977. *Vegetable Oils. Part VIII.* The Separation of Fatty Acids by Reversed-phase Chromatography: An Empirical Approach and a Mathematical Treatment.*

By F. D. Gunstone and P. J. Sykes.

In connection with the separation of fatty acids by reversed-phase chromatography between paraffin and a range of aqueous acetones, a method of measuring the partition coefficient is described. This can be used empirically to indicate the eluting solvent of optimum efficiency. Various oxygenated acids can be separated by using castor oil or acetylated castor oil as stationary phase. A mathematical treatment of the experimental system is given.

In both partition chromatography and counter-current distribution, compounds are distributed between two immiscible solvents according to their several partition coefficients. In Craig's apparatus for counter-current distribution the separation is effected after a number of discrete transfers have been made, whereas in partition chromatography there is continuous flow of mobile phase over the stationary phase, held by an inert support and contained in a column. For satisfactory partition chromatography the stationary phase should be the solvent in which the compounds to be separated have greater solubility; they are then slowly eluted by the greater volume of the mobile phase in which they are less soluble.

Partition chromatography has been widely and effectively used for the separation of fatty acids. For acids of shorter chain length the stationary phase is the more polar solvent and the mobile phase is the less polar, and several systems of this type suitable for the C_1—C_{10} acids have been described.[1] Since the longer-chain acids are considerably more soluble in the less polar phase, it is more convenient to reverse this system, and Howard and Martin [2] were the first to show how the higher acids (C_{12}—C_{18}) could be so separated. The stationary phase, medicinal paraffin, is held on kieselguhr made non-wetting by treatment with dichlorodimethylsilane, and the mobile phase is a range of aqueous acetones of increasing acetone concentration.

Several investigators [3] have used this method with only minor modifications and have extended the range of acids which can be separated to C_8—C_{24}, with aqueous acetone solutions of concentration varying between 40% and 90%. Unsaturated acids are eluted along with lower saturated acids (oleic acid with palmitic acid, and linoleic acid with myristic acid, etc.); and the best separations are obtained when each acid, or group of saturated and unsaturated acids, is eluted with the aqueous acetone of optimum efficiency. There is, however, only a very general agreement among other investigators [2,3] about which is the best eluting solvent, and a method has now been developed for deriving this from a knowledge of the partition coefficient of the acid between paraffin and a range of aqueous acetones. The same method has been used to investigate the possible separation of oxygenated acids by using other stationary phases. Finally, a mathematical description of the experimental system is reported; this gives results in general accord with those obtained experimentally.

* Part VII, *J. Sci. Food Agric.*, 1959, **10**, 522.

[1] Mitchell, Montague, and Kinsey, "Organic Analysis," Interscience Publ. Inc., New York, 1956, Vol. III, 71.

[2] Howard and Martin, *Biochem. J.*, 1950, **46**, 532.

[3] (*a*) Silk and Hahn, *Biochem. J.*, 1954, **56**, 406; (*b*) Crombie, Comber, and Boatman, *Biochem. J.*, 1955, **59**, 309; Steinberg, Slaton, Howton, and Mead, *J. Biol. Chem.*, 1956, **220**, 257; Kapitel, *Fette u. Seifen*, 1956, **58**, 91; Popjak and Tietz, *Biochem. J.*, 1954, **56**, 46; Garton and Lough, *Biochim. Biophys. Acta*, 1957, **23**, 192; Lough and Garton, *Biochem. J.*, 1957, **67**, 345; Riley and Nunn, *Biochem. J.*, 1960, **74**, 56.

Experimental

Materials.—Samples of lauric, myristic, palmitic, stearic, arachidic, oleic, and linoleic acid were supplied from the Research Laboratories of Unilever Ltd., Port Sunlight. All the other acids used were available as research specimens or were prepared from such by standard procedures.

Aqueous acetone designated $Z\%$ was prepared by diluting acetone (Z ml.) with water until the volume of the mixture was 100 ml. All acetone solutions are equilibrated with the appropriate stationary phase before use.

