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Plenary Lectures and Reviews

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CHROMATOGRAPHIC REVIEWS (Vol. 23, No. 1)

edited by

Michael Lederer

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Biomedical Applications		162/1	162/2	162/3	162/4	163/1	163/2	163/3	163/4	164/1	164/2	164/3	164/4

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(Chromatographic Reviews, Vol. 23, No. 1)

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(*Nobel Prize laureates)

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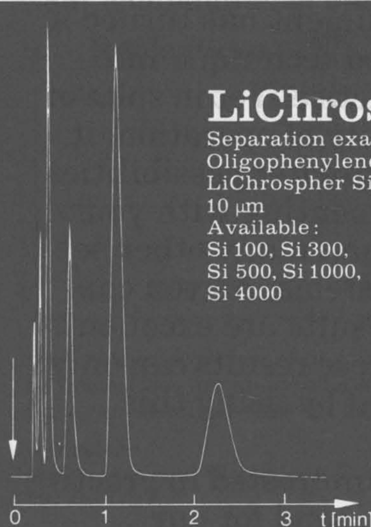

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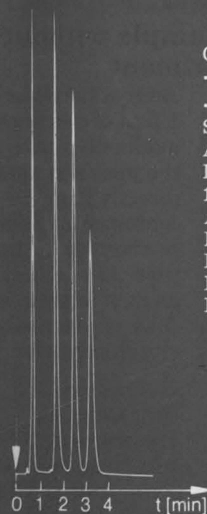

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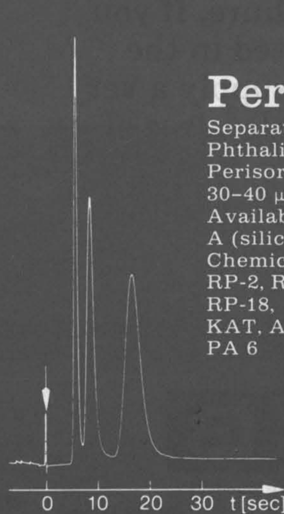

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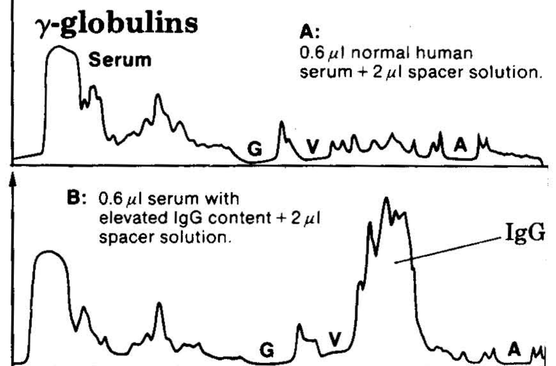

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Delmotte, P., Science Tools 24 (1977) 33–41.

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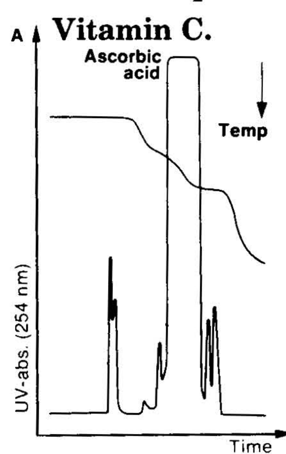

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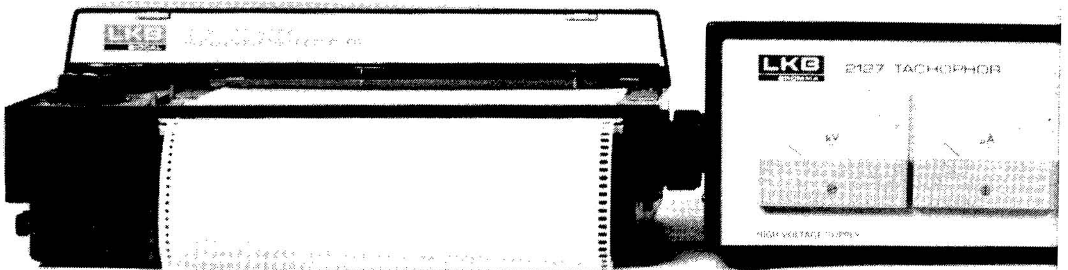
Baldesten, A., Hjalmarsson, S-G and Neumann, G., Fres. Z. Anal. Chen (1978) 148–149.



