THE RESOLVING POWER OF CHROMATOGRAMS

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While many detailed treatments of partition chromatography exist[1–5] it may be useful to put together a simple version of the approach that regards a chromatographic column as equivalent to a counter-current system of a finite number of perfect equilibrations, *i.e.* as containing a number of theoretical plates (MARTIN AND SYNGE[1], CRAIG[6]). The treatment presented consists essentially of the approach of STACK-DUNNE (cited by DIXON AND STACK-DUNNE[7]) for the first part, followed by that of TAIT AND TAIT[8] who continued from STACK-DUNNE's position.

In this treatment chromatography is the classical "elution analysis" of TISELIUS (*e.g.* ref.[9]) where the sample is added in a small volume of mobile phase to a column containing two phases in equilibrium, and elution is then continued by passing more of the mobile phase. The solutes in the sample are assumed to have fixed partition coefficients (K) in favour of the stationary phase. This definition of partition chromatography does not exclude cases where the stationary phase with which the solute equilibrates occupies only a small fraction of the total volume of the stationary material with which the column is packed, *i.e.* adsorption chromatography, nor does it exclude cases where any particular type of force may be predominant in determining partition coefficients, *e.g.* ion-exchange chromatography. It does exclude separations based on stepwise or continuous changes in the mobile phase.

PROCESS OF CHROMATOGRAPHY

The process by which separations are obtained may be expressed as follows. If equilibrium is at all times approached for each solute between the mobile and stationary phases, there will be a fixed fraction of the molecules of each solute in the mobile phase, a fraction determined by the partition coefficient and the volume ratio of the phases. This fraction is constant from time to time and from one section of the column to another. The maintenance of a constant fraction of the molecules of one solute in the moving phase, when each molecule is passing back and forth between the two phases, means that each must spend that same fraction of the time in the moving phase. It will therefore, like all other identical molecules, move along the column at that fraction of the speed of the moving phase. Thus the band of this substance moves at a fixed fraction of the speed of the moving phase, a fraction which differs for substances of different partition coefficients.

NUMBER OF THEORETICAL PLATES

Owing to imperfections in a column such as slowness of equilibration and uneven flow, the mobile phase at a given level has an average solute content, not in equilibrium with the stationary phase at that level, but in equilibrium with the stationary phase at a slightly higher level.

MARTIN AND SYNGE[1] therefore treated the column as a series of plates each comprising a layer of such thickness that "the solution issuing from it is in equilibrium with the mean concentration of solute in the non-mobile phase throughout the layer". After perfect equilibration of the phases in the plates, quantitative transfer of the mobile phase to the next plate was imagined.

GLUECKAUF[4], however, pointed out that a given number of theoretical plates gives less efficient separation when used with continuous flow than as a discontinuous system in which the contents of each plate are transferred to the next after equilibration. Since nevertheless the elution curves of chromatograms are very similar to those of counter-current distributions, the degree of departure of a column from perfection may be measured by how few plates the ideal counter-current system *of equivalent performance* possesses. In this treatment, therefore, the theoretical plates referred to are those of the discontinuous counter-current system that is equivalent to the chromatogram with respect to the elution curves produced, and they are not those of MARTIN AND SYNGE's definition.

Both the real column and the equivalent series of plates contain a volume B of stationary phase, a volume F of mobile phase, and the solute considered has a partition K in favour of the stationary phase.

(a) *Ideal column*

Then if the column operates ideally, there will be F parts solute in the mobile phase for every BK parts in the stationary phase, at any given distance from the origin. The fraction of solute in the mobile phase is therefore:

$$\frac{F}{F + BK} = R_F \tag{1}$$

where R_F is defined as the ratio of the speed the band moves in the column to the speed of eluent in the column, because each solute molecule spends this fraction of the time in the mobile phase.

If E is the volume of eluent emerging from the point of application of the sample to the point of emergence of the solute in maximal concentration (see Fig. 1), then:

$$\frac{F}{E} = R_F \tag{2}$$

since E of eluent flows while the band moves through F of mobile phase.