Columns for Reversed-phase Chromatography.—Throughout this work columns 35 cm. long and 1·3 cm. in diameter were used according to the general directions of Howard and Martin [2] and incorporating the improved suggestions of Silk and Hahn.[3a] (Fuller details are given in ref. 4) Each column contained 25 g. of packing made of siliconised Hyflo Supercel (14·6 g., d 2·1) [5] and neutralised liquid paraffin (10·4 g., d 0·88) or siliconised Hyflo Supercel (14 g.) and acetylated castor oil (11 g., d 0·97).

Medicinal castor oil was extracted with light petroleum (b. p. 40—60°) to purify the triricinolein.[6] If castor oil was required, the product was neutralised by percolation as a solution in ether through a column of alumina (Peter Spence, Type H); otherwise the triricinolein was acetylated by boiling acetic anhydride, and the acetylated product, after recovery, was finally neutralised in the same way.

Determination of the Partition Coefficient (K).—The acid (*ca.* 2 mg., accurately weighed) under investigation is dissolved in the stationary phase (*ca.* 0·5 ml., accurately weighed) by warming them together in a centrifuge tube to 100° or, if this fails, by addition of a mutual solvent (ether, alcohol, or acetone) which is subsequently completely removed under reduced pressure. Siliconised Hyflo Supercel (1·3—1·4 times the weight of stationary phase) is then added along with the chosen acetone (5·00 ml.). The tube is corked and, after equilibration at the desired temperature (35° with paraffin, 20° with castor oil and acetylated castor oil), is centrifuged. Two 2 ml. portions are removed with a pipette and titrated against 0·01N-methanolic potassium hydroxide (A ml.), a nitrogen-stirred titration cell and micrometer syringe [2,3a] being used. To the remaining mixture of acid, stationary phase, inert support, and aqueous acetone (1 ml.), 95% aqueous acetone (5·00 ml.) is added, and after equilibration two further 2 ml. portions are removed and titrated (P ml.).

The partition coefficient, which is the concentration of acid in the mobile phase divided by that in the stationary phase is given by the following expression, in which V is the volume of the stationary phase (the density of stationary phase is determined):

$$K = AV/(6P - A)$$

In this calculation it is assumed that the 95% acetone, even though diluted by 1 ml. of more aqueous acetone, removes all the acid from the stationary phase. There is good evidence that K is very large and as a further check the recovery of acid is calculated. This should be $100 \pm 5\%$ for the value of K to be significant:

$$\text{Recovery } (\%) = 100\text{N}E(2A + 3P)/W$$

where N is the normality of the alkali and W and E are the weight and equivalent of the acid used.

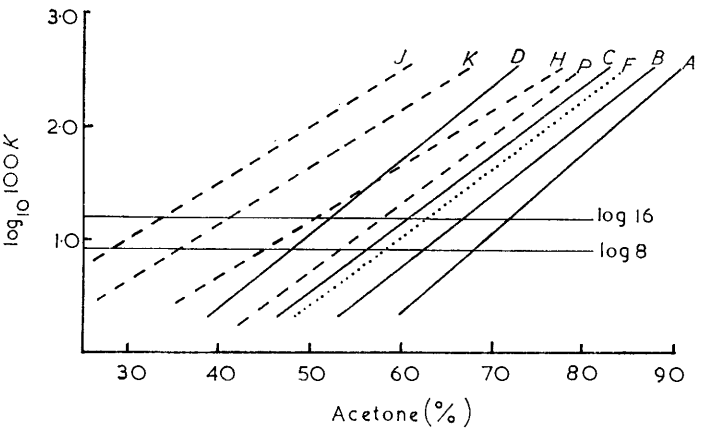
Results.—Values of K for a number of saturated and unsaturated acids and for various oxygenated acids have been measured, several aqueous acetones being used as one liquid phase and liquid paraffin, castor oil, or acetylated castor oil as the other. The results are given in Figs. 1—3 where $\log_{10} 100K$ is plotted against the percentage of acetone in the aqueous acetones. The plots are straight lines, most of which are approximately parallel. The lines for non-separable groups of saturated and unsaturated acids lie very close together and some of these have been omitted from the figures.