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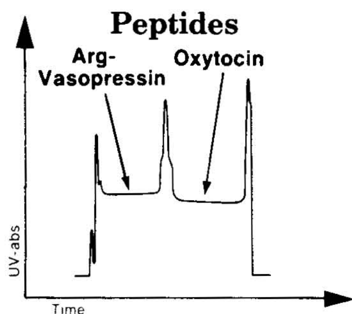

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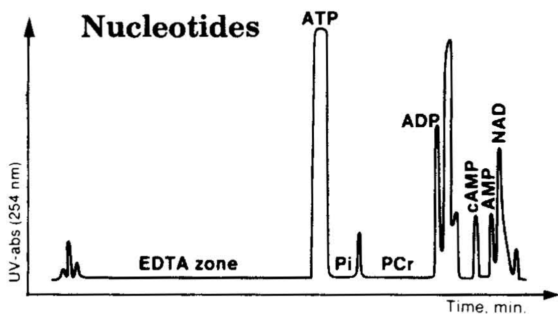

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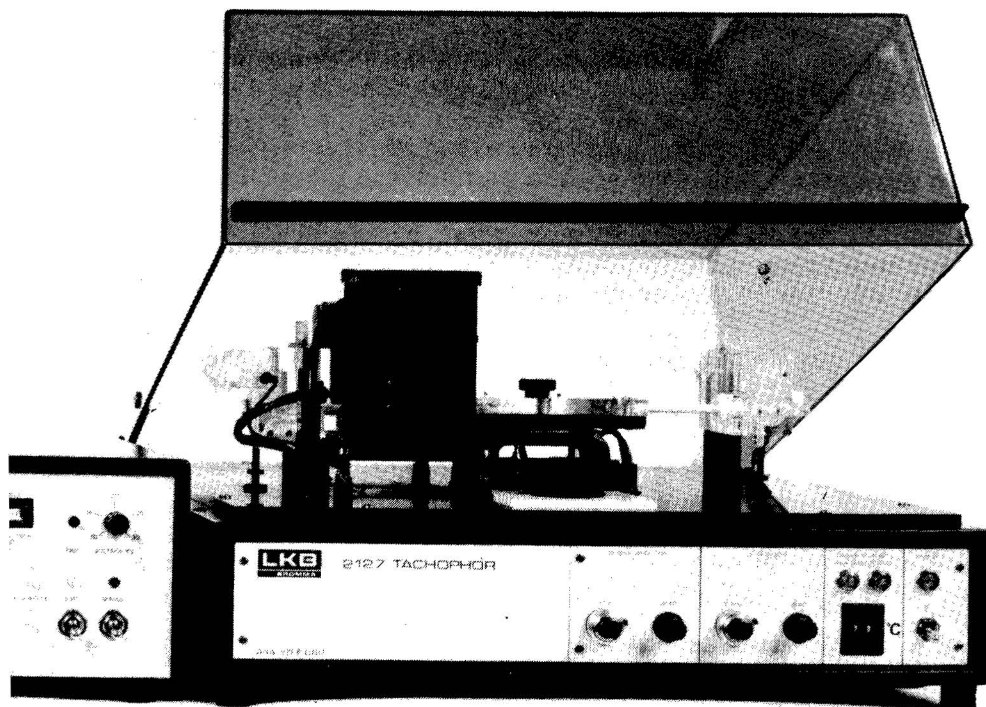
Gower, D.C. and Woledge, R.C., Science Tools 24 (1977) 17–21.



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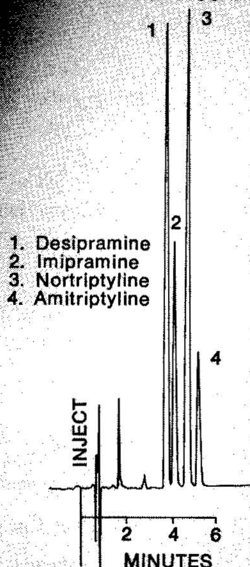

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CHROMATOGRAPHIC REVIEWS

edited by

Michael Lederer

VOL. 23 1979

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Baden-Baden, September 25–29, 1978

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PREFACE

The five manuscripts published in this issue are the Plenary Lectures and Reviews read at the *Twelfth International Symposium on Chromatography, Baden-Baden, September 25–29, 1978*, which were received by the Editor by the agreed deadline (December 1st, 1978).

