THE PAPER PARTITION CHROMATOGRAPHY OF UNSATURATED LIPIDS AS THEIR π-COMPLEXES WITH SILVER IONS

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The ability of olefin π-bonds to form unstable co-ordination complexes with silver ions has been known for many years[1]. More recently these π-complexes have been widely used for the separation of unsaturated lipids by adsorption chromatography[2,3]. We decided to study the application of π-complexes to the reversed-phase partition chromatography of lipids since no reports on this subject have been published so far.

EXPERIMENTAL

The general chromatographic procedure employed has been described earlier[4-6]. Chromatographic paper strips (27 × 4.5 cm) were impregnated with a 2 % solution of the stationary non-polar phase (dodecane). Fatty acid methyl esters (500–5000 μg) prepared by methanolysis of linseed, cottonseed, mustard and parsley oils[7] were applied along a baseline on the paper strips. The chromatograms were developed for 12–48 h with 70–90 % aqueous methanol saturated with dodecane and silver nitrate at room temperature, washed with 5 % aqueous nitric acid and water, and kept in the hot air current for 5–10 min[7] to remove dodecane. The lipid spots were visualized by staining with Sudan black B, and eluted with *n*-hexane. The methyl esters free from dodecane were identified by gas–liquid chromatography[6]. The methyl ester retention volume relative to methyl myristate, the number of carbon atoms and double bonds and the position of double bonds in the aliphatic chain were determined as described earlier[8]. The structure of fatty acids was referred to by shorthand designation[9].

The cottonseed oil triglycerides were separated into fractions of different polarity[6], hydrocarbons, b.p. 230–260°, being the stationary phase. Single fractions[4] were then separated in a 97.5–100 % methanol–$AgNO_3$/dodecane solvent system for 24–36 h[5]. Individual triglycerides were converted into fatty acid methyl esters[10]. The latter were determined by gas–liquid chromatography[6].

RESULTS AND DISCUSSION

The R_2 values (R_F ratio of a given substance and of butyl hexabromostearate[4]) and relative retention volumes of saturated and unsaturated fatty acid methyl esters are shown in Table I. The acids were identified using a linear relationship between the carbon number in the aliphatic chain and the $\log_{10}$ (relative retention volume) for a homologous series (Fig. 1).

One can conclude from these results that "critical pairs" of saturated and unsaturated esters having the same polarity constant $K_2 = 100 - m + 2e$, where $m =$ carbon number and $e =$ double bond number[4,5], can be separated in the silver nitrate-containing solvent system. The same effect was achieved by quantitative double bond bromination of lipids[5], but in the present solvent system the saturated lipids always remain on a baseline of the chromatogram.

