemical structures such as *trans*-retinoic acid and its photoisomers. The chromatographic separation of seven geometric isomers of *trans*-retinoic acid was then carried out with good efficiency in half the time required in previous work.

INTRODUCTION

Argentation chromatographic methods are useful for the analysis of unsaturated compounds. Many publications have described the use of metal ions to improve the separation of analytes by thin-layer chromatography [1], gas–liquid chromatography [2] and high-performance liquid chromatography (HPLC) [3], especially for *cis–trans* geometric isomers [1,4–6].

First, silver ion complexing liquid chromatography was performed using adsorption chromatographic systems with silica gel impregnated with various amounts of silver salts as stationary phases [7,8]. This technique was then transposed successfully to reversed-phase liquid chromatographic systems for the separation of various compounds such as vitamin D, estrogenic derivatives, unsaturated hydrocarbons, triglycerides and fats [9–11], but a strong interaction takes place in the mobile phase. It was shown previously [9] that the presence of a silver salt in the mobile phase leads to the formation of a m-complex. The bonding is considered to involve an interaction between very electrophilic silver ions and filled π orbitals of unsaturated compounds.

Two conclusions on the behaviour of n-complexes in reversed-phase liquid chromatography were drawn by Schomburg and co-workers [10,11]: the polarity of the Ag^+ r-complex is

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higher than the polarity of the initial compound, so its elution is quicker and a decrease in its capacity factor is observed with silver salt in the mobile phase; and the retention times depend on the number, type and position of the unsaturations, according to the complexation equilibrium constants. *Cis* complexes, generally more stable than *trans* complexes, show a greater affinity with the mobile phase and elute earlier. This explains the high separation efficiencies observed with argentation chromatographic systems in the analysis of geometric isomers.

In this study, we first confirmed the previously published data on the chromatographic separation of fatty acid isomers. We then investigated the potential of this method for improving the separation of photoisomers of retinoic acid. The photodegradation of trans-retinoic acid leads to the formation of numerous isomers. Until now, their separation has been carried out by reversed-phase liquid chromatography [12–16], but the methods showed poor resolution and/or very long analysis times. With regard to the presence of conjugated double bonds in the structure of retinoic acid, we also studied the behaviour of the *cis–trans* isomer mixture in argentation liquid chromatography and the benefits obtained in terms of resolution and analysis time.

EXPERIMENTAL

Chemicals

Fatty methyl esters and silver and potassium salts were purchased from Sigma (La Verpillibre, France). *trans*-Retinoic acid was kindly donated by Laboratories Roche (Neuilly sur Seine, France). HPLC-grade methanol was purchased from Prolabo (Paris, France) and perchloric acid from Merck (Nogent sur Marne, France). Water for HPLC was doubly distilled.

The photoisomers of retinoic acid were obtained by irradiation of a 0.05 g per 100 ml solution of trans-retinoic acid in ethanol solution. Aliquots of 5 ml of this solution were poured into a Pyrex crystallizing dish (50 mm diameter), then irradiated with a Biotronic UV crystallizing dish (50 mm diameter), then irradiated with a Biotronic UV system (Vilber Lourmat, Torcy, France) controlled by a microcomputer. Irradiations were performed at 365 nm (UVA) and 312 nm (UVB) simultaneously.

HPLC of fatty acid methyl esters

The chromatograph consisted of a solvent-delivery pump from Altex (Touzart et Matignon, Vitry sur Seine, France), a Rheodyne injection valve (20-μl loop), a Shimadzu SPD-2A variable-wavelength detector (Touzart et Matignon) operating at 210 nm and a Kipp & Zonen BD-40 recorder (Cunow, Cergy St. Christophe, France). The column was 300 mm $\times$ 4 mm I.D. stainless-steel packed with Nucleosil C_{18} stationary phase (7 μm particle size) from Touzart et Matignon. The initial eluent was methanol–water-perchloric acid (85:15:0.01, v/v/v) with the addition of either silver perchlorate (concentration range $1 \cdot 10^{-4}$–$5 \cdot 10^{-3}$ *M*) or potassium perchlorate (concentration range $1 \cdot 10^{-4}$–$1 \cdot 10^{-3}$ M). These mobile phases were degassed by sonication and filtered through a 0.22-μm membrane (Millipore, St. Quentin en Yvelines, France). The flow-rate was set at 1 ml min^{-1}.