[4] Gunstone and Sykes, *J. Sci. Food Agric.*, in the press.
[5] "International Critical Tables," ed. Washburn, McGraw-Hill Book Co. Inc., 1927, Vol. II, p. 87.
[6] Achaya and Saletore, *J. Sci. Ind. Res., India*, 1952, **11**, *B*, 471.

Discussion

The Partition Coefficient.—In seeking to relate the partition coefficients of these acids to their chromatographic behaviour, values of K must be measured under conditions which resemble those operating during column elution and are also practically convenient. The change in the ratio of the two liquid phases from 2·5 : 1 to the larger value (10 : 1) used in determining K has little effect on the result and is made on practical grounds. The addition of kieselguhr on the other hand makes a marked difference to the measured values of K and has the effect of increasing the proportion of acid in the stationary phase. It follows, from the general agreement between practical results and those derived from the theoretical treatment described later, that absorptive forces due to kieselguhr which have

Fig. 1. *Partition coefficient for paraffin–aqueous acetone.*



The lines for the following acids have been omitted for clarity. They lie on or very close to those indicated in parentheses: *E*(*B*), *G*(*C and F*), *R and S* (*K*), *T*(*H*), *and U*(*P*).

A, Stearic acid. *B*, Palmitic acid. *C*, Myristic acid. *D*, Lauric acid. *E*, Oleic acid. *F*, Linoleic acid. *G*, Hexadec-*cis*-9-enoic acid. *H*, 12-Hydroxystearic acid. *J*, 9-Hydroxyoctadec-*cis*-12-enoic acid. *K*, *threo*-9,10-Dihydroxystearic acid. *L*, *erythro*-9,10-Dihydroxystearic acid. *M*, *erythro*-9,10-Dihydroxypalmitic acid. *N*, *threo*-9,10,12-Trihydroxystearic acid (m. p. 106—108°). *O*, *threo*-9,10-*threo*-12,13-Tetrahydroxystearic acid (m. p. 144—146°). *P*, 12-Acetoxystearic acid. *R*, *threo*-9,10-Diacetoxystearic acid. *S*, *erythro*-9,10-Diacetoxystearic acid. *T*, 9,10-Epoxystearic acid. *U*, 12-Oxostearic acid.

been neglected in the theoretical treatment are in fact included in the K values determined in the presence of kieselguhr. These points are illustrated by the following data:

Acid	Ratio Aq. acetone : paraffin	Kieselguhr	Aq. acetone (%) 62	67	73
Palmitic	10 : 1	Present	0·10	0·16	—
	2½ : 1	Present	—	0·19	—
	2½ : 1	Absent	0·57	1·03	—
Stearic	10 : 1	Present	—	0·07	0·18
	2½ : 1	Present	—	0·09	—
	2½ : 1	Absent	—	0·44	0·85

The Optimum Concentration of Aqueous Acetone for Elution.—Before the partition coefficients were measured some saturated and unsaturated acids had been run on paraffin columns and the best eluting solvents for lauric, myristic (also hexadecenoic and linoleic), palmitic (also oleic), stearic, and arachidic acid were found to be 56, 62, 67, 73, and 78% aqueous acetone respectively. Not surprisingly, each acid is now seen to have about the same partition coefficient at the chosen aqueous acetone concentration (Table 1).

TABLE 1.