From equations (1) and (2):

$$E = F + BK \tag{3}$$

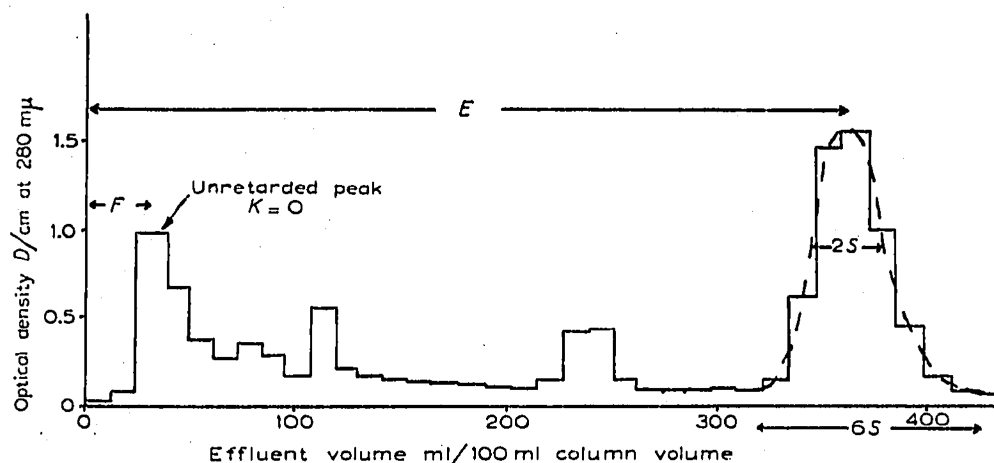

Fig. 1. Elution diagram of a chromatogram showing, E, F and S. A dotted line has been drawn through the centres of the blocks for the determination of S (see text and Fig. 3). Ion-exchange chromatogram of pig β-melanocyte-stimulating hormone, a pituitary peptide of 18 amino-acid residues (from DIXON[10]).

(b) *Equivalent series of plates*

The treatment of CRAIG[6] is applied as follows:

Let p be the fraction of solute in the mobile phase at equilibrium, *i.e.*

$$p = \frac{F}{F + BK} \tag{4}$$

Then after t transfers, the fractions of solute in plates, 0, 1, 2, 3, . . . r . . . t are given by the terms of the binomial expansion of $([1 - p] + p)^t$ (Fig. 2). Of these the largest is the $(pt + 1)$th term.

This is because the term corresponding to plate number "r", *i.e.* the $(r + 1)$th term

$$= \frac{t!}{r!\,(t - r)!}\,(1 - p)^{t-r}p^r.$$

The maximum term is the $(r + 1/2)$th when the (r)th equals the $(r + 1)$th.

$$\frac{t!}{r!\,(t - r)!}\,(1 - p)^{t-r}p^r = \frac{t!}{(r - 1)!\,(t - r + 1)!}\,(1 - p)^{t-r+1}p^{r-1}.$$

Hence $$\frac{1}{r} \cdot p = \frac{1}{t - r + 1}\,(1 - p)$$

or $$pt - pr + p = r - pr$$

i.e. $$r = pt + p,$$

so $$r + 1/2 = pt + p + 1/2 \simeq pt + 1$$

The solute therefore travels in maximal concentration at p times the rate of the mobile phase, as in the ideal column, and eqns. (1), (2) and (3) therefore apply.

The standard deviation of the distribution in the plates in terms of plate number is:

$$\sqrt{tp\,(1 - p)}$$

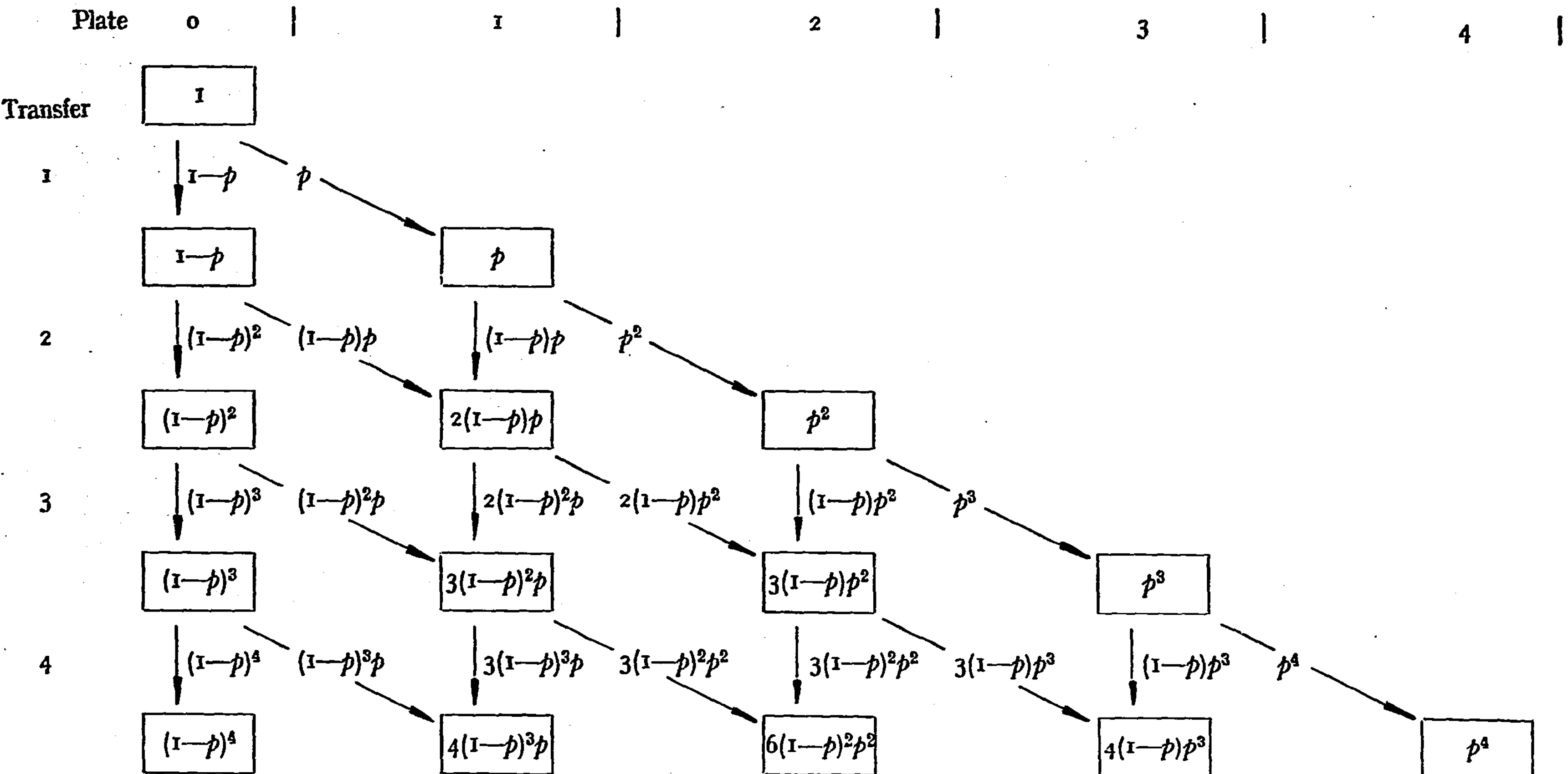

Fig. 2. Diagram of counter-current distribution. Each box represents a plate. The numbers in the boxes represent the fraction of one solute in that plate, starting from the top when all the solute is in plate 0. The diagonal lines show the fraction of the total transferred to the next plate, always p times the amount in the previous plate, and the vertical lines the fraction of the total remaining in the plate, *i.e.* $(1 - p)$ times the amount previously present. By adding the amount transferred to a plate and the amount remaining there, its content after the transfer is found. The amounts in the plates after t transfers are given by the binomial expansion of $([1 - p] + p)^t$.

Let $t = T$ at the point when a volume E of effluent has emerged, *i.e.* when the solute emerges in maximal concentration.

The standard deviation of the distribution in the plates,

$$S_T = \sqrt{Tp\,(1 - p)},$$

or by using eqns. (3) and (4),

$$S_T = \sqrt{T\frac{F}{E}\frac{E - F}{E}} \tag{5}$$

In this it is imagined that further tubes were present so that the solute remained distributed in plates instead of emerging in the effluent.

Now when it emerges in the effluent, its distribution reflects the distribution in the plates. All the material in the peak can be taken to have undergone T transfers if T is large, although strictly the leading edge of the peak will have undergone fewer and the trailing edges more.

If N is the total number of plates, and it takes volume E of effluent to move the peak through the N plates, a volume E/N will move the peak through one plate. Thus the distribution in terms of effluent volume reflects the distribution in the plates if plate number is converted into effluent volume by multiplying by E/N.