HPLC of retonoic acid isomers

HPLC was performed using a Knauer pump (Cunow) equipped with a Rheodyne injection valve (5-μl loop). Detection was carried out with a Shimadzu SPD-2A variable-wavelength detector (Touzart et Matignon) set at 345 nm and connected with a Perkin-Elmer (Z.I. de Courtaboeuf, France) LCI 100 computing integrator. A column (150 mm $\times$ 4.6 mm I.D.) packed with a Spherisorb ODS-2 stationary phase (3 or 5 μm particle size) from SFCC (Neuilly Plaisance, France) was used. The mobile phases consisted of methanol-water-perchloric acid (80:0:0.02, v/v/v) containing either a silver salt (nitrate or perchlorate) (concentration range $1 \cdot 10^{-3}$–$5 \cdot 10^{-2}$ *M*) or a potassium salt (nitrate or perchlorate) (concentration range $1 \cdot 10^{-3}$–$5 \cdot 10^{-2}$ and $1 \cdot 10^{-3}$–$1 \cdot 10^{-2}$ *M*, respectively). The flow-rate was set at 1 ml min^{-1}.

RESULTS AND DISCUSSION

π-Complex formation

The effectiveness of **π-complexation** was demonstrated by comparing the behaviour of saturated and unsaturated fatty acid methyl esters with silver salts and by comparing the behaviour of the retinoic acid photoisomers with both potassium and silver salts. The chemical structures are illustrated in Fig. 1.

Fig. 2 shows the variation of the capacity factors of the saturated and unsaturated fatty acid methyl esters with the silver perchlorate concentration in the mobile phase. At low concentrations ($<10^{-3}$ M) no significant change is observed in the capacity factors for either saturated or unsaturated compounds. Indeed, the ionic strength remains very weak. Using silver perchlorate concentrations higher than 10^{-3} ***M*** in the mobile phase, a significant decrease in the capacity factors is observed for unsaturated compounds since the retention times of saturated

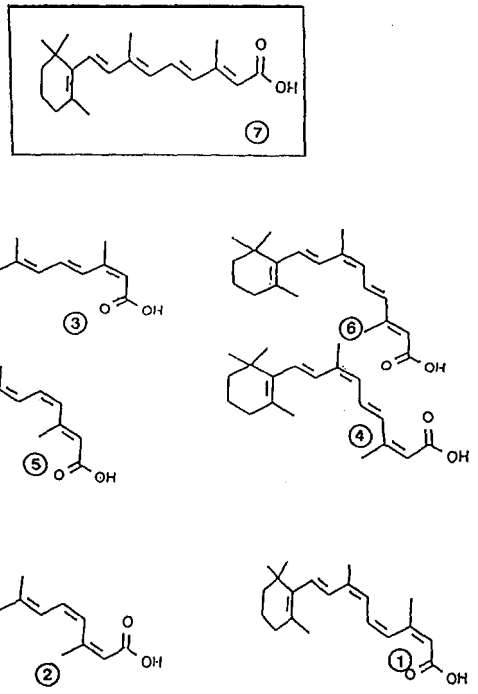

Fig. 1. Structures of ***trans*-retinoic** acid ***(7)*** and its **photo**-isomers **13-*cis* (3), 9-*cis* (6), 11-*cis* (5), 9,13-di-*cis* (4), 11,13**-di-cis (2) and **9,11,13-tri-*cis*** (1).

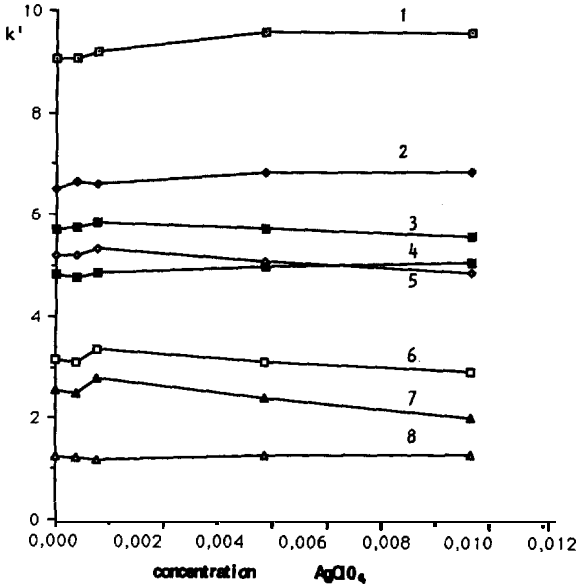

Fig. 2. Effect of the silver perchlorate concentration in the mobile phase on the capacity factors of unsaturated and saturated fatty acid methyl esters: **1** = methyl stearate; 2 = methyl heptadecanoate; 3 = methyl ***trans*-9-octadecenoate**; 4 = methyl **palmitate**; **5** = methyl oleate; 6 = methyl ***trans*-9,*trans*-12-octadecadienoate**; 7 = methyl **linoleate**; 8 = methyl **laurate**.

methyl esters increase owing to the stronger "salt" effect.