Several lecturers (Dr. E. Jellum, Professor G. Guiochon and Professor J. F. K. Huber) have informed us that they do not intend to publish their lectures. The remaining papers will be considered for publication in later issues of *Chromatographic Reviews* if and when they are submitted.

Rome (Italy)
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THE EDITOR

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GRADIENT ELUTION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

I. THEORETICAL BASIS FOR REVERSED-PHASE SYSTEMS

L. R. SNYDER, J. W. DOLAN and J. R. GANT
Technicon Instrument Corp., Tarrytown, N.Y. 10591 (U.S.A.)

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1. INTRODUCTION

At the present time it is probable that 60–80% of all separations by high-performance liquid chromatography (LC) are carried out in the reversed-phase mode with bonded-phase (*e.g.*, C_{18}) packings, generally using 5–10-μm totally porous particles. When gradient elution is added to this technique, which is increasingly the case,

the resulting separation system offers the same broad applicability for non-volatile samples that temperature-programmed gas chromatography (GC) provides for volatile mixtures. Gradient elution is a powerful separation procedure, but one that is at best difficult to understand. As a result, its optimal application in a given situation would be facilitated by a simple set of guidelines which relate various separation characteristics to the different operating parameters or experimental variables. That is the aim of the present and the following[1] papers.

Over the past few years, a general theory of gradient elution separation has been developed (*e.g.*, refs. 2–8), but many of the practical implications of this work seem to have gone largely unnoticed. More recently, the special case of reversed-phase gradient elution (RP-GE) has been examined theoretically[7,9,10]. Most of this work, however, has focused on detailed mathematical expressions for sample retention times and resolution. While these approaches in principle allow precise calculations of separation in a given RP-GE system for a particular sample (of known composition), they offer little help for the case of unknown samples. Further, such calculations require detailed studies of the elution of the sample in question under isocratic conditions, in order to specify the dependence of isocratic sample retention on mobile phase composition.

Our intention in this paper is to provide an approximate theoretical treatment for understanding and controlling separation in RP-GE for the general case, where the composition of the sample may not be known, and where no information is available on the variation of sample capacity factor (k') values with mobile phase composition. In Part II[1] we shall illustrate some of our theoretical conclusions with experimental examples, and offer a detailed set of rules for optimizing RP-GE separations. Our primary emphasis will be on resolution and detection sensitivity as a function of experimental conditions.

2. EXPERIMENTAL

This paper is almost entirely theoretical, and any new experimental data referred to are described in detail in Part II[1]. Most of the figures and some of the tables presented here are based on rigorous calculations, in most instances involving numerical integration of eqn. i-1 (Appendix I). Some assumptions are implicit not only in these calculations, but in much of the attendant discussion. These include the following:

(1) It is assumed that the sample is injected as a very small volume, relative to the larger volume of later eluting bands; *i.e.*, the sample volume does not affect the final band width.

(2) It is assumed that there is no time delay between the gradient generator and sample injector; *i.e.*, the gradient program begins at the column inlet at the moment of sample injection. This is generally not true of actual gradient elution systems, where a finite volume exists in the tubing connecting the gradient mixer and the column inlet.

(3) It is assumed that the mobile phase gradient entering the column is not affected by any sorption of mobile phase components on the column. In actual systems involving reversed-phase operation this is probably a reasonable approximation.

3. GRADIENT ELUTION

As far as possible, our approach here will be to explain gradient elution in terms of isocratic elution, and to transfer the various concepts to gradient elution which are applicable for isocratic separation. While it can be argued that some mathematical precision is sacrificed in this procedure, this is inevitable in terms of our goal of developing general guidelines for carrying out RP-GE separations. To follow more easily the discussion in the balance of this paper, it will prove useful to first look at what happens in gradient elution in qualitative terms. We shall also define several retention parameters that play a key role in any theory of gradient elution.

