Silicic Acid Column Chromatography: Parameters for a Binary Solvent System

D. G. McCONNELL, R. L. HOFFMANN, G. J. ELMAN and C. D. EVANS,
Northern Regional Research Laboratory,[1] Peoria, Illinois

Abstract

The elution characteristics of methanol-benzene solvent systems were determined by separating a mixture of polar and nonpolar fatty methyl esters by liquid chromatography on silicic acid columns. A series of curves were plotted showing the relationship between the elution volume of each component and methanol concentration of the stationary phase. The resulting graphs serve as a basis for predicting elution conditions for separating other polar materials. Adsorption isotherms were plotted from equilibrium studies of methanol-benzene systems on silicic acid. Methanol concentrations of the effluents from various columns were determined by refractive index. An abrupt concentration change occurs in the methanol content of the effluent when the mobile solvent is either richer or poorer in methanol than the equilibrated solvent. Elution position of this abrupt change depends upon the concentration of methanol in both the mobile and the stationary phases. The procedure has been rigorously standardized because small variations in the amount of methanol on the column create large differences in elution volumes.

Introduction

LIQUID CHROMATOGRAPHY is a valuable tool for fractionating natural and oxidative polar lipids. Fatty acids and their methyl esters, partial glycerides and triglycerides with various functional groups, such as hydroxy, hydroperoxide and carbonyl, have been successfully fractionated (2–5). Significant improvements have been made in preparation of the column and regulation of the fractionation procedure by controlling small changes in methanol concentration. These improvements have increased the usefulness of the method.

Experimental

Chromatography

Stepwise preparative details of our improved chromatographic procedure are as follows: (a) Slurry 50 g silicic acid (Mallinckrodt 100-mesh, suitable for chromatography by the method of Ramsey and Patterson), after drying it at 100C for 16 hr, with 100 ml of benzene in a 250-ml Erlenmyer flask. (b) Slowly add the calculated amount (see Discussion) of methanol in benzene as a 1:1 v/v mixture to the vigorously swirled slurry. (c) Allow the slurry to equilibrate with occasional shaking for at least 1 hr at ambient temperature. (d) Pour the entire slurry into a 24 × 400 mm glass tube fitted with a porous glass disc at the bottom and a 500-ml reservoir at the top. (e) Rinse the remaining slurry into the column with a methanol-benzene solution that has the same composition as the equilibrated, supernatant solvent. If benzene or a mobile solvent of a composition different from the equilibrated supernatant of the slurry is used for rinsing, the concentration of adsorbed methanol will be altered and the separation characteristics of the chromatographic system will change. (f) Apply 12 psig air pressure to pack the column to constant depth and then remove any excess solvent. To prevent channeling always release the pressure very slowly. (g) Add the sample dissolved in 5 ml or less of mobile solvent and stir it into the top 0.5 in. of the column. Allow the sample and solvent to drain into the column under pressure. (h) Rinse the sides of the tube with 2 to 3 ml of mobile solvent and allow it to drain into the column. (i) Add a sufficient amount of mobile solvent for chromatogram development and adjust the air pressure so that the flow rate is 3.3 ml/min. (j) Collect effluent in 5-ml fractions.

Methanol Concentration vs. Refractive Index

Solutions of methanol (0 to 50%, by volume) in benzene were prepared and the refractive index of each was determined at 30C with a refractometer. Methanol concentration was plotted versus refractometer scale readings, and lines were fitted to the points by linear regression. Lines representing concentrations of 0 to 5% and 10 to 50% had slopes of −37.1 and −30.8, respectively. This significant difference in the slopes indicates that the line for the total concentration range is curved. Methanol concentrations of all unknown solutions in the 0 to 5% methanol range were accurately determined from the standard plot. The standard error of the slope for this range is ±0.5.