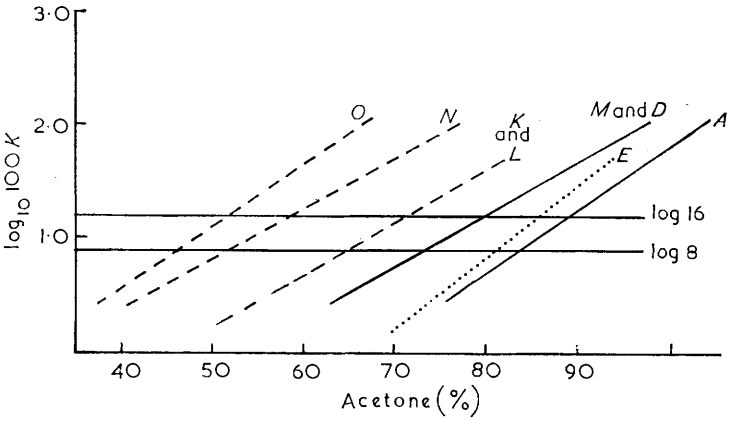
(*a*) The optimum concentration of acetone for the elution of the acid concerned from a paraffin column.
(*b*) The partition coefficient of the acid for this aqueous acetone.
(*c*) The partition coefficient of the acid for the aqueous acetone used for the elution of the next lower group of acids; *i.e.*, 53% for the myristic acid group, 62% for the palmitic acid group, and 67% for stearic acid.

Acid *	12 : 0	14 : 0	16 : 1	18 : 2	16 : 0	18 : 1	18 : 0
(*a*)	53 †	62	62	62	67	67	73
(*b*)	0·16	0·18	0·15	0·15	0·16	0·18	0·18
(*c*)	—	0·05	0·05	0·04	0·10	0·08	0·07

* The numerals used to designate the acids indicate the number of carbon atoms and the number of double bonds per molecule. Thus 12 : 0 is lauric acid, etc.
† The value of 56% found empirically and reported in the text was changed to 53% on the strength of these results.

Consider the separation of two acids A and B of which A has the higher value of K. At low acetone concentrations both acids will be eluted very slowly from the column and, though they are separable, the system will be inconvenient: at higher acetone concentrations both acids will be eluted very quickly and separation will be impracticable.

FIG. 2. *Partition coefficient for castor oil–aqueous acetone.* (*Key as* Fig. 1.)



Between these two values, however, there is a range of acetone concentations for which the acid A is eluted at a convenient rate whilst the acid B is only eluted after prolonged percolation. The solvent of choice for this separation will lie within this range. It is clear from the empirical observations and the data in Table 1 and Fig. 1 that, for the paraffin system being used, an acid is satisfactorily eluted when its partition coefficient is equal to or greater than about 0·16 and it will be conveniently separated from other acids having a partition coefficient not greatly above about 0·08. These two values have been used to draw the two horizontal lines in Fig. 1.

The same horizontal lines were drawn on Figs. 2 and 3 for castor oil and acetylated castor oil, leading to useful practical conclusions concerning which acids can be separated and under what conditions. As an illustration of this it was inferred from Fig. 3 that it should be possible to separate *threo*-9,10-dihydroxystearic acid (*K*, 61%), 9-hydroxyoctadec-12-enoic acid (*J*, 67%), *threo*-9-10-diacetoxystearic acid (*R*, 74%), and 12-acetoxystearic acid (*P*, 80%) on an acetylated castor oil column by using the solvents indicated to elute each acid. The results in Fig. 4, obtained with solvents very close to those recommended, are entirely satisfactory.

Separation of Oxygenated Acids.—Part of the purpose of this investigation was to extend the techniques developed by others for the separation of saturated and unsaturated fatty acids to various oxygenated acids. Seed oils containing such acids could then be examined by this procedure as an alternative to gas–liquid chromatography which is reported [7] to be unsuitable for esters containing the system –CH:CH·CH:CH·CH(OH)–.

When paraffin columns are to be used it is to be expected that oxygenated acids having higher values of K would be eluted before non-oxygenated acids of comparable chain

FIG. 3. *Partition coefficient for acetylated castor oil–aqueous acetone.* (*Key as* Fig. 1.