The following examples of isocratic and gradient elution are calculated as described under Experimental. The particular conditions assumed (*e.g.*, gradient shape and steepness) are for the most part both practical and reasonable. Fig. 1 shows an isocratic separation of an eight-component mixture, for the experimental conditions summarized in the figure caption. The k' values for bands 1, 2, 3, ..., 8 increase in geometric progression: 0.5, 1.0, 2.0, ..., 64. The bands are shown as triangles rather than Gaussian bands, with the base of each band equal to 4σ.

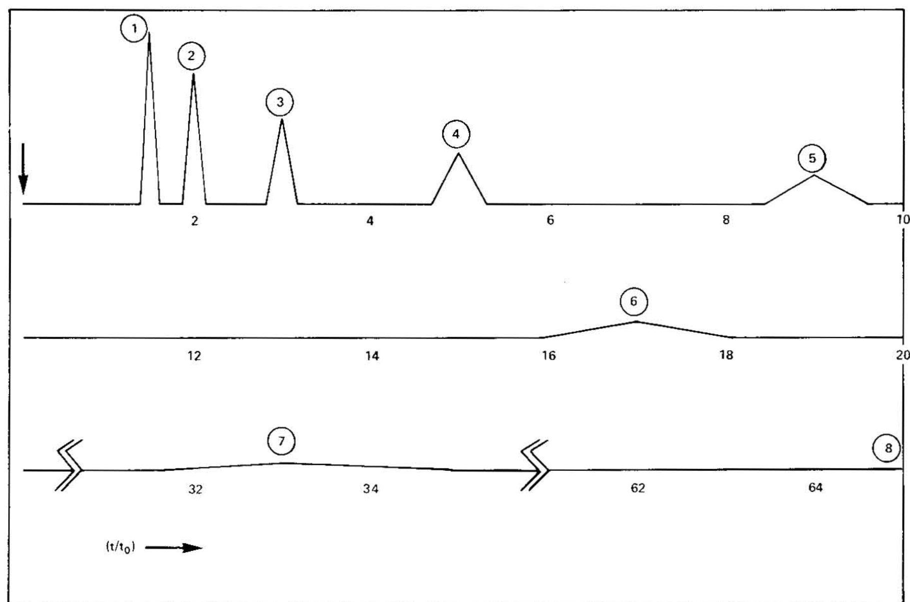


Fig. 1. Calculated isocratic separation of eight-component sample by reversed-phase LC. Mobile phase, 20% methanol–water; $N = 1000$; k' values for bands 1–8 are 0.5, 1.0, 2.0, ..., 64.

Fig. 2 shows a proposed gradient or solvent program for the RP-GE separation of the same sample as in Fig. 1. A linear gradient from 20 to 80% methanol–water is assumed, and the light solid curve shows the change in mobile phase composition at the column inlet as a function of time t after sample injection (expressed in units of t/t_0, which we shall comment on shortly). The dashed curve in Fig. 2 (labeled "outlet")