Adsorption Isotherm

Stationary phases are reported hereafter as the percentage of methanol by weight of silicic acid. Slurries containing stationary phases of 8 to 22% methanol were allowed to equilibrate at room temperature (26C), and aliquots of the supernatant solvents were analyzed for methanol concentration. These analyses showed that 81 to 97% of the methanol added to the slurry was adsorbed on the silicic acid. An isotherm for adsorption of methanol on silicic acid is plotted in Fig. 1A. The straight line for the function $\frac{c}{x/m} = 1/a + \frac{\beta}{a}c$ (Fig. 1B) shows that the isotherm

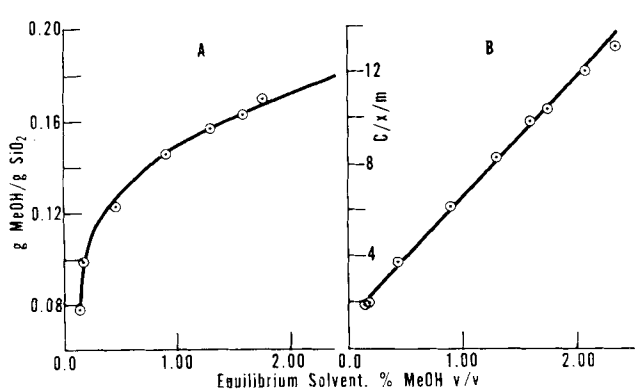

FIG. 1. *A*, adsorption isotherm for methanol on silicic acid. *B*, a plot of Langmuir's equation, $c/x/m = 1/a + \beta c/a$, c = conc. of MeOH in solution, x/m = g MeOH adsorbed.

g SiO_2

[1] No. Util. Res. and Dev. Div., ARS, USDA.

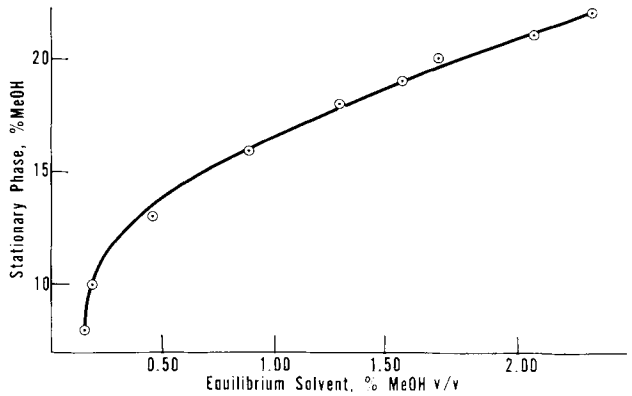

FIG. 2. Methanol concentrations of equilibrated solvents from slurries of methanol-benzene silicic acid.

is a true Langmuir type adsorption (1). In column preparation it is more convenient to have the methanol concentration of the stationary phase plotted against that of equilibrated solvent (Fig. 2). For any desired stationary phase, this plot gives the equilibrated solvent concentration that does not alter methanol equilibrium. The equilibrated solvent is used to rinse slurries into the column, so that there will be no change in separation characteristics of the chromatographic system.

Gradient

Column effluents of chromatograms without samples are easily monitored for methanol concentration by determining the refractive index of each 5-ml fraction. An abrupt change in effluent concentration, hereafter referred to as the gradient (Fig. 3), occurs at elution volumes that vary according to the concentrations of adsorbed methanol and methanol in the mobile phase (Fig. 4). When 1 to 8% water was added to 16% methanol stationary phases and when chromatograms were developed by 1% methanol phase, gradient elution volumes were gradually increased by 15 to 75 ml.

Compounds Eluted Before Gradient

As a representative lipid sample, a mixture of oiticica methyl esters and methyl ricinoleate was prepared that contained nonpolar, keto and hydroxy methyl esters. Fractionation was accomplished on stationary phases containing 8, 10, 13 and 16% methanol and eluted with 1% and 2%, v/v, methanol in benzene. Peak positions were determined by plotting fraction number against fraction weight and reported as elution volume in milliliters (Fig. 3). Peak elution

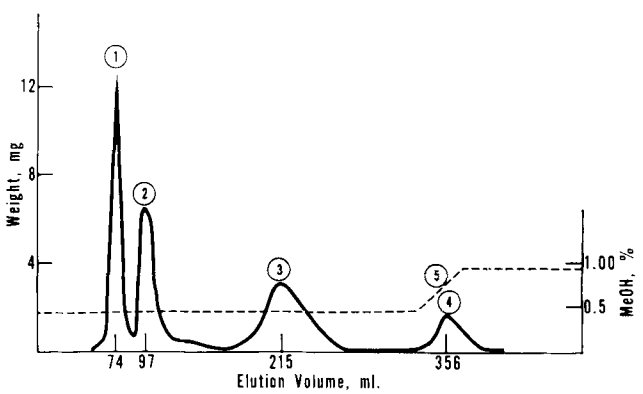

FIG. 3. Chromatographic separation of a fatty acid ester mixture: *1*, nonpolar; *2*, keto; *3*, hydroxy; *4*, oxidation products and *5*, gradient. Mobile solvent: 1% methanol in benzene by volume. Stationary phase: 13% MeOH to SiO_2 by weight.