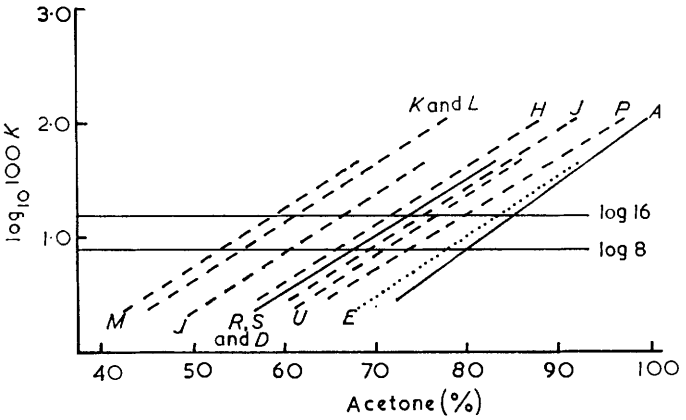
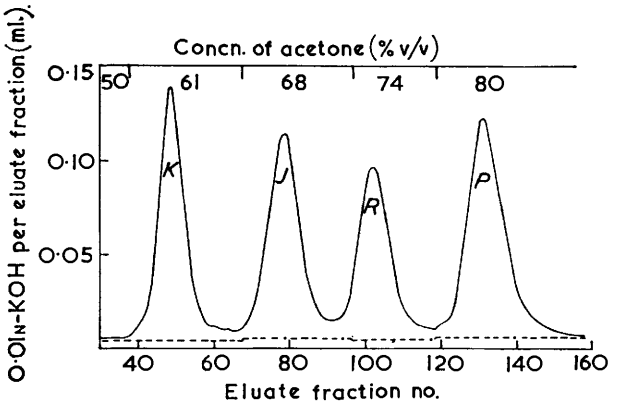


FIG. 4. *Elution curve for the separation of* threo-9,10-*dihydroxystearic acid* (*K*), 9-*hydroxyoctadec*-12-*enoic acid* (*J*), *threo*-9,10-*diacetoxystearic acid* (*R*), *and* 12-*acetoxystearic acid* (*P*).



Acid	*K*	*J*	*R*	*P*
Calc.	22·6	22·2	25·1	30·1
Obs.	22·9	21·7	25·2	30·2

length. Fig. 1 provides quantitative evidence of this, and the results indicate the possibility of certain separations and the impossibility of others by using the chromatographic system here described. Thus hydroxystearic acid can be separated from hydroxyoleic acid but not from epoxystearic acid: *threo*-9,10-dihydroxystearic acid and the *threo*- and *erythro*-9,10-diacetoxystearic acids can be separated from various mono-oxygenated acids but not from one another; other similar relationships can be derived from Fig. 1.

Even when the partition coefficient indicates the possibility of separation, other factors may limit the practical value of the system. A mobile phase of acetone content so high

[7] Morris, Holman, and Fontell, *J. Lipid Res.*, in the press.

that it removes the stationary phase from the column cannot be used (aqueous acetone up to 90% can be safely used with paraffin); also, difficulties arise when the solute has very low solubility in the stationary phase. The only stationary phases used in this type of work are paraffin, castor oil,[8,9] and mixtures of these two.[9] Mixtures are unsatisfactory because they are difficult to reproduce, and because they are frequently unstable on account of the differential removal of the two components by the mobile phase.

In an attempt to increase the usefulness of this procedure, some potential stationary phases other than paraffin have been investigated, including dinonyl phthalate, six polyesters of Reoplex type, castor oil, and acetylated castor oil. All except the last two were considered to be too soluble in the higher aqueous acetones; castor oil can be used with aqueous acetones up to 75—80%, and acetylated castor oil up to 80—85%. The partition coefficients measured on a number of acids for these solvents are plotted in Figs. 2 and 3.

Since solvents with an acetone content greater than 80% cannot be used with castor oil, saturated acids higher than lauric, and the unsaturated acids which separate with them, cannot be conveniently removed from a column without also removing so much of the stationary phase that it is unfit for further use; this also applies to monohydroxystearic acid. Di-, tri-, and tetra-hydroxystearic acids, however, should be separable by this system and this has been confirmed.

