Argentation High Performance Liquid Chromatography of Methyl Esters[1]

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ABSTRACT

The technique of argentation chromatography with silver ion on a macroreticular exchange resin has been applied to high performance liquid chromatography (HPLC) to separate fatty methyl esters and their isomers. Elution of methyl linolenate from the column and more rapid separation of dienes are made possible by programming column temperature from 25 to 70 C. Samples from ca. 0.025 μ to 8 μl can be analyzed on a 2-mm id x 61-cm column. Two 7-mm id x 61-cm columns in series have been used to separate 100-μl samples into fractions for further analysis by capillary gas chromatography. Various forms of argentation chromatography have been widely used for analysis and for separation of compounds from oils and fats including hydrogenated soybean oil. This paper describes the application of argentation procedures to modern HPLC to obtain faster, more efficient separations. It also describes the application of temperature programming to give more rapid separations and to extend our previous method to include methyl linolenate and its isomers.

INTRODUCTION

Silver ion complexing or argentation chromatography has been widely used to separate fatty esters. A recent review summarizes its application (1). We have used column chromatography with silver ion bound to macroreticular sulfonic acid resins since 1964 (2) and we have recently found improved separations with newer resins of greater surface area and with smaller particles obtained by grinding the resin (3). Houx et al. (4) and Warthen (5) have used argentation chromatography with other resins to separate alkenol acetates. Houx (6) used high performance liquid chromatography (HPLC) to separate methyl oleate and elaidate, and Lam and Grushka (7) used a silver-loaded aluminosilicate for HPLC separation of a *cis,trans* 9,11-octadecenoate mixture into its 4 components.

Our previous work was done in glass columns at pressures no greater than 15-20 psi and it seemed likely that even better results could be achieved under the conditions used in modern HPLC. In this paper we describe such separations of greater speed and improved resolution. We also describe the use of higher column temperatures and of temperature programming which have made possible separations of methyl linolenate isomers that were not eluted in practical times under our previous conditions.

EXPERIMENTAL

In previous work (3), grinding Amberlite XE-284 resin to obtain 100-200 mesh material had produced a large proportion of fines. The fines left from grinding the resin in a mortar were sieved in U.S. Standard Sieves to give a 270-350 mesh fraction (53-44 micron), which was washed with water until the supernatant liquid was almost clear. This resin fraction was treated with silver nitrate as previously described (3), washed with methanol, air-dried and packed in a 2-mm id x 61-cm stainless steel column. The larger amount of fines from ball mill grinding of the resin was processed in a similar way and packed in 2 7-mm id x 61-cm columns. Columns were packed by adding small portions of dry resin and tapping them on a table as used for gas chromatographic columns. Methanol was pumped through the columns until good baselines were obtained and was used as the solvent for the chromatograms.

Chromatograms were run on a Waters ALC-202 chromatograph with U6K sample injector and refractometric detector. Temperatures were controlled with Waters temperature-control blocks attached to a constant temperature water bath. When temperature programming was used, cooling water was shut off and the bath heater was run continuously. Temperature rise was ca. 0.78 C/min and approximated a linear gradient, but no control of the rate was attempted. A thermocouple near the column in the block indicated very nearly the water bath temperature but we have no measure of the actual methanol temperature in the column.

For quantitative runs, the refractometer signal was fed directly to a computer programmed to sum areas under curves and to report peak area percentage. Attenuation was changed as necessary to give suitable peak areas and percentages were adjusted for the changes in attenuation.

Most samples other than common saturated methyl esters were produced in the course of other work at the Northern Regional Research Center (NRRC). Unconjugated positional diene isomers other than 9,12, 9,15 and 12,15 were a gift from F.D. Gunstone.

