

High-performance liquid chromatography of fatty acid derivatives in the combined silver ion and reversed-phase modes

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ABSTRACT

In order to learn more of the mechanism of silver ion complexation in chromatographic systems, the retention characteristics of some unsaturated fatty acid esters (phenacyl and phenethyl) were determined using reversed-phase high-performance liquid chromatography with silver ions in the mobile phase. It was possible to calculate equivalent chain length and fractional chain length values, which appeared to indicate that double bonds and silver ions formed simple 1:1 complexes in the mobile phase. The overall lipophilicity of the complex had an effect in determining the resolution of positional isomers. The equilibrium constant for the formation of phenacyl oleate (or "argentation constant") was calculated to be in the range 0.059–0.067, depending on the experimental conditions. The mechanism is compared with that for systems in which silver ions are bound to the stationary phase.

INTRODUCTION

In a previous paper [1], we described the retention characteristics of derivatives of unsaturated fatty acids in silver ion high-performance liquid chromatography (Ag-HPLC). A column was used with silver ions linked via ionic bonds to phenylsulphonic acid moieties, which were in turn bound to a silica matrix [2]. As there were appreciable differences in retention of the phenacyl derivatives of isomeric monoenoic fatty acids, base-line resolution of the natural 6-, 9-

and 11-18:1 isomers was possible at ambient temperature. The results enabled some suggestions to be made about the probable complexation mechanism.

In an effort to understand better the nature and strength of the interaction between a double bond in a lipid and silver ions, we have turned to another method of introducing silver ion into an HPLC system, *i.e.* reversed-phase HPLC (RP-HPLC) with a mobile phase containing a silver salt. This modification of silver ion HPLC had been applied to the separation of simple fatty acid derivatives by others [3–6], but has been of rather limited use because of numerous technical problems, as discussed elsewhere [7,8]. However, we considered that this form of silver ion chromatography might be a suitable tool for estimation of the interaction between a double bond in a fatty acid derivative and a silver ion,

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distinct from hydrophobic and other effects. By this means, it is possible to compare the retention characteristics of a solute in the presence and absence of silver ions in the mobile phase, while keeping all other chromatographic conditions constant.

The chromatographic behaviour of derivatives of some positional isomers of monoenoic fatty acids and common polyunsaturated fatty acids were investigated in this study in an attempt to reveal the contribution of the position and number of double bonds in the complexation reaction. The equilibrium constant of the reaction between silver ions and phenacyl oleate was determined quantitatively by using an approach suggested by Horváth *et al.* [9].

EXPERIMENTAL

Materials and reagents

Free fatty acids with 0 to 6 double bonds, including the three naturally occurring *cis*-octadecenoic positional isomers (oleic, petroselinic and *cis*-vaccenic acids) were purchased from Sigma (Poole, UK). The rest of the isomeric *cis*-octadecenoic acids had been prepared earlier by total synthesis [10]. All solvents were analytical or HPLC grade and were supplied by FSA Scientific Apparatus (Loughborough, UK). Silver nitrate (AnalaR), silver perchlorate (Sigma), ammonium nitrate (AnalaR) and potassium nitrate (FSA) were used to modify the mobile phase.

High-performance liquid chromatography

A Spectra-Physics (St. Albans, UK) Model 8770 solvent-delivery system was used, together with a Pye Unicam Model 4025 UV detector and an octadecyl (Spherisorb S50DS2, Hichrom, Reading, UK) or an octyl (Spherisorb S5C8, Hichrom) column (250 mm × 4.6 mm I.D.). Methanol–water mixtures were used as mobile phase at a flow-rate of 1 ml/min. The proportion of water was varied depending on the column used and the derivative studied. Silver, ammonium and potassium salts (0.0025 to 0.08 M) were added to the mobile phase as aqueous solutions in such proportions that their final

concentration in the mobile phase was in the range 0.0025 to 0.08 M.

The phenacyl and phenethyl derivatives were detected at 257 nm, as this wavelength was less sensitive toward the presence of silver salts in the mobile phase [3].