The chromatographic elution of retinoic acid photoisomers was studied using a mobile phase containing either potassium nitrate or perchlorate (Fig. 3A) or with silver nitrate or perchlorate (Fig. 3B). As expected, only the presence of silver salts led to a decrease in the capacity factors of the seven compounds, whereas the retention times increased with potassium salts. The phenomenon is more important than with the fatty acid methyl esters because of the wider concentration range used.

Two phenomena occur when a silver salt is added in the mobile phase: a "salt" effect, ***i.e.,*** a simple increase in the ionic strength of the medium, which was observed for low concentrations of silver perchlorate in the fatty acid methyl esters study and for potassium salts in the retinoic acid photoisomers study; and a **π-complexation** effect due to [Ag-molecule] $^{+}$ **form-**

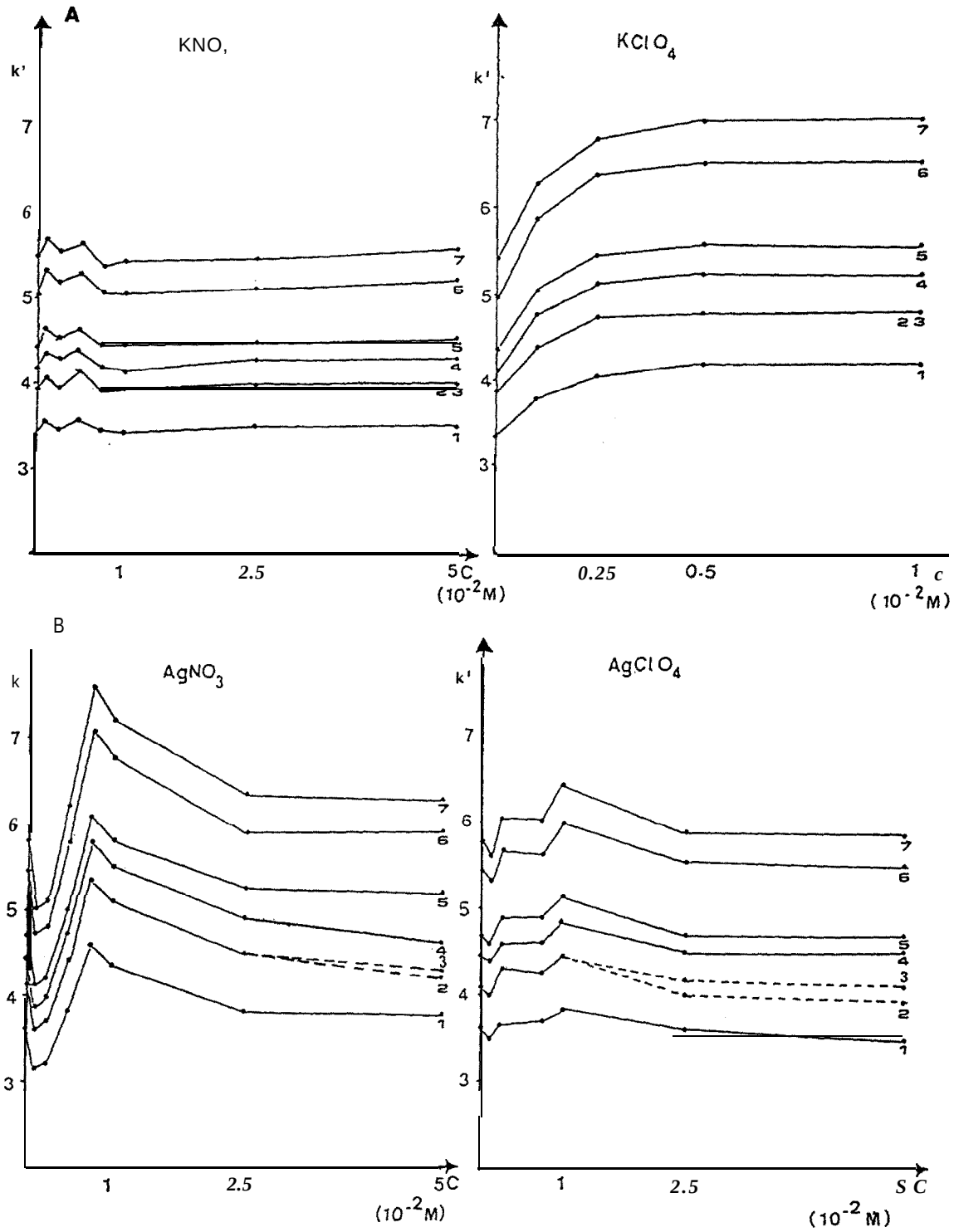

Fig. 3. Effect of (A) potassium salt and (B) silver salt concentrations in the mobiie phase on the capacity factors of ***trans*-retinoic** acid photoisomers: **1 = 9,11,13-tri-*cis*-retinoic** acid; 2 = **11,13-di-*cis*-retinoic** acid; 3 = **13-*cis*-retinoic** acid; 4 = **9,13-di-*cis*-retinoic** acid; 5 = **11-*cis*-retinoic** acid; 6 = **9-*cis*-retinoic** acid; **7** = ***trans*-retinoic** acid.

ation, which was observed only with silver salts at a defined concentration and with compounds containing π-electrons of various origins, particularly double bonds. This effect is observed not only with the fatty acid methyl esters but also with more complex chemical structures such as *trans*-retinoic acid and its photoisomers.

Influence of the kind and concentration of silver salts