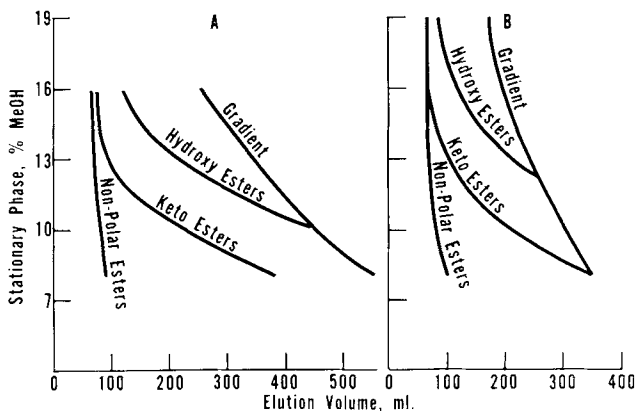

FIG. 4. Effects of different concentrations of stationary phase methanol on elution volumes of nonpolar, keto and hydroxy fatty acid methyl esters and on the gradient. Mobile solvent: *A*, 1% methanol in benzene by volume. *B*, 2% methanol in benzene by volume.

volumes from the eight chromatograms were plotted vs. stationary phase composition for both 1% and 2% methanol mobile solvents (Fig. 4).

Compounds Eluted After Gradient

Soybean oil monoglycerides, prepared by partial lipase hydrolysis of refined soybean oil (6), were chosen as typical compounds that would be eluted after the gradient. Monoglycerides were fractionated into their component α and β structural types on columns containing stationary phases of 18, 19, 20, 21 and 22% methanol on silicic acid. Mobile solvents were chosen from Fig. 2, so that there would be no gradient. These nongradient columns represent the portions of chromatograms that normally occur after the gradient. Peak elution volumes were determined from weight curves (Fig. 5) and were plotted vs. the percentage of methanol in the stationary phases (Fig. 6).

Estimation of Error

For reproducibility of peak elution volume as in collecting individual components without resorting to the use of weight curves, the same stock of silicic acid was used for each set of experiments. However, a weight curve should be established as the control. Table I shows that batches of silicic acid from three separate drums of the same label exhibit slightly different fractionating properties. When separation is

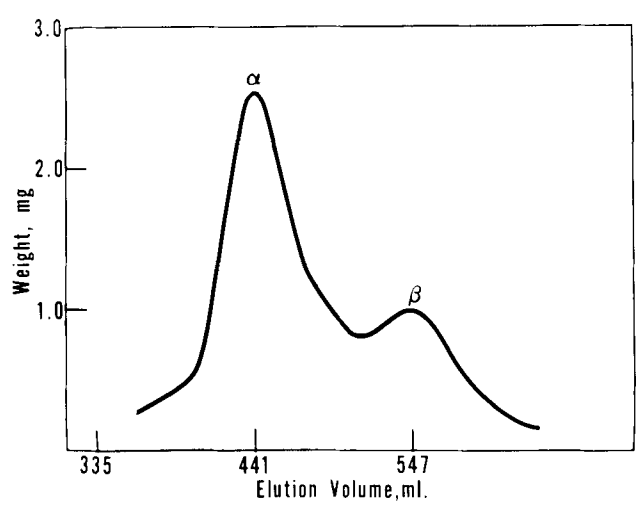

FIG. 5. Chromatographic separation of α- and β-monoglycerides of soybean oil. Mobile solvent: 1.6% methanol in benzene by volume. Stationary phase: 19% MeOH to SiO_2 by weight.