Acetylated castor oil is considered to be more suitable because aqueous acetone solutions up to 85% can be used and, at the same time, the values of K are slightly higher than those with castor oil. Thus *threo-* and *erythro*-9,10-dihydroxystearic acid are best eluted from castor oil with 72% acetone and from acetylated castor oil with 61% acetone, and the corresponding values for 12-hydroxystearic acid are 80 and 72%. Reference has already been made to a successful separation of the acids in Fig. 4 and many others can be predicted from Fig. 3. It is noteworthy that the relative values of K for a range of acids is not the same in different stationary phases. This is illustrated by the fact that 9,10-dihydroxystearic acid can be separated from its diacetyl derivative on an acetylated castor oil column but not on a paraffin column.

The use of paraffin and of acetylated castor oil columns, and occasionally of castor oil columns, provides a valuable technique for the analytical separation of a wide range of saturated, unsaturated, and oxygenated acids.

Theoretical Treatment of Experimental System.—Theories of chromatography relevant to this discussion have been put forward by Martin and Synge,[10] Mayer and Tompkins,[11]

TABLE 2. *Symbols used in the mathematical treatment.*

K'	The partition coefficient of the acid under chromatographic conditions.
p	The number of theoretical plates (or tubes) in the column.
a	The number of an eluate fraction where each fraction has the same volume as that of the mobile phase in a theoretical plate (or tube).
E_a	The fraction of solute present in the ath eluate.
V_m	The volume of mobile phase contained in the column.
v	The volume of each experimental eluate (2·0 ml.).
f	The number of the experimental eluate fraction containing most solute counted from the point where the solvent front emerges from the column.
$E'_{max.}$	The fraction of solute in the eluate f.
c	$= V_m/v$, *i.e.*, the volume of mobile phase in the column expressed as a number of experimental eluate fractions.
$A_{\epsilon\{x\}}$	The area of the normal curve of error for the argument $\{x\}$.

and Glueckauf.[12] The following treatment provides a reasonable explanation of the results obtained in the present study. The symbols used are given in Table 2. From the experimentally determined position and height of the eluate maximum, it has been possible

[8] Savary and Desnuelle, *Bull. Soc. chim. France*, 1953, 939.
[9] Matic, *Biochem. J.*, 1956, **63**, 168.
[10] Martin and Synge, *Biochem. J.*, 1941, **35**, 1358.
[11] Mayer and Tompkins, *J. Amer. Chem. Soc.*, 1947, **69**, 2866.
[12] Glueckauf, *Trans. Faraday Soc.*, 1955, **51**, 34.

to derive values of K' and p, to plot a theoretical eluate curve, and to determine the partition coefficient K'_B which an acid B must have if it is to be separated from acid A of partition coefficient K'_A. Though some of the experimental results are obtained by using only one solvent, rather than the usual range of solvents, the application of the theory to the more usual experimental conditions is also considered.

The column is considered to contain a number of plates (p) each of which acts as a single tube in a counter-current distribution apparatus. When mobile phase flows through the column, the amount of solute in any plate can be derived from the general formula (1) for calculating the fraction ($T_{n,r}$) of solute of partition coefficient K, in the rth tube after n transfers:

$$T_{n,r} = \frac{n!}{r!(n-r)!}\left(\frac{1}{K+1}\right)^n K^r \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

After p transfers the solvent front reaches the pth plate and thereafter a portion of the solute in this plate emerges with the eluate. This is given by equation (2):

$$E_{a+1} = \frac{(p+a)!}{p!a!}\frac{(K')^{p+1}}{(K'+1)^{p+a+1}} = \frac{(p+a)}{a(K'+1)}E_a \quad . \quad . \quad . \quad . \quad (2)$$

The fraction of solute in the eluate rises to a maximum when $K' = p/a$. The ratio a/p is equivalent to the total volume of eluate required to attain the maximum expressed in volumes of mobile phase on the column:

$$1/K' = a/p = fv/V_m \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (3)$$