Figs, 2 and 3B show that a defined silver salt concentration has to be reached to induce the complexation. This critical salt concentration changes with the chemical nature of both the analytes and silver salt used: using silver perchlorate, the complexation was observed for a lower silver concentration with fatty acid methyl esters ($5 \cdot 10^{-3}$ M) than with retinoic acid photoisomers ($1 \cdot 10^{-2}$ M).

The comparison between silver nitrate and perchlorate was performed only with retinoic acid photoisomers. The chromatographic analysis of fatty acid methyl esters using silver nitrate was not carried out because there was too much noise at the detection wavelength (210 nm). The decrease in retention times related to the π-complexation is amplified with silver nitrate but the phenomenon is more selective with silver perchlorate. When the silver salt concentration in the mobile phase is higher, the "salt" effect increases with increasing ionic strength and compensates for the "complexation" effect (Fig. 3B). The retention times of the compounds become constant.

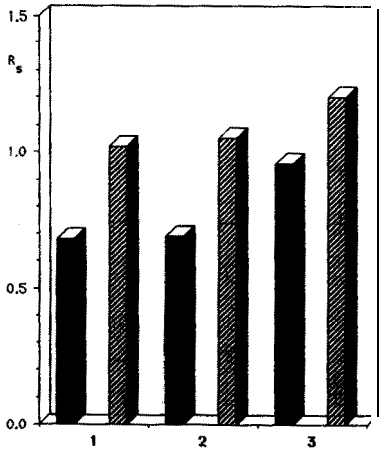

Fig. 4. Variation of R_s with silver percblorate concentration in the mobile phase in chromatographic separations of the *cis–trans*-linoleic acid methyl esters and the *cis–trans*-oleic acid methyl esters. Mobile phase (1) without salt, (2) containing $5 \cdot 10^{-3}$ *M* $KClO_4$ and (3) containing $5 \cdot 10^{-3}$ *M* $AgClO_4$. Black bars, C''_{18_c}/C''_{18_t}; hatched bars, C'_{18_c}/C'_{18_t}.

improvement of the separation of retinoic acid photoisomers

The chromatographic analysis of a *cis–trans*-linoleic acid and *cis–trans*-oleic acid mixture shows the improvement of the resolution, R_s, in the presence of silver perchlorate by a factor of 1.4 for C''_{18_c}/C''_{18_t} and 1.2 for C'_{18_c}/C'_{18_t} (Fig. 4). These results confirm those previously published for oleic and elaidic acid isomers [10]. As the capacity factor seems to be a function of the number of double bonds, the improvement in