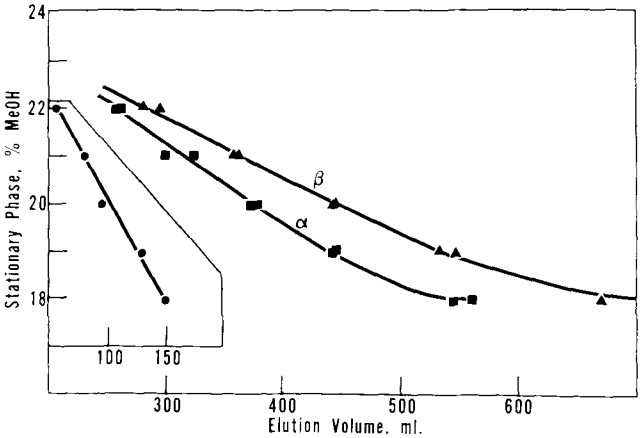

FIG. 6. Effects of different concentrations of stationary phase methanol on elution volumes of α- and β-monoglycerides of soybean oil. Insert: Peak separation between α- and β-monoglycerides of soybean oil.

the only criterion, small variations in silicic acid are not important.

The average tube volume, delivered by a 5-ml siphon, and its standard deviation were calculated to be 4.74 ± 0.06 ml. This error in tube volume, 1.3%, and errors involved in preparation of methanol-benzene solutions, 0.03 to 0.06%, would be insignificant in determining peak elution volumes.

Discussion

Column Equilibria

Determinations of methanol concentration in the supernatant solvent from silicic acid slurries containing benzene and 8 to 22% methanol, by weight of the adsorbent, show that an equilibrium is established between methanol adsorbed on the silicic acid and the methanol remaining in solution. When more methanol is added to an equilibrated slurry, a new equilibrium is established, and the amount of methanol adsorbed on the silicic acid increases.

Results from equilibrated columns show that at first the effluent methanol concentration remains constant and equal to that of the solvent originally in equilibrium with adsorbed methanol (Table II) until appearance of the gradient (Fig. 3). After the gradient, the effluent has the same methanol concentration as the mobile solvent (Table II). The effluent methanol concentrations shown in Table II are lower than the actual concentrations because methanol is lost by evaporation as the effluent falls from the column into the receiving vessel.

A positive gradient occurs if the mobile solvent is richer in methanol than the equilibrated solvent. When mobile solvent enters the first plate of the column

TABLE I
Effect of Various Batches of SiO_2 on Chromatographic Separation

SiO_2 batch number	Mobile phase[a] volume % MeOH in benzene	Methyl ester peaks elution volume, ml		
		Nonpolar	Keto	Hydroxy
1	1	74	107	250
2	1	74	97	215
3	1	74	106	238
3	1	74	103	238
2	2	73	97	191
3	2	74	107	222
3	2	74	108	223

[a] All stationary phases 13% MeOH by weight of SiO_2.

TABLE II
Methanol Concentration of Chromatographic System Components (% MeOH)

Stationary phase[a]	Equilibrated solvent[b]	Effluent[b]		Mobile solvent[b]
		Before gradient	After gradient	
10	0.21	0.21	0.94	1
10	0.19	0.16	1.81	2
13	0.48	0.46	0.90	1
13	0.48	0.43	1.75	2
16	0.88	0.80	0.90	1
16	0.92	0.78	1.73	2

[a] By weight of SiO_2.
[b] By volume in benzene.

to mix with the equilibrated solvent already present, the adsorbed methanol is no longer in equilibrium with the methanol solution around it. Silicic acid in the first plate adsorbs methanol from the mobile solvent until that plate has reached a new equilibrium, at which time the methanol concentration of the solvent in contact with adsorbed methanol equals the concentration of the mobile solvent. The mobile solvent that has been reduced in methanol while in the first plate continues down the column. In passage through succeeding plates it suffers further methanol depletion until its concentration equals that of the original equilibrated solvent. This process continues until the entire column is at the new equilibrium, and the methanol concentration of the effluent now equals that of the mobile solvent. No change occurs in the effluent methanol concentration until the "front" of the new equilibrium passes through the last plate. This elution front is described as the gradient. In effect, the column embodies two separate chromatographic processes, each with a separate methanol-benzene solvent system. The gradient is the transition between them. When there is less methanol in the mobile than in the equilibrated solvent, methanol is desorbed from the silicic acid until the entire column is at a new, but lower, equilibrium at which time a negative gradient is eluted. No gradient will be produced when methanol concentration is the same in both equilibrated and mobile solvents.