The dead volume was measured using acetone, which is not retained in the column and absorbs strongly below 330 nm. The appropriate myristic acid (14:0) derivative was used as an internal standard in each run. Experiments were performed at constant temperature, 20°C, by fitting the column into a water jacket, through which ethyleneglycol was pumped from a Chrompack Model RTE-110B temperature control unit (Chrompack UK, London, UK).

Derivatization

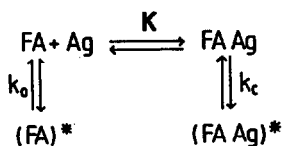
Fatty acids were converted into phenacyl [11] and phenethyl derivatives. To produce the phenethyl derivatives, the free acids (10 mg) were first converted to the corresponding acid chlorides by reaction with oxalyl chloride (0.5 ml) for 36 h at room temperature; the excess reagent was evaporated in a stream of nitrogen and finally by a rotary evaporator. The residue was dissolved in toluene (0.5 ml), and phenethyl alcohol (10 mg) in toluene (1 ml) and pyridine (0.2 ml) were added. The mixture was heated overnight at 50°C, then the excess solvent and pyridine were removed in a rotary evaporator. Hexane (5 ml) was added to the residue and the solution was washed with water (2 × 5 ml). Finally, the product was cleaned by elution through a Florisil column (0.5 g) with hexane–acetone (5 ml; 99:1, v/v). The solvents were evaporated under nitrogen and the required phenethyl derivatives were re-dissolved in methanol for analysis (0.02 to 0.2 mg/ml). Phenacyl and phenethyl derivatives were injected onto the column as methanolic solutions (10 μl).

RESULTS AND DISCUSSION

Retention characteristics of fatty acid derivatives

The interactions that take place in reversed-phase HPLC with a mobile phase containing

silver ions may be presented as:



where FA denotes a fatty acid molecule, FFAg is the complex with a silver ion, and * denotes a molecule retained by the support. If the concentration of the complexing ion in the mobile phase is sufficiently high, the solute (e.g. a fatty acid derivative) should be in the form of a complex, and the capacity factor (k'_c) would represent the retention of the complex in the RP-HPLC system. When the mobile phase contains no silver ions, the retention factor (k'_0) would represent the retention of the uncomplexed fatty acid derivative. The difference between the two values corresponds to the effect of complex formation [9].

Phenacyl esters of unsaturated fatty acid were studied, because of the interesting results reported earlier [1] for their retention characteristics on a silver loaded column and because of the convenience of using UV detection. Phenethyl esters were prepared as they are similar to phenacyl esters in molecular weight, shape and properties, but lack a second carbonyl group thought to participate in complexation [1].

Preliminary experiments showed that the most suitable mobile phase was a mixture of methanol and water in proportions that depended on the nature of the fatty acid derivative and the chain-length of the bonded stationary phase. The composition of the mobile phase was considered to be optimum when the best resolution of 6-18:1 and 9-18:1 derivatives, used as test substances, was achieved. Mobile phases based on acetonitrile were not suitable because of their strong interaction with silver ions.

The retention characteristics were measured by the capacity factor k' , or, as specified below, by the ratio of the capacity factor of the analyte to that of the same myristic acid (14:0) derivative, and denoted here as k'' . k' values were measured with a standard deviation of ± 0.01 .

Phenacyl derivatives of fatty acids, including *cis*- and *trans*-isomers, have been resolved with

high selectivity by RP-HPLC on octadecyl columns [3,11,12]. A column with an octyl bonded phase was less selective because of the lower lipophilic interaction, and separations of positional isomers of fatty acids could not be achieved with the common mobile phases. On the other hand, the effects of adding silver ions in the mobile phase were expected to be more distinctive.

The temperature was maintained at 20°C. Although preliminary experiments with lower temperatures (10°C and 0°C) improved the resolution, inconvenient increases in the retention time and peak broadening also occurred. When the composition of the mobile phase was adjusted to give comparable retention times and peak shapes, no effect of temperature on resolution was apparent. This confirmed our earlier conclusion [1] that the whole chromatographic system responds to the change in the temperature.