If S is the standard deviation of the distribution of solute in the effluent (Fig. 1):

$$S = \frac{E}{N} S_T \tag{6}$$

From eqns. (5) and (6):

$$S = \frac{E}{N}\sqrt{T\frac{F}{E}\frac{(E - F)}{E}}$$

or

$$S = \frac{1}{N}\sqrt{T\,F\,(E - F)} \tag{7}$$

Since T transfers move the effluent T plates and move the peak N plates,

$$\frac{N}{T} = R_F,$$

or by eqn. (2),

$$\frac{N}{T} = \frac{F}{E}. \tag{8}$$

Using eqn. (8) to eliminate T from eqn. (7):

$$S = \sqrt{\frac{E\,(E - F)}{N}} \tag{9}$$

i.e.

$$N = \frac{E\,(E - F)}{S^2}. \tag{10}$$

This equation allows determination of the number of plates of any column from the plot of concentration of solute against effluent volume. For this S must be measured. Since the binomial distribution approximates to the normal distribution when N is large, S may be taken as (i) one sixth of the peak width (strictly the width containing 99.7 % of the solute), or (ii) half the width of the peak at e^{-1} (0.607) of the maximum height[4], or (iii) half the width at the point of inflexion or, (iv) quarter the width of the triangle formed by producing tangents to the curve at the points of inflexion to the baseline[5] (Fig. 3). Methods (i) and (ii) are preferred as if the actual peak is skewed, methods (iii) and (iv) may be unsatisfactory. There is, however, a danger that with

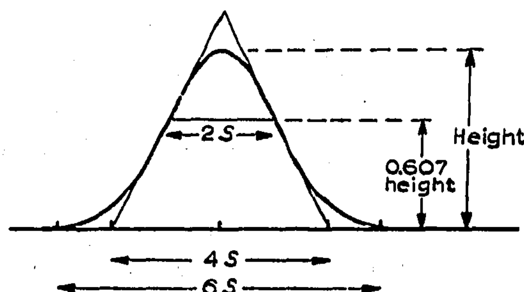

Fig. 3. Normal distribution curve with tangents to the points of inflexion, showing methods of determining S.

methods (ii)—(iv) the effect of tailing may be neglected. Thus S and hence N may be determined from a published elution diagram, and the units of effluent volume do not need to be known.

In Fig. 1, for example, both methods (i) and (ii) give $S = 18$. Since $F = 30$ and $E = 360$, N by eqn. (10), is 368. As the column was 38.5 cm long, it exhibited about 9.6 plates/cm. Such a determination of N allows one to judge whether a column is operating as satisfactorily on a given occasion as the system is known to do in other cases, so may be helpful in repeating published work. One could also judge, for example, whether a given rise in flow rate or particle size were having appreciable effect on the elution pattern. A lower value of N per unit length for apparently similar conditions reveals some deterioration of performance. To go further it is necessary to consider the resolving power of columns.

RESOLVING POWER OF COLUMNS

(a) *Derivation*

A fixed fraction of a normal distribution lies on one side more than tS from the mean, where t has any given value. Thus if $t = 2$, the fraction is 2.3 %, and if $t = 3$, it is 0.14 %. If we consider two peaks with elution volumes E and $E + \Delta E$, they may be regarded as just resolved when $\Delta E = t\,(S_1 + S_2)$, where S_1 and S_2 are the standard deviations of their distributions, and we assign a value to t which shows what we take to be resolution[1]. In the case where the column performs equally well for the two substances, *i.e.* manifests an equal value of N for both, and if ΔE is small compared

with E, as it will be if the column resolves reasonably well, $S_1 \simeq S_2$. Thus the condition for separation becomes $\Delta E \geqslant 2 t S$, or $\Delta E \geqslant A S$ where $A = 2 t$.

So long as the peaks approximate to normal distributions, taking $A = 4$ as the definition of resolution means that we will tolerate up to 2.3 % mutual contamination. The band 4 S wide centred on the centre of the peak should contain 95.4 % of the peak and only 2.3 % of the neighbouring peak from which it is just resolved. The more stringent definition of resolution of putting $A = 6$ diminishes the maximum permissible mutual contamination from 2.3 % to 0.14 %.

Let the resolving power, P, of a column be defined as the ratio $K/\Delta K$ for two substances of partition coefficients K and $K + \Delta K$ that the column can just resolve to the extent given by assigning an arbitrary value to A. It may be noted that when both R_F and ΔK are small, $K/\Delta K$ is approximately $R_F/\Delta R_F$. This is because differentiating eqn. (1) gives:

$$\frac{K}{\Delta K} = -(1 - R_F)\frac{R_F}{\Delta R_F} \text{ when } \Delta K \longrightarrow 0$$

The above definition of P is the inverse of TAIT AND TAIT's[8] definition of resolving power, while the rest of the treatment is theirs. This definition is preferred because an increase in resolving power thus defined means that the column is more rather than less powerful.