RESULTS AND DISCUSSION

Resolution and Retention Volumes

A series of chromatograms of a methyl palmitate, stearate, elaidate and oleate mixture on the 2-mm x 61-cm column at 25 C with 0.5 ml/min methanol flow showed no trend of theoretical plate number or resolution with sample sizes from 0.025 μl to 2 μl. A typical chromatogram is shown in Figure 1. In the nonlinear range of the refractometer, almost baseline separation of saturates and elaidate was found up to 8 μl and of elaidate and oleate up to 16 μl. However, performance was affected by flow rate. With a 0.5-μl sample as flow rate increased from 0.2 ml/min to 2 ml/min, theoretical plates for saturates decreased from 635 to 210, for elaidate from 520 to 130 and for oleate from 540 to 100. For measurement of retention volumes and composition of mixtures, flow rates of 0.5 ml/min were chosen for samples containing only saturates and monoenes and 1 ml/min for samples containing polyunsaturates to give good resolution in reasonable elution times.

Retention volumes of a number of esters listed in Table I are consistent with previous observations on factors influencing retention in argentation methods. As expected, retention volumes of saturates are all nearly the same; as discussed previously (3), the volume for long-chain saturates is believed to correspond to V_O, the volume for an unretarded substance. Generally, retention volumes for monoenes are much less than dienes and depend mainly on the configuration of the double bond. Retention volume is decreased when the double bond is conjugated with the carbonyl and increased by a terminal double bond. With

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dienes, retention depends on the number of methylene groups between double bonds, as well as on configuration. As found by Rakoff and Emken (8), a hydroxy group increases retention. We have noted that water is eluted just after methyl elaidate and may cause a spurious peak if a sample is diluted with methanol of slightly different moisture content than the eluting solvent.

Effect of Temperature on Retention Volume

A continuing difficulty with silver resin chromatography has been the tenacity with which polyunsaturates have been held. In previous work (3) with XE-284, ca. 30 hr was required to remove methyl linoleate from a column suitable for monoene separations. A shorter column with 100-200 mesh resin reduced this time to 4 hr (3), and our present HPLC column eluted linoleate in 30 min. Methyl linolenate eluted so slowly that it never gave a discernible peak. Emken et al. have removed linolenate from a silver resin column with 10% 1-hexene in methanol (9), and Adlof et al. (10,11) achieved more rapid elution of polyunsaturates from resins only partially saturated with silver ion.

With our present HPLC equipment, temperature of the column can be conveniently increased by increasing the temperature of the water bath. When the column temperature was increased, methyl linoleate was eluted still more rapidly at the expense of poorer resolution of monoenes, and methyl linolenate gave a discernible peak at 50 C. The relationship between temperature and retention time is illustrated by the Arrhenius-type plot in Figure 2. Capacity ratios, $k' = (V-V_O)/V_O$, which are proportional to adjusted retention times for any one column, are used so that the data may be compared with those from the larger columns. A temperature-programmed separation is shown in Figure 3, in which the temperature started at 25 C to obtain better separation in the monoene region and increased to 60 C for elution of methyl linolenate. The rise in temperature during the run is shown on the figure.

Quantitative Measurements

As shown in Table II, the composition of elaidate-oleate mixtures and of commercial standard mixtures correspond well with the weight percentage. Since linolenate was not eluted at 25 C, for 15A—the standard mixture run at 25 C—weight percentage was recalculated to omit linolenate.

Although area percentages of the commercial standard mixtures corresponded fairly well to weight percentages, the agreement was considerably improved by correcting for difference in refractive index of the components, and corrected values are listed in the Table. For the elaidate-oleate