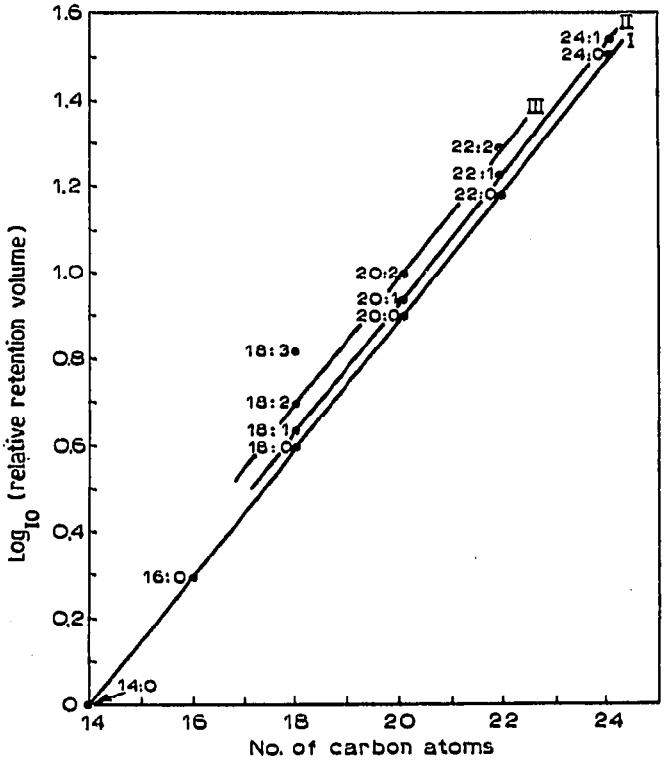

Fig. 1. The relationship between the logarithm of the relative retention volume of fatty acid methyl esters and the number of carbon atoms and double bonds in the aliphatic chain. (I) Saturated fatty acids; (II) monounsaturated fatty acids; (III) diunsaturated fatty acids.

The relative mobility of complexes is directly proportional to the number of double bonds and inversely proportional to carbon atoms number (Table I); therefore, the separation in a silver nitrate system may be regarded as the true partition chromatography[7]. Nevertheless unsaturated esters behave differently in the reversed-phase systems of different composition. Unsaturated esters having the same K_2 value do not separate in a system which does not contain complex-forming agents[11]. In a silver nitrate partition system such "critical pairs" ($20:1^{11}$ and $22:2^{13,16}$, $18:1^{9}$ and $20:2^{11,14}$) separate readily (Table I).

The chromatographic mobility of esters depends also upon the number of methylene groups between the double bonds. For example, the great difference between the R_2 values of $18:1^{9}$ and $18:2^{9,12}$, or of $18:2^{9,12}$ and $18:3^{9,12,15}$ (Table I) may result because methyl linoleate or methyl linolenate, besides having an additional double bond, have two of their double bonds widely separated and are in a more favourable position for π-complex formation[12]. Unsaturated esters are also separated according to the position of the double bonds in the chain. So methyl oleate ($18:1^{9}$) may be completely separated from methyl petroselinate ($18:1^{6}$) (Table I).

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TABLE I

RELATIVE MOBILITY OF FATTY ACID METHYL ESTERS IN PARTITION AND GAS–LIQUID CHROMATOGRAPHIC SYSTEMS

Methyl esters	*16:0*	*18:0*	*18:1*^{9}	*18:1*^{6}	*18:2*^{9,12}	*18:3*^{9,12,15}	*20:0*	*20:1*^{11}	*20:2*^{11,14}	*22:0*	*22:1*^{13}	*22:2*^{13,16}	*24:0*	*24:1*^{15}
R_2	0	0	1.01	1.29	4.66	6.24	0	0.85	1.66	0	0.32	1.41	0	0.22
Relative retention volume	1.99	3.89	4.31	4.33	5.23	6.66	7.75	8.31	9.96	14.72	16.02	21.32	28.26	30.72

TABLE II

SEPARATION AND IDENTIFICATION OF TRIGLYCERIDES

K_2	R_2	K_3	R_2'	*Fatty acid composition (mole %)**												*Triglyceride identification*
				14:0		*16:0*		*16:1*^{9}		*18:0*		*18:1*^{9}		*18:2*^{9,12}		
				f	*c*	*f*	*c*	*f*	*c*	*f*	*c*	*f*	*c*	*f*	*c*	
51	0.21	51	0.34	1.0	—	65.7	66.7	—	—	—	—	—	—	33.3	33.3	*PLL***
		54	0.50	0.2	—	33.6	33.3	—	—	—	—	33.7	33.3	32.5	33.3	*POL*
		56	0.62	0.9	—	0.5	—	0.6	—	32.4	33.3	—	—	65.5	66.7	*SLL*
		57	0.70	—	—	1.8	—	0.9	—	0.5	—	65.0	66.7	31.8	33.3	*OOL*
53	0.25	54	0.70	0.7	—	32.4	33.3	—	—	—	—	1.3	—	65.6	66.7	*PLL*
		57	0.89	0.3	—	0.7	—	0.6	—	—	—	32.8	33.3	65.6	66.7	*OLL*
55	0.32	57	1.02	0.3	—	—	—	—	—	—	—	0.5	—	99.2	100	*LLL*

* f = found fatty acid composition; c = composition calculated for identified triglycerides.