A column of resolving power P requires a $1/P$-fold change in K before it can separate two substances.

Rearranging eqn. (2) for the two substances

$$E - F = BK \tag{2a}$$

$$(E + \Delta E) - F = B(K + \Delta K) \tag{2b}$$

Hence

$$\Delta E = B\Delta K \tag{11}$$

Dividing eqn. (2a) by eqn. (11)

$$\frac{K}{\Delta K} = \frac{E - F}{\Delta E} \tag{12}$$

$$P = \frac{K}{\Delta K}$$

for the condition that

$$\Delta E = AS$$

$$\therefore P = \frac{E - F}{A\,S} \tag{13}$$

Substituting S from eqn. (9),

$$P = \frac{\sqrt{N}}{A}\sqrt{1 - \frac{F}{E}} \tag{14a}$$

or

$$P = \frac{\sqrt{N}}{A}\sqrt{1 - R_F} \tag{14b}$$

(b) Discussion

As TAIT AND TAIT[8] point out, there are two main implications of this equation. Firstly the resolving power is proportional to $\sqrt{N}$, as previously noted by SCHUBERT[11] and emphasized by HAMILTON *et al.*[5]. Thus if two peaks are just resolved to an acceptable degree, it will require four times as long a column (assuming the same number of theoretical plates per unit length can be achieved) to separate them to the same degree from a peak half way between them. Secondly the R_F, *i.e.*, F/E, should be kept low, but no great advantage is gained by further diminution below about 0.3, because by then the term $\sqrt{1 - R_F}$ has reached 84 % of the maximum value it can attain (Fig. 4). TAIT AND TAIT[8] also point out that the requirements of the $\sqrt{N}$ and

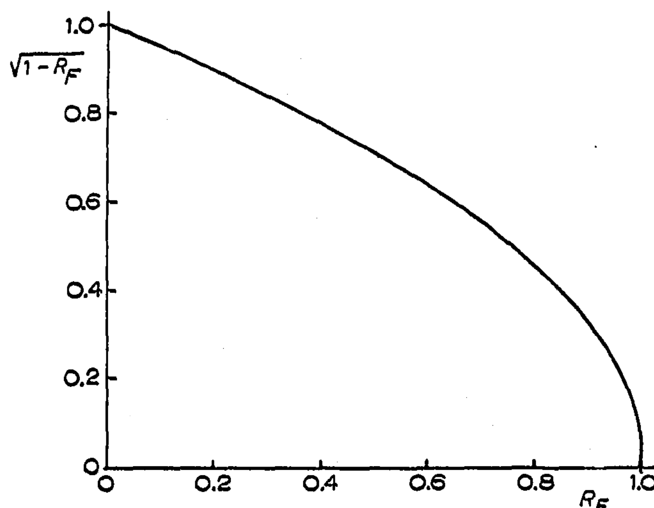

Fig. 4. Graph of $\sqrt{1 - R_F}$ against R_F.

the $\sqrt{1 - R_F}$ terms conflict in paper chromatography unless over-running of the paper by the eluent be allowed.

In the example in Fig. 1, $P_{A=4} = 4.6$ (by eqn. (14a)). Thus the band containing 95.4 % of the peak should contain less than 2.3 % of the original amount of any substance present as an impurity in the sample applied to the chromatogram for which the resolving power of the chromatogram is as great, if such substances differ from the peak in partition coefficient by more than 1/4.6-fold or 22 %. Similarly $P_{A=6} = 3.1$, so 99.7 % of the peak should contain less than 0.14 % of any such impurity which differs from the peak substance by more than 1/3.1-fold in K, or 32 %.

An index reflecting the resolving power of the column without the disadvantage of the arbitrary assignment of a value to A would be the product PA. Since it would be less easily visualized in terms of the column's separating power, there is probably little gain in using it rather than its components N and R_F.

The practice of taking the ratio $K/\Delta K$ instead of an absolute value of K or its inverse as a measure of the difficulty of separation of substances is justified by the fact that in a given system the substitution of one group by another should produce a

given absolute change in ln K, not in K, more or less independently of the rest of the solute molecule (MARTIN[12]). This arises from the fact that each group contributes more or less independently to the difference in standard free energy of the solute between the two different phases.