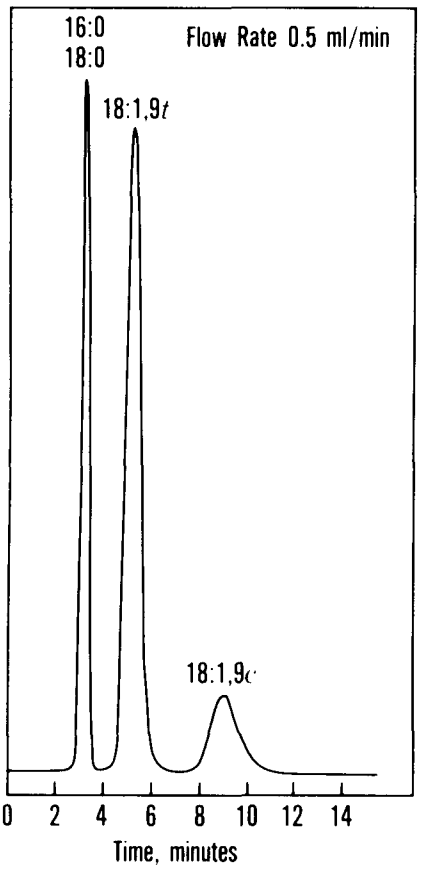

FIG. 1. Chromatogram of 5 μl of 10% methyl palmitate, stearate, elaidate, oleate mixture on 2 mm id x 61 cm column. Flow rate: 0.5 ml/min methanol. Refractometer attenuation: 16X.

TABLE I

Retention Volumes of Methyl Esters at 25 C on 2 mm id X 61 cm Column

Saturates		Monoenes		Dienes		Conjugated esters	
Ester	Retention volume (ml)	Ester	Retention volume (ml)	Ester	Retention volume (ml)	Ester	Retention volume (ml)
2:0	2.08	18:1, 2*c*	2.72	18:2, 5*c*,12*c*	36.64	18:2, 9*c*,11*c*	7.04
3:0	2.02	18:1, 2*t*	1.75	18:2, 6*c*,12*c*	46.42	18:2, 9*c*,11*t*	4.74
4:0	1.96	18:1, 9*c*	4.52	18:2, 9*c*,15*c*	59.25	18:2, 9*t*,11*t*	2.96
6:0	1.90	18:1, 9*t*	2.89	18:2, 9*c*,15*t* / 9*t*,15*c*	22.46	18:3, 9*c*,11*t*,13*t*	8.24
7:0	1.90	18:1, 15*c*	4.95	18:2, 9*t*,15*t*	9.34	18:3, 9*t*,11*t*,13*t*	5.37
8:0	1.81	18:1, 15*t*	2.78	18:2, 6*c*,11*c*	47.02		
9:0	1.84	18:1, 17	6.16	18:2, 7*c*,12*c*	49.78		
10:0	1.84	16:1, 9*c*	4.80	18:2, 6*c*,10*c*	44.09		
12:0	1.80	20:1, *c*	4.22	18:2, 8*c*,12*c*	45.98		
14:0	1.75	18:1, 9*c*,12OH	7.34	18:2, 9*c*,12*c*	27.81		
16:0	1.77			18:2, 9*c*,12*t* / 9*t*,12*c*	12.50		
18:0	1.74			18:2, 9*t*,12*t*	5.86		
20:0	1.70			18:2, 12*c*,15*c*	33.17		
				18:2, 12*c*,15*t* / 12*t*,15*c*	12.02		