The adsorption isotherm (Fig. 1) shows that the weight ratio of adsorbed methanol to adsorbent increases with the methanol concentration of equilibrated solvent. Added mobile solvent that differs in methanol concentration from the equilibrated solvent will cause a change in equilibrium, and the parameters of the new equilibrium will also lie on the isotherm curve. Since the isotherm follows Langmuir's equation, the adsorption of methanol on silicic acid is chemisorptive; little or no benzene is adsorbed.

Separation of Polar Materials by Silicic Acid Chromatography

Fractionation of polar materials depends primarily on the amount of methanol adsorbed on the silicic acid. Since the equilibrium does not change appreciably before the gradient, mobile concentration has little effect on elution volumes of nonpolar, keto and hydroxy methyl esters. However, increasing the amount of adsorbed methanol reduces the elution volumes of these polar materials (Fig. 4). The more polar the material, the greater its elution volume is influenced by the change in adsorbed methanol. Decreasing the amount of adsorbed methanol will, therefore, increase the separation of closely related polar compounds. Changes in methanol concentration of the stationary phase will also increase or decrease the elution volume of the gradient. In some chromatograms two or more

components may be eluted at the gradient position because the abrupt increase in adsorbed methanol rapidly displaces compounds that would be eluted at a greater volume if the gradient did not occur. This "double peaking" effect can be overcome by displacing or eliminating the gradient.

The chromatographic system described above offers a convenient means by which the heretofore difficult separation of α and β monoglycerides may be achieved. Results of α- and β-monoglyceride separation on non-gradient chromatograms are similar to those described for the separation of nonpolar, keto and hydroxy methyl esters. Decreasing methanol concentration in the stationary phase increases peak elution volume and peak separation (Fig. 6). At large elution volumes however, the peaks tend to be broad (Fig. 5). The degree of separation between α- and β-monoglycerides has an inverse linear relationship to adsorbed methanol within the concentration range studied. (See insert graph, Fig. 6).

Compounds used to obtain general separation curves (Fig. 4 and 6) are representative of those found in natural vegetable oils. Stationary- and mobile-phase methanol concentrations for separation of lipid mixtures similar to those discussed can be ascertained by comparing polarities of individual components to the representative curves. A trial chromatogram, such as one with a 16% methanol stationary phase, would indicate polarities of unknown compounds. Proper methanol concentrations to give optimum separation of each compound could be determined by comparing the results of the trial run to the general separation curves. When the gradient is not needed or when it interferes with separation, solvent concentrations for a column with no gradient can be taken from the general separation curve (Fig. 4 and 6) and the equilibrated solvent curve (Fig. 2).

The highly reproducible chromatographic method based on silicic acid is simple because only one mobile solvent system is used to fractionate materials of widely differing polarities. Nonpolar triglycerides, diglycerides, hydroxy fatty acids and monoglycerides can be easily separated on one column. Separations of several polar compounds have already been achieved, and with the information reported here it is believed that other investigators may find additional applications.

ACKNOWLEDGMENT

Helpful advice was given by Prof. B. Singh, Bradley University.

REFERENCES

1. Daniels, F., J. W. Williams, P. Bender, R. A. Alberty and C. D. Cornwell, "Experimental Physical Chemistry," 6th ed., McGraw-Hill Book Co., Inc., New York, 1953, p. 330.
2. Evans, C. D., D. G. McConnell, E. M. Frankel and J. C. Cowan, JAOCS *42*, 764–770 (1965).
3. Frankel, E. N., C. D. Evans, D. G. McConnell anl E. P. Jones, Ibid. *38*, 134–137 (1961).
4. Frankel, E. N., C. D. Evans, Helen A. Moser, D. G. McConnell and J. C. Cowan, Ibid. *38*, 130–134 (1961).
5. Frankel, E. N., D. G. McConnell and C. D. Evans, Ibid. *39*, 297–301 (1962).
6. Mattson, F. H., and R. A. Volpenhein, J. Lipid Res. *2*, 58–62 (1961).

[Received July 21, 1967]