The silver salt concentration was varied between the relatively narrow limits of 0.0025 M to 0.08 M. Measurements above this range were not possible because of the background UV absorption of the silver ions and the salt anions.

When silver ions were added to the mobile phase, the capacity factors (i.e. k'_{Ag} and k''_{Ag}) decreased gradually with the increasing silver salt concentration. In Fig. 1, the k''_{Ag}/k''_0 ratio is

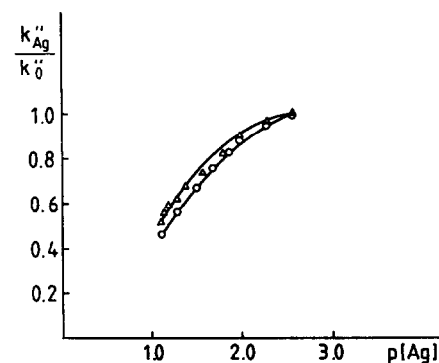


Fig. 1. The dependence of the k''_{Ag}/k''_0 of phenacyl oleate on the negative logarithm of the silver ion concentration (molar), $p[\text{Ag}^+]$, in the mobile phase. Δ = Spherisorb S5C8 column and a mobile phase of methanol–water (87:13, v/v); \circ = Spherisorb S5ODS2 column with mobile phase 92.5:7.5 (v/v).

plotted against the silver ion concentration with the phenacyl ester of 9-18:1 as test compound for both columns. It has been suggested [9] that this ratio is a measure of the complexation effect. Evidently, since the complex is retained less strongly than the non-complexed derivative (*i.e.* $k'_{Ag} < k'_o$), the ratio is less than 1 and its value decreases with an increasing degree of complexation. The effect of complexation was slightly stronger for the octadecyl bonded phase and this was probably connected with the stronger response of the octadecyl moieties toward the changed physical characteristics of the complexed fatty acid derivative.

Although other monoene isomers were studied, the retention behaviour of the three naturally-occurring isomers, 6-, 9- and 11-18:1, was most important from a practical point of view. Under all condition, the 6-18:1 isomer was retained more than was 9-18:1. However, 6-18:1 was also found to be retained more in the silver-loaded column, presumably as a result of the formation of a stronger silver ion complex [1], so it was expected that it would be retained less in a reversed-phase system with silver ions in the mobile phase. In fact, the phenacyl derivatives of the monoenoic fatty acid isomers followed the elution order reported by Wood [12] for a RP-HPLC system (Fig. 2) with two important exceptions. The 4- and 5-18:1 isomers were retained more than their neighbours, and this was ascribed to an interaction between the carbonyl oxygen and the double bond that lowered the

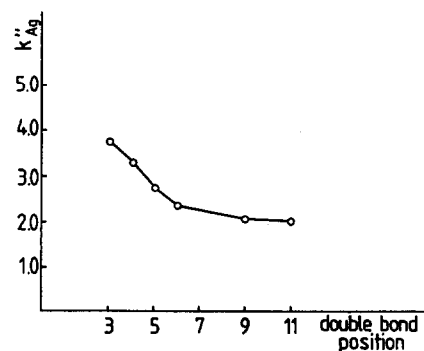


Fig. 2. The relative capacity factors, k'_{Ag} , of some isomeric C_{18} monoenoic phenacyl derivatives with the position of the double bond.

overall polarity of the molecule. In the presence of silver ions (0.05 M) in the mobile phase, the capacity factors of these isomers fell on their "correct" places on the graph. Fig. 2 reveals that the lipophilic interaction governs the elution order. Thus, 6-18:1 and 9-18:1 were separated completely with an R_s value of 2.5 (with 0.075 M silver nitrate in the mobile phase). No conditions were found under which 9- and 11-18:1 were separated. Comparative experiments with the same mobile phase and no silver nitrate showed that 6-18:1 and 9-18:1 were readily resolved (with an R_s value close to 1.0) on an octadecyl but not on an octyl column. Thus, silver ions in the mobile phase enable or improve the resolution but do not affect the elution order.