BATE-SMITH AND WESTALL's[13] concept of R_M follows from this. If eqn. (1) is rearranged to find K, $K = F/B(1/R_F - 1)$.

Since the contributions of different groups to ln K are constant, so are the contributions to $R_M = \log (1/R_F - 1)$.

A recent application of this to analytical chemistry by MILSTEIN AND SANGER[14] follows PARDEE's[15] application of the constancy of increments in ln K to relate the R_F values of peptides to those of their constituent amino acids.

The assumption that a column will exhibit the same number of plates for the different substances being separated may often be unjustified. The peak of one substance may trail badly, so that it cannot be entirely removed from a substance for which the column shows a high resolving power as defined above. In many such cases the substances may differ greatly in chemical nature, so that a preliminary separation may be possible before chromatography.

Finally it should be noted that successful separation does not depend solely on the resolving power of the column, but also on the choice of a system such that a large value of $\Delta K/K$ will be obtained for the substances to be separated. Table I gives an example showing how choice of the right conditions greatly increased the difference in partition between two different substances.

The literature of more detailed treatments of chromatography, including those which avoid the model of an equivalent counter-current system, is cited together with their own contributions by HAMILTON *et al.*[5].

TABLE I

PARTITION COEFFICIENTS BETWEEN WATER AND *n*-BUTANOL

	ACTH	*α-MSH*
Acid system	3	1
Neutral system	13	0.3

ACTH: Pig corticotropin A_1; MSH: Melanocyte-stimulating hormone.
Acid system: 115 ml water; 85 ml *n*-butanol; 20 ml acetic acid; 1.6 g NaCl.
Neutral system: 50 ml water; 50 ml *n*-butanol; 0.5 g $NaHCO_3$; 1 g Na *p*-toluenesulphonate.
Partition coefficients given are those in favour of the aqueous layer. Both systems contain anions suitable for complex formation with peptides and salts, both of which are likely to lower the partition coefficients. The large difference is probably because the acid system is below the isoelectric points of both substances, whereas the neutral system is between them.
From unpublished data of H. B. F. DIXON AND L. R. WEITKAMP.

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SUMMARY

The resolving power, P, of a chromatogram is defined as the inverse of the fractional change in partition coefficient by which two substances must differ before the chromatogram can separate them. It is shown that $P = \sqrt{N}/A \sqrt{1 - R_F}$, where N is the number of plates of the discontinuous counter-current systems of equivalent performance and A is a constant to which a value must be assigned in the definition of P and which reflects the degree of separation required between the substances. A useful definition of resolving power is $P_{A=4}$, when 2.3 % is the maximum mutual contamination of substances permitted.

Methods of determining N and R_F from the elution diagrams of chromatograms are quoted.

REFERENCES

1 A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, 35 (1941) 1358.
2 S. W. Mayer and E. R. Tompkins, *J. Am. Chem. Soc.*, 69 (1947) 2866.
3 J. Beukenkamp, W. Rieman and S. Lindenbaum, *Anal. Chem.*, 26 (1954) 505.
4 E. Glueckauf, *Trans. Faraday Soc.*, 51 (1955) 34.
5 P. B. Hamilton, D. C. Bogue and R. A. Anderson, *Anal. Chem.*, 32 (1960) 1782.
6 L. C. Craig, *Anal. Chem.*, 22 (1950) 1346.
7 H. B. F. Dixon and M. P. Stack-Dunne, *Biochem. J.*, 61 (1955) 483.
8 S. A. S. Tait and J. F. Tait, *Quantitative Paper Chromatography of Steroids, Memoirs of the Society of Endocrinology*, No. 8, University Press, Cambridge, 1960, p. 40.
9 A. Tiselius, *Advances in Protein Chem.*, 3 (1947) 67.
10 H. B. F. Dixon, *Biochim. Biophys. Acta*, 37 (1960) 38.
11 J. Schubert, in F. C. Nachod, *Ion Exchange*, Academic Press, Inc., New York, 1949, p. 167.
12 A. J. P. Martin, *Partition Chromatography, Biochem. Soc. Symp.* (*Cambridge, Engl.*), No. 3 (1949) 4.
13 E. C. Bate-Smith and R. G. Westall, *Biochim. Biophys. Acta*, 4 (1950) 427.
14 C. Milstein and F. Sanger, *Biochem. J.*, 79 (1961) 456.
15 A. B. Pardee, *J. Biol. Chem.*, 190 (1951) 757.