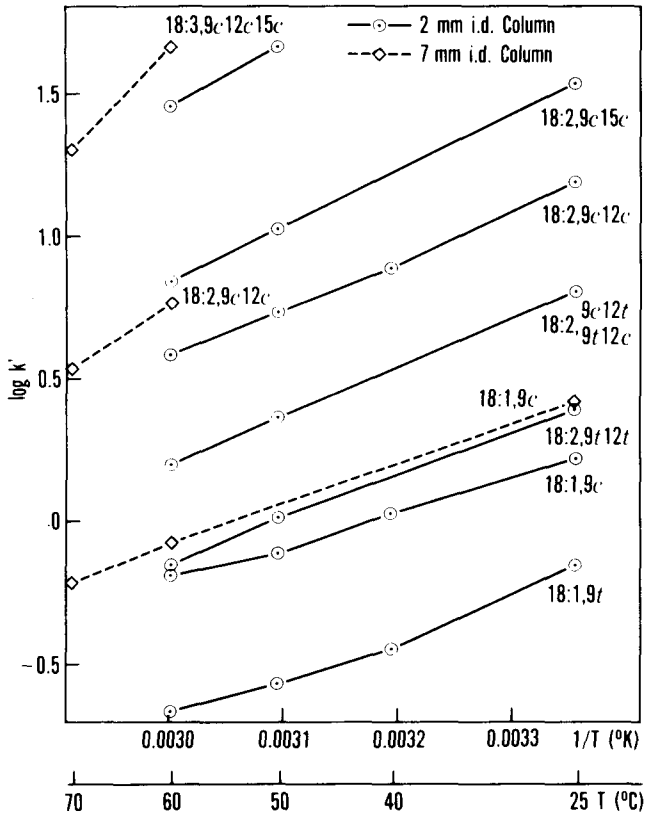

FIG. 2. Arrhenius-type plot of log capacity ratio vs reciprocal of absolute temperature for silver resin columns. Solid line: 2 mm id column. Dotted line: 7 mm id column.

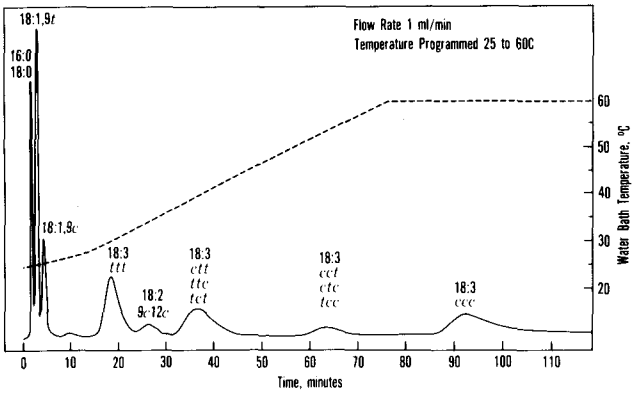

FIG. 3. Chromatogram of 25 μl of 10% methyl ester mixture on 2 mm id x 61 cm column. Flow rate: 1 ml/min methanol. Refractometer: 16X. Temperature programmed 25-60 C.

mixtures, on the other hand, area and weight percentages agreed well and attempted corrections made little difference. Consequently, for unknown mixtures the same correction factor may be used for oleate and elaidate, and the HPLC percentages for these samples in Table II are actual area percentages.

Correction factors at 25 C were calculated as described before (12), with N_D^{25} for methanol taken as 1.3265. Because proportions of palmitate and stearate differ greatly among the samples, a different saturate correction factor was necessary for each standard. With samples of the same type—such as vegetable oils or shortenings—where proportions of palmitate and stearate are more similar, an approximate correction factor might be satisfactory for the whole group. The correction factor for all *cis* linolenate was used for its *trans*-containing isomers.

Because *cis* and *trans* monoenes and dienes are sepa-

TABLE II

Quantitative Analysis of Mixtures on 2 mm id X 61 cm Column

Sample	Saturates (%)	Elaidate (%)	Oleate (%)	Linoleate (%)	Linolenate[a] c_2t (%)	Linolenate[a] ccc (%)	Linolenate[a] Total (%)
Mixture 1							
Weight	0.1	49.6	50.3				
HPLC	0.1	50.7	49.2				
Mixture 2							
Weight	0.1	49.8	50.1				
HPLC	0.1	50.5	49.4				
Standard 15A[b]							
Weight	12.4		36.1	51.5			
HPLC	11.5		35.8	52.7			
Standard 16A[b]							
Weight	12.0		18.0	36.0			34.0
HPLC	13.0		18.3	35.5			33.2
Standard 1A[b]							
Weight	40		20	20			20
HPLC	40		20.5	22.3			17.2
Standard 1B[b]							
Weight	20		20	25			35
HPLC	19.4		19.9	23.9	6.2	30.6	36.8
Standard 1C[b]							
Weight	60		20	15			5
HPLC	57.5		21.4	15.3	1.2	4.6	5.8

[a] c_2t is mixture of linolenate isomers with one *trans* double bond.