Silver perchlorate was found to be superior to nitrate, with lower concentrations needed to separate 6- and 9-18:1 isomers completely on the octadecyl column; an R_s value of 3.5 was obtained with 0.05 M silver perchlorate *versus* 2.1 with 0.05 M silver nitrate. The type of salt had no significant effect with the octyl column.

Capacity factors decreased with an increasing number of double bonds in the fatty acid. To demonstrate the degree of retention, the capacity factors were determined relative to those of the corresponding oleate derivatives with the results listed in Table I. Evidently, the nature of the ester moiety had little influence on the relative degree of retention of the polyunsaturated fatty acids, as the two sets of results are almost the same. The C_{18} derivatives with two to four double bonds eluted from 2 to 5 times faster, respectively, than did the monoene. Because of the differences in chain lengths, eicosapentaenoate and docosahexaenoate eluted as quickly as the corresponding 18:4 derivatives.

A more impressive presentation of the effect of the complexation on retention is the plot of the $k''_{Ag} - k''_o$ values (the fraction of the capacity factor that corresponds directly with the effect of complexation) *versus* the concentration of silver ion in the mobile phase. Fig. 3 illustrates this effect for the same fatty acids as in Table I. With a greater degree of unsaturation, higher silver ion concentrations increased the effect of the complexation. The greater the number of double bonds, the smaller was the effect of the silver

TABLE I

RATIO OF THE RELATIVE CAPACITY FACTORS (k''_{Ag}) OF PHENETHYL AND PHENACYL ESTERS OF SOME POLYUNSATURATED FATTY ACIDS TO THAT OF THE OLEATE DERIVATIVE

The Spherisorb S5C8 column was used with a mobile phase of methanol–water (88:12, v/v) for the phenethyl esters and (81:19, v/v) for the phenacyl esters with 0.08 M silver perchlorate.

Fatty acid	$k''_{pufa}/k''_{18:1}$	
	Phenethyl ester	Phenacyl ester
9-18:1	1.0	1.0
9,12-18:2	0.6	0.5
9,12,15-18:3	0.3	0.3
6,9,12,15-18:4	0.2	0.2
5,8,11,14-20:4	0.4	0.3
5,8,11,14,17-20:5	0.2	0.2
4,7,10,13,16,19-22:6	0.2	0.2

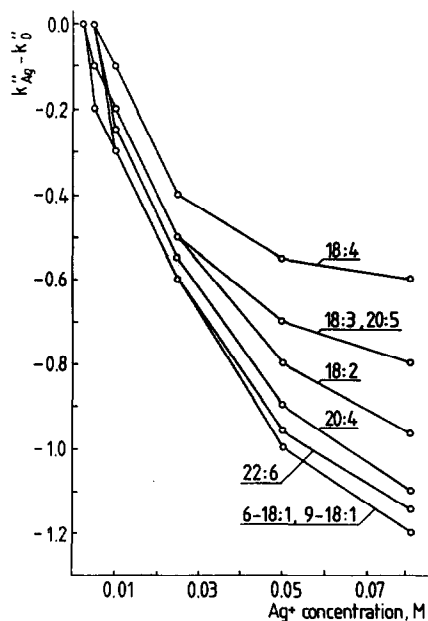


Fig. 3. The $k''_{Ag} - k''_0$ values for phenacyl derivatives of some polyunsaturated fatty acids on a Spherisorb S5C8 column and mobile phase methanol–water (87:13, v/v); k''_0 was measured with 0.08 M ammonium nitrate in the mobile phase; k''_{Ag} was measured with 0.08 M silver nitrate in the mobile phase.

ions on the capacity factor. Finally, the retention of the two monoenoic isomers was affected equally by the presence of silver ions in the mobile phase, suggesting that retention order is not determined by a difference in the strength of the complexes but by the general lipophilicity of a molecule. This property should be greater for 6-18:1 as it possesses a longer lipophilic “tail”. It is evident that 18:3 and 20:5 fatty acids have the same $k''_{Ag} - k''_0$ values, while those of 22:6, 20:4 and 18:2 are also similar, over the range of silver ion concentrations.