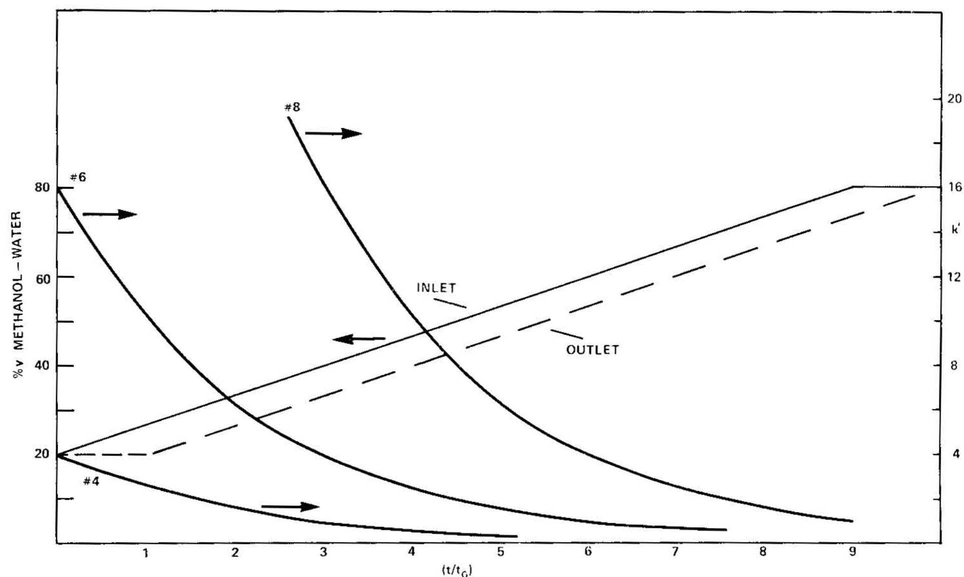


Fig. 2. Linear solvent program for separation by reversed-phase gradient elution. Program runs from 20 to 80% methanol–water; k_i values assume a 2-fold decrease for each 10% increase in methanol concentration.

shows the composition of the mobile phase as it leaves the column; this lags the inlet composition by one column volume or a time equal to t_0. Also superimposed on Fig. 2 are heavy curves for the k' values of bands 4, 6 and 8 as they would exist at the column inlet as a function of time (t/t_0). That is, these latter inlet k' values (k_i) refer to the isocratic k' for a mobile phase with the given inlet composition at time t/t_0. The actual bands will have migrated some distance across the column, and at any given time t/t_0 they will be moving in a somewhat weaker solvent. The instantaneous or actual k' value for the band (k_a) will therefore be slightly larger than k_i at any given time.

The resulting gradient elution separation of the sample of Fig. 1, using the solvent program of Fig. 2, is shown in Fig. 3a. Here, it is assumed again that the various sample bands are of equal area, corresponding, *e.g.*, to the case of equal concentrations and equal detector sensitivities. Comparison of Figs. 1 and 3a shows that the first-eluted bands (1–3) are eluted in a similar fashion by either isocratic or gradient elution; the retention times are similar, the band widths are comparable and the peak heights are about the same. The reason is that in gradient elution the bands elute within the first 3 column volumes, before the mobile phase composition leaving the column has changed much (*cf.*, Fig. 2), and before the k_i values for these bands have decreased greatly from the starting isocratic values (k_0) as in Fig. 1. Note that the mobile phase composition for Fig. 1 is the same (20% methanol–water) as for the start of the gradient elution separation in Fig. 3a (see Fig. 2).

The appearance of the last eluted bands (5–8) differs greatly in gradient (Fig. 3a) from isocratic (Fig. 1) elution. The reason is that the initial k' or k_0 values for these bands are fairly large, and as a result the bands elute under essentially