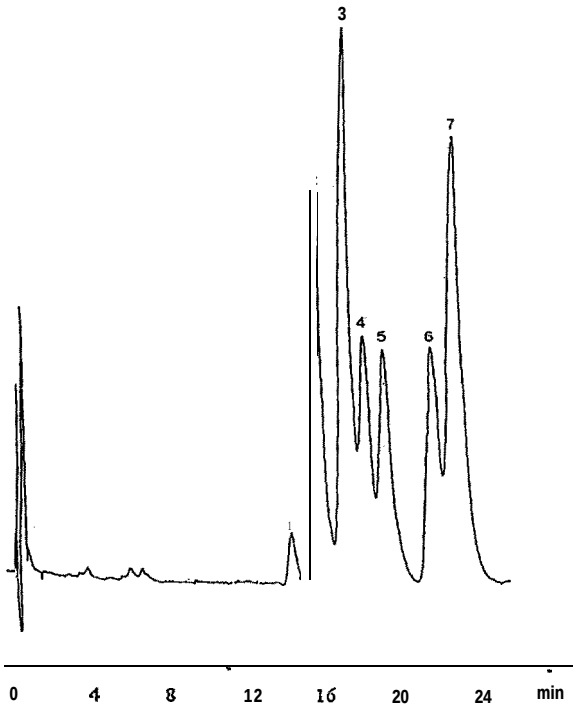

Fig. 5. Ag^+ complexation reversed-phase liquid chromatogram of *tram-retinoic* acid photoisomers: 1 = 9,11,13-tri-cis-retinoic acid; 2 = 11,13-di-*cis*-retinoic acid; 3 = 13-*cis*-retinoic acid; 4 = 9,13-di-*cis*-retinoic acid; 5 = 11-cis-retinoic acid; 6 = 9-*cis*-retinoic acid; 7 = *trans*-retinoic acid. For chromatographic conditions, see Experimental (particle size of stationary phase = 3 μm).

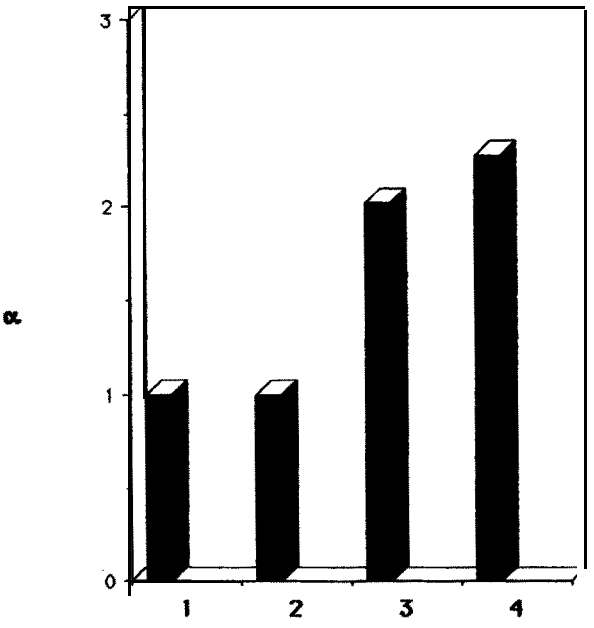

Fig. 6. Dependence of selectivity (α) for 13-*cis* and 11,13-di-*cis* photoisomers on silver salt concentration. Mobile phase (1) without salt, (2) containing $1 \cdot 10^{-2}$ *M* $KClO_4$ and (3 and 4) containing $1 \cdot 10^{-2}$ *M* $AgClO_4$. Particle size of the stationary phase (1,2,3) 5 μm and (4) 3 μm.

the separation depends on the steric configuration.

Likewise, the separation of the seven geometric isomers was achieved with the addition of silver salts to the mobile phase (Fig. 5). For example, Fig. 6 illustrates the twofold improvement in selectivity obtained between the 13-*cis* and the 11,13-di-*cis* isomers using mobile phases of similar ionic strength but containing Ag^+ ions instead of K^+ ions.

All these results demonstrate the improvement of the efficiency of the silver ion complexing liquid chromatographic system. Compared with the previously published data, the present analysis provides efficient separations of *trans*-retinoic acid and its photoisomers in half the time. The selectivity per unit time is enhanced with respect to the other proposed chromatographic separations.

CONCLUSIONS

This work confirmed the chromatographic behaviour of fatty acid methyl esters with silver salts. We showed that the formation of π-complexes with *trans*-retinoic acid photoisomers can occur. A comparison of the behaviour of these molecules with similar chemical structures showed the necessity to determine accurately two critical parameters, the nature and the concentration of the silver salt added to the mobile phase. Moreover, this method leads to very efficient separations and is able to resolve a complex mixture of retinoic acid photoisomers. This method is particularly adapted for the study of *trans*-retinoic acid photodegradation.

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