The contribution of the double bonds and the nature of the ester moiety in the retention characteristics of the fatty acids with different degrees of unsaturation and chain length are better understood by calculating the equivalent chain length (ECL) values [13,14]. This was possible because of the linear relationship between the logarithms of the capacity factors (k') and the chain length of the saturated fatty acid derivatives (both phenethyl and phenacyl esters), as has been shown by others [15,16] (Fig. 4). Fractional chain length (FCL) values, *i.e.* the increment in ECL value of a given fatty acid derivative below that of the saturated derivative of the same chain length, were also calculated. As in GLC [17], the FCL value is influenced by the number and position of the double bonds in the aliphatic chain. Because of their higher lipophilicity, phenethyl esters were retained more than were phenacyl esters.

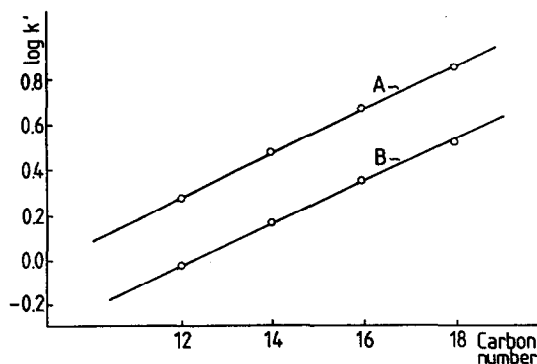


Fig. 4. The capacity factors (k') of saturated fatty acids as phenethyl (A) and phenacyl (B) derivatives in relation to chain length as measured on a Spherisorb S5C8 column with a mobile phase of methanol–water (88:12, v/v).

TABLE II

THE EQUIVALENT CHAIN LENGTH (ECL) AND FRACTIONAL CHAIN LENGTH (FCL) VALUES OF SOME POLYUNSATURATED FATTY ACIDS AS PHENETHYL (PHE) AND PHENACYL (PHA) DERIVATIVES ON A RP-HPLC COLUMN

The Spherisorb S5C8 column was used with a mobile phase of methanol–water (88:12, v/v); the water contained 0.08 M ammonium nitrate.

Fatty acid	ECL		FCL	
	PhE	PhA	PhE	PhA
6-18:1	16.87	17.00	-1.13	-1.0
9-18:1	16.71	16.94	-1.26	-1.06
9,12-18:2	15.63	15.72	-2.37	-2.28
9,12,15-18:3	14.22	14.61	-3.78	-3.39
6,9,12,15-18:4	13.60	13.50	-4.68	-4.50
5,8,11,14-20:4	15.21	15.28	-4.79	-4.72
5,8,11,14,17-20:5	14.16	13.94	-5.84	-6.06
4,7,10,13,16,19-22:6	15.00	14.39	-7.00	-7.61

When silver ions were absent from the mobile phase, irrespective of the nature of the ester moiety, the corresponding ECL values were similar, as were the relevant FCL values listed in Table II. For the C₁₈ fatty acids, monoene:diene:triene:tetraene, the ratio of the FCL

values was close to 1:2:3:4, indicating that the double bonds have an equal contribution to the overall polarity of the fatty acid–silver ion complex. The effect of the ester group on ECL values is small in this system.