[b] Commercial Nu-Chek-Prep., Inc. Standards: 15A run isothermally at 25 C weight percent recalculated to omit linolenate. Others programmed 25-60 C. HPLC values corrected for differences in refractive index of components. Weight percent of saturates: (15A) 16:0 6.2, 18:0 3.1, 20:0 3.1; (16A) 16:0 7, 18:0 5; (1A) 16:0 20, 18:0 20; (1B) 16:0 5, 18:0 15; (1C) 16:0 35, 18:0 25.

rated, this technique provides additional information over the usual packed column gas chromatogram in the analysis of samples like methyl esters from hydrogenated oils.

Preparative Scale Separations

Although sufficient material for capillary gas chromatograms has been recovered in some fractions from the 2-mm id x 61-cm column, larger scale separations with the 2 7-mm id x 61-cm columns in series provide more material for subsequent analyses. With 5 ml/min methanol flow at 25 C, a 0.1-ml sample, the largest which allowed use of a linear range on the refractometer, gave resolution of 1.6 between saturates and elaidate and 1.9 between elaidate and oleate. Good resolution was still obtained with 0.4 ml of the mixture.

Retention was greater than on the small column, as shown by comparison of capacity factors in Figure 2, and temperature was raised to 70 C for good elution of linolenate. Although this is above the atmospheric boiling point of methanol, the solvent is under pressure in the column and apparently cools rapidly as it elutes. The elevated temperatures did not hinder refractometric detection.

The separation of linseed oil esters that was obtained by programming from 25 C to 70 C is shown in Figure 4. Samples of methyl esters from commercial hydrogenated vegetable oil shortening (Iodine Value 90) and hydrogenated winterized liquid oil (Iodine Value 116) have been chromatographed in the same way, and fractions have been collected for capillary gas chromatography on a BDS column at 190 C (13). Some isomers that are present in too small amounts to be easily detected in the original sample are concentrated in small fractions and are easily detected. Among such isomers are the *9-trans,12-trans*-octadecadienoate found just after oleate. From the weight percentages of material recovered by evaporation of eluates from this region and from capillary gas chromatographic analyses of these eluate fractions, we estimate *9-trans,12-trans*-octadecadienoate content to be 0.57% in the shortening and 0.16% in the liquid oil. Although the linolenate content of the products was too low to be detected refractometrically, solvent from the triene regions was evaporated and left a small residue giving linolenate and 2 other octadecatrienoate gas chromatograph peaks, all of approximately equal area.

The application of HPLC and temperature programming increases the value of argentation chromatography for analysis and separation of methyl esters. Linolenate elution times are longer than desirable but as can be seen from the long spaces between peaks in Figures 3 and 4, time could be reduced with equipment allowing a higher temperature gradient. Linolenate tailing also increases time needed for its elution and the skewed peak results in a lower theoretical plate count than found for earlier components. The method described here still uses particles larger than modern high-efficiency columns, and further improvement might be expected with 5- or 10-μ particles. Elevated temperatures have been used before to reduce solvent viscosity and improve separation efficiency. Examples of this are the work of Pei et al. (14) and the recent publication of Perchalski and Wilder (15). However, we are not aware of temperature programming to obtain useful separations over a range of retention times. The technique may be useful for other HPLC systems. Where it is applicable, it avoids additional equipment for solvent programming and makes possible the use of refractometer as detector.

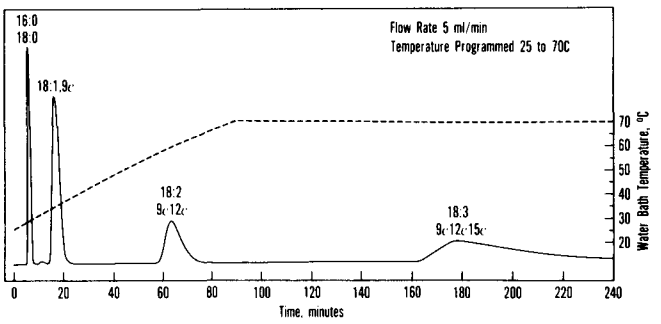

FIG. 4. Chromatogram of 1 ml of 20% linseed oil methyl esters on two 7 mm id x 61 cm columns. Flow rate: 5 ml/min methanol. Refractometer: 64X. Temperature programmed 25-70 C.

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