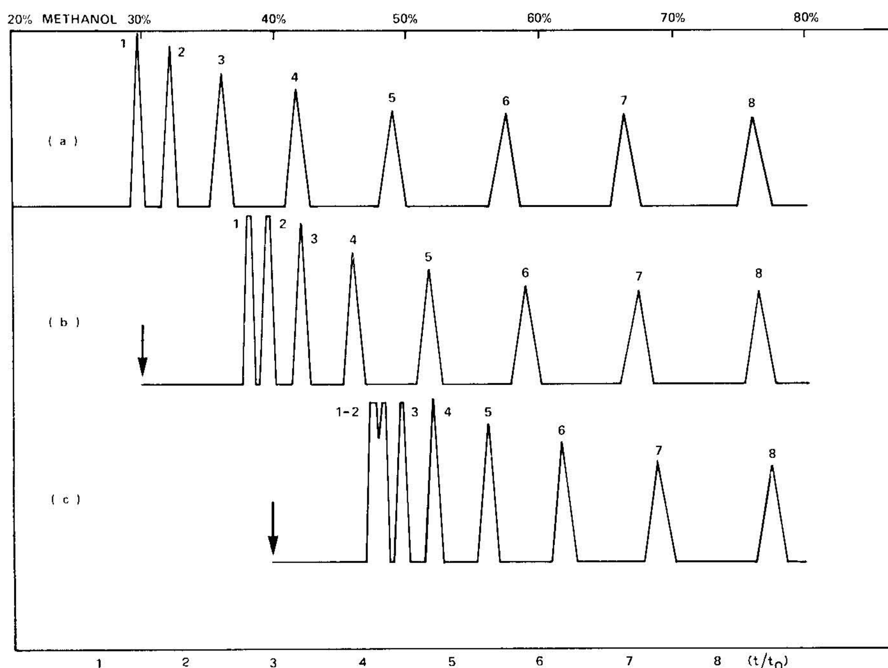
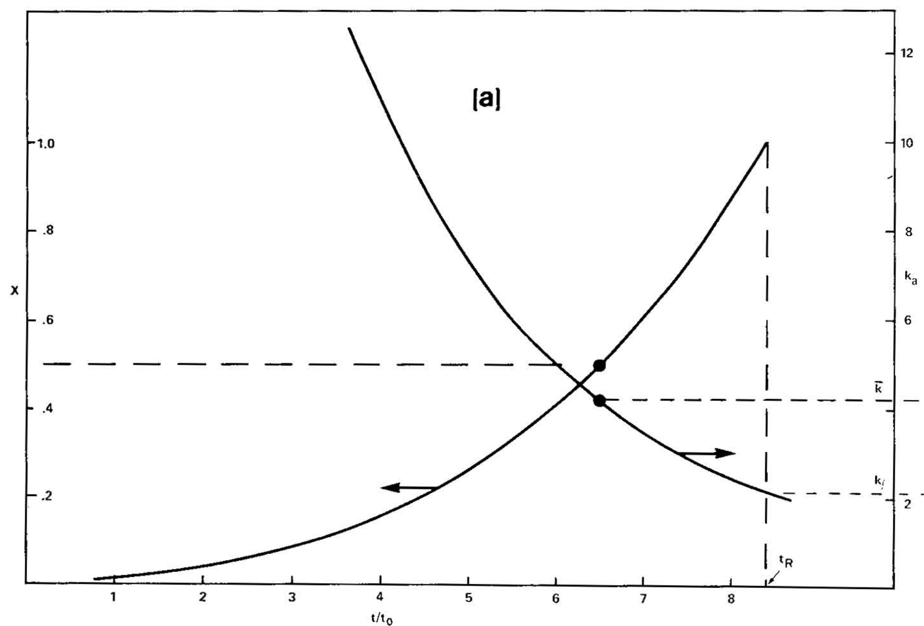


Fig. 3. Calculated gradient elution separation for sample and column of Fig. 1. (a) Using solvent program of Fig. 2 ($b = 0.2$); (b) same, except program starts at 30% methanol–water; (c) same, except program starts at 40% methanol–water.

gradient conditions, rather than near-isocratically as for bands 1–3. As a result, these later eluted bands in gradient elution have approximately equal band widths (and similar detection sensitivities), and are more closely spaced in the chromatogram. When the sample k_0 values are in geometric progression as in this example, equal spacing of the bands results. Let us look more closely at the elution of these late bands in RP-GE, as in Fig. 4a. Here, the fractional movement x of band 8 across the column is shown as a function of time t/t_0. It is seen that the movement of band 8 along the column is negligible ($x \leq 0.1$) prior to $t/t_0 \approx 3$. At this point, band 8 begins to move across the column increasingly rapidly, until its elution at $t/t_0 = 8.4$. The instantaneous k' value for band 8 (k_a) during this RP-GE separation is also shown in Fig. 4a. The value of k_a remains large up to $t/t_0 \approx 3$, then drops rapidly as band 8 begins to migrate. The average or effective value of k_a during elution of the band determines the resolution of that band, just as k' determines the resolution of a band in isocratic elution. If we define this average k_a as $\bar{k}$, equal roughly to the value of k_a at $x = 0.5$ ($\bar{k} = 4.2$ in Fig. 4a), then the resolution, R_s, for the band in gradient elution will be given as

$$R_s = \tfrac{1}{4}(\alpha - 1)\, N^{\frac{1}{2}}\, [\bar{k}/(1 + \bar{k})] \tag{1}$$

which is exactly analogous to the corresponding relationship for isocratic elution (*e.g.*, ref. 11). Here, α is the separation factor for two adjacent bands and N is the column