The picture was different when silver ions were added to the mobile phase, and the ECL and FCL values are listed in Table III. ECL values of phenethyl derivatives were lower, implying a stronger effect of the silver ions. Also, the ratio of the FCL values of the phenacyl derivatives of the monoenoic to tetraenoic C₁₈ fatty acids were the same as in RP-HPLC, *i.e.* 1:2:3:4, but that of the phenethyl esters was 1:1.3:1.9:2.4. More information was obtained by comparing the Δ FCL values, where

$$\Delta\text{FCL} = \text{FCL}_0 - \text{FCL}_{\text{Ag}}$$

and represents the effect of complexation alone on the chromatographic behaviour of the fatty acid derivative. The Δ FCL values of the C₁₈ phenethyl derivatives are higher than those of phenacyl esters derivatives by a value of about the same magnitude. Evidently, a further factor contributed to the complexation and must be connected with the phenethyl moiety. As the ratio of the FCL values for the C₁₈ monoene:diene:triene:tetraene was not far from

TABLE III

THE EQUIVALENT CHAIN LENGTH (ECL) AND FRACTIONAL CHAIN LENGTH (FCL) VALUES FOR SOME POLYUNSATURATED FATTY ACIDS AS PHENETHYL (PHE) AND PHENACYL (PHA) DERIVATIVES ON AN RP-HPLC COLUMN IN THE PRESENCE OF SILVER IONS

The Spherisorb S5C8 column was used with a mobile phase of methanol–water (88:12, v/v); the water contained 0.08 M silver nitrate.

Fatty acid	ECL		FCL		Δ FCL	
	PhE	PhA	PhE	PhA	PhE	PhA
6-18:1	13.73	15.50	-4.27	-2.50	-3.14	-1.50
9-18:1	13.10	15.06	-4.90	-2.94	-3.64	-1.88
9,12-18:2	10.79	12.39	-7.21	-5.61	-4.84	-3.33
9,12,15-18:3	7.29	9.28	-10.71	-8.72	-6.93	-5.33
6,9,12,15-18:4	4.50	6.28	-13.50	-11.72	-8.82	-7.22
5,8,11,14-20:4	8.58	10.28	-11.42	-9.72	-6.63	-5.00
5,8,11,14,17-20:5	5.63	7.50	-14.37	-12.50	-8.53	-6.44
4,7,10,13,16-22:6	5.63	7.50	-16.37	-14.50	-9.37	-6.89

2:3:4:5, the phenethyl moiety appeared to be roughly equivalent to one double bond in its effect. A confirmation of this assumption is given by the results in Table IV, where the ECL and FCL values of the phenethyl and phenacyl derivatives were calculated using the relative capacity factor, k'' (note that this leads to small differences from analogous values in Tables II and III). As the contribution of the ester moiety was eliminated, ECL, FCL and Δ FCL values of both types of derivative were very close.

Complexation of oleic acid with silver ions

The data presented in Fig. 1 can be utilised to evaluate the equilibrium constant (K) of the silver ion complex of phenacyl oleate by the method described by Horváth *et al.* [9]. The approach has several limitations, the most important being that a correct evaluation can only be made when a 1:1 complex is formed. In the reaction between an olefinic double bond and silver ions, the information is contradictory. It has been reported that silver ions can form a *bona fide* complex with two ethylene molecules [18] or with a diene system [19], but other data show that 1:1 complexes can also be formed [20]. In this system, the simple relationship between the number of double bonds and ECL values suggests the latter. Our calculations assume that phenacyl oleate and a silver ion form a 1:1

complex in the mobile phase that interacts with the hydrocarbon moieties of the bonded phase, and that k'' can be substituted for k' values.

Another requirement was to perform the measurements over as broad a range of silver ion concentrations in the mobile phase as possible, but as discussed above the upper limit was only 0.08 M unfortunately. With these provisos, the results allowed a quantitative evaluation of the equilibrium constant. The equation used was [9]:

$$k''_{Ag} = (k''_o + K[Ag^+]) / (1 + K[Ag^+])$$

which can be linearized [9] for graphical evaluation of the constant K in the form

$$Y = A + BX$$

where

$$Y = k''_{Ag} - k''_o; X = (k''_{Ag} - k''_o) / [Ag^+] \text{ and}$$

$$K = -1/B$$

The experimental conditions and the results are listed in Table V. The K values are very close despite the different columns, silver salts and phenacyl oleate concentrations. To our knowledge there is only one study, which deals with measurements of the so-called "argentation constant" [21] for a fatty acid derivative, when a value of 8.4 was found for methyl oleate. In this