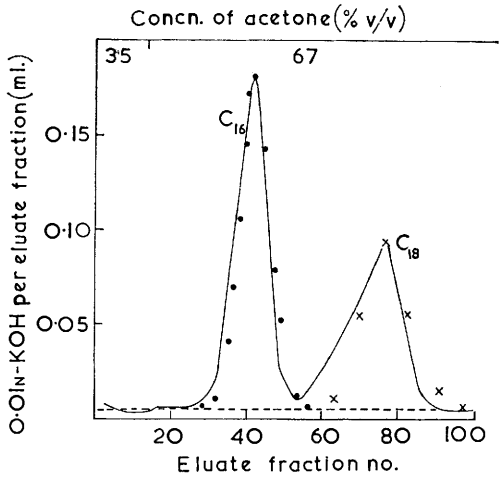


FIG. 5. *Elution of palmitic and stearic acid from a paraffin column by use of* 35% *and* 67% *acetone.*

The solid line is that measured practically by titration of successive 2 *ml. portions of eluate. Individual points are those derived mathematically from equation* (2) *with the values indicated:* ● $p = 70$, $K = 0{\cdot}50$; × $p = 114$, $K' = 0{\cdot}22$.

From eluate curves obtained with palmitic and stearic acid using only 35% and 67% acetone, palmitic acid has an operating partition coefficient of 0·50 and stearic acid of 0·22 (Fig. 5). It is considered that the 35% acetone has practically no effect on the subsequent behaviour of the acids. After allowance for the relative volumes of the two liquid phases in the column (*i.e.*, 2·5), partition coefficients of 0·20 and 0·09 are obtained; these are to be compared with experimentally measured values of 0·16 and 0·07 (Table 1).

From equations (2) and (3) it is possible to derive (4):

$$p = 2\pi(1+K')\left(\frac{E'_{\max.}V_m}{K'_V}\right)^2 = 2\pi f(f+c)(E'_{\max.})^2 \quad . \quad . \quad (4)$$

A value of p may thus be derived from the position and height of the eluate maximum. Values of 70 and 110 are obtained from Fig. 5, and many other curves have given values around 100. By substituting derived values of K' and p in equation (2), some selected points from the theoretical curve have been obtained (Fig. 5).

Glueckauf's equation [12] may be used to discuss the separation of two solutes:

$$\% \text{ impurity} = 100\left(0{\cdot}5 - A\epsilon\left\{\frac{\sqrt{p}(\sqrt{B} - \sqrt{A})}{\sqrt[4]{AB}}\right\}\right)$$

where A is $(1 + 1/K'_A)$ and B is $(1 + 1/K'_B)$. This equation relates the efficiency of separation to the K' values of the two solutes and to the number of theoretical plates in the column being used. To separate a solute of K' 0·5 from a second solute in a system having $p = 100$ so that the two overlap by less than 0·2%, the second solute must have K' less than 0·23. These K' values of 0·50 and $<0{\cdot}23$ correspond to 0·20 and $<0{\cdot}09$ for the experimentally determined values and are in agreement with the empirically selected values of 0·16 and 0·08.

When the column is developed with a single solvent, acid of K' *ca.* 0·5 reaches its maximum eluate after $2{\cdot}0V_m$ of eluate has been collected, in line with the requirement of equation (3). Under normal running conditions the acids have this K' value for the optimum solvent, but they appear to reach their maximum eluate after about $1{\cdot}0V_m$ of eluate has appeared (cf. Fig. 4). This apparent anomaly is related to the use of a range of solvents to elute the acid mixture. Even those solvents which are not ideal for the elution of a particular acid will cause it to move slowly down the column so that when the correct solvent is applied the acid is already part of the way down the column and is therefore eluted more quickly, generally after $1{\cdot}0V_m$ or less.

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CHEMISTRY DEPARTMENT, UNIVERSITY OF ST. ANDREWS. [*Received, April 29th,* 1960.]