** P, S, O and L = acyls of palmitic, stearic, oleic and linoleic acids.

The separation of lipids on silver nitrate-impregnated silicic acid depends only upon differences in the degree of unsaturation[13,14,15], the position of double bonds in chain[16], and the geometric configuration of the double bonds[2,17]; the aliphatic chain length has no effect on the adsorption behaviour of lipids. In the present method the R_2 value depends both on the number and position of double bonds and on the chain length of unsaturated lipids. Therefore, this method combines the advantages of Ag^+–silicic acid chromatography and ordinary reversed-phase partition chromatography.

More than 10 mg of methyl esters can be separated on the standard strip (27 × 4.5 cm); so the present procedure may be used on a preparative scale. The gas-chromatographic data show that eluted esters are more than 99 % pure.

The results of the separation of cottonseed oil triglycerides are shown in Table II. The properties of unsaturated triglyceride co-ordination complexes were characterized by equation $K_3 = m - s$, where K_3 = polarity constant of triglycerides with substituted double bonds and s = number of saturated acyls in a triglyceride molecule[4]. As shown in Table II, the original non-substituted glycerides with the same polarity constant K_2 have the same R_2 value and form a separate chromatographic zone[4,5].

In the silver nitrate-containing reversed-phase system a new polarity gradient depending upon differences in the number of carbon atoms and saturated acyls in a triglyceride is established. Each new polarity constant K_3 corresponds to its own R_2' coefficient value. It follows that single zones obtained by partition chromatography of π-complexes are individual triglycerides. The fatty acid composition and identification of separated glycerides are given in Table II. There is a satisfactory agreement between calculated and found values.

By comparison of the data of Table II with those obtained on separation of brominated triglycerides[4–6] it may be concluded that there is a marked analogy in the chromatographic behaviour of bromides and π-complexes of triglycerides. This conclusion seems to disagree with findings of DUTTON *et al.*[12], who studied the separation of unsaturated lipids by countercurrent distribution in the system hexane/0.2 *M* silver nitrate in 90 % methanol. These workers claim that differences in partition coefficients of substances being fractionated depend upon the tendency of the substances to complex with the silver ion and remain in the lower polar phase. No silver ion is present in the upper non-polar phase where only free unsubstituted lipids are contained. Our results demonstrate that separation of unsaturated lipids in the silver nitrate-containing reversed-phase system depends upon polarity differences of π-complexes. These complexes, like the brominated glycerides, are partitioned continuously between polar and non-polar phases of the chromatographic system. The advantage of the present method over the method of bromination lies in the possibility of analytical and preparative isolation of native individual triglycerides from natural sources.

Thin-layer chromatography on silicic acid impregnated with silver nitrate makes possible the separation of triglycerides into classes according to the number of double bonds[14,15]. The same separation may be obtained by the present method; moreover, our system permits the separation of the triglycerides with the same degree of unsaturation and same polarity constant but differing by the saturated fatty acid acyl content, *e.g.*, dioleolinolein and stearodilinolein (Table II).

The results obtained suggest that the proposed separation method of complex lipid mixtures may be used on an analytical and preparative scale, particularly in conjunction with partition, adsorption, and gas–liquid chromatography, as well as other modern methods of lipid analysis.

SUMMARY

Unsaturated fatty acid methyl esters and triglycerides were separated as their silver ion π-complexes by reversed-phase partition chromatography in the system 70–100 % aqueous methanol, saturated with silver nitrate and dodecane/dodecane. The fatty acid composition of single fractions was determined by gas–liquid chromatography. The unsaturated methyl esters were completely separated from saturated ones of the same polarity, and from unsaturated esters with different carbon atom or double bond number and different position of the double bonds in the aliphatic chain. Triglyceride fractions of the same polarity were fractionated into individual compounds according to double bond or saturated fatty acyls number. The method may be used on an analytical or preparative scale.

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