TABLE IV

THE EQUIVALENT CHAIN LENGTH (ECL) AND FRACTIONAL CHAIN LENGTH (FCL) VALUES OF SOME POLYUNSATURATED FATTY ACIDS AS PHENETHYL (PHE) AND PHENACYL (PHA) DERIVATIVES ON SPHERISORB S5C8 COLUMN CALCULATED BY USING THE RELATIVE CAPACITY FACTORS k'' ($k'_{FA}/k'_{14:0}$)

The subscript "o" denotes measurement with ammonium nitrate and "Ag" with silver nitrate in the mobile phase.

Fatty acid	ECL _o		FCL _o		Δ ECL _{Ag}		FCL _{Ag}		Δ FCL	
	PhE	PhA	PhE	PhA	PhE	PhA	PhE	PhA	PhE	PhA
6-18:1	16.58	16.66	-1.42	-1.34	13.79	13.80	-4.21	-4.20	2.79	2.86
9-18:1	16.47	16.56	-1.53	-1.44	13.15	13.38	-4.85	-4.62	3.22	3.18
9,12-18:2	15.29	15.22	-2.71	-2.78	10.80	10.22	-7.20	-7.78	4.49	5.00
9,12,15-18:3	14.00	14.00	-4.00	-4.00	7.40	7.11	-10.60	-10.89	6.60	6.09
6,9,12,15-18:4	12.83	12.89	-5.17	-5.11	4.50	4.11	-13.50	-13.89	8.33	8.78
5,8,11,14-20:4	14.86	14.89	-5.14	-5.11	8.77	8.22	-11.23	-11.78	6.09	6.67
5,8,11,14,17-20:5	13.69	13.78	-6.31	-6.22	5.78	5.78	-14.22	-14.22	7.91	9.00
4,7,10,13,16,19-22:6	14.65	13.78	-7.35	-8.22	5.67	4.89	-16.33	-17.11	8.98	8.89

TABLE V

EQUILIBRIUM CONSTANT OF THE PHENACYL OLEATE COMPLEX WITH SILVER IONS AS MEASURED BY RP-HPLC UNDER DIFFERENT EXPERIMENTAL CONDITIONS

Column	Mobile phase ^a	Silver salt	Sample concentration, <i>M</i>	Equilibrium constant	Regression coefficient
Spherisorb S5ODS2	92.5:7.5	AgNO ₃	6.7.10 ⁻⁴	0.067 ± 0.002	0.988
Spherisorb S5C8	87.0:13.0	AgNO ₃	6.7.10 ⁻⁵	0.059 ± 0.002	0.971
Spherisorb S5C8	81.0:19.0	AgClO ₄	6.7.10 ⁻⁵	0.062 ± 0.002	0.953

^a Methanol–water.

instance, the distribution of methyl oleate was measured between isoctane and aqueous methanol containing silver nitrate by means of iodine values rather than by modern chromatographic methods. This very high value does not agree with other data on the equilibrium constants of monounsaturated hydrocarbons and other derivatives [20], which did not exceed 1.0. We believe that our value is more accurate than that reported earlier [21].

CONCLUSIONS

In silver ion absorption chromatography (TLC or HPLC), silver ions are held directly by the support [8], and complexation takes place on the surface. In silver ion chromatography in a reversed-phase mode, complexation occurs in the mobile phase and the complex interacts with the bonded phase. Complexation with silver ions is important in both instances, but it seems that the selectivity for the separation of positionally-isomeric monoenes, for example, depends more on the other factors. In adsorption silver ion chromatography, steric factors and the molecular conformation are more important; the ester moiety and one double bond, or two double bonds, in a single molecule appeared to interact simultaneously with one silver ion in a system in which silver ions were attached via ionic bonds to the stationary phase [1]. In RP-HPLC in the reversed-phase mode, it appeared that 1:1 complexes with double bonds and silver ions only were formed. If the ester moiety interacted with

silver ions, it did so independently of complexation with double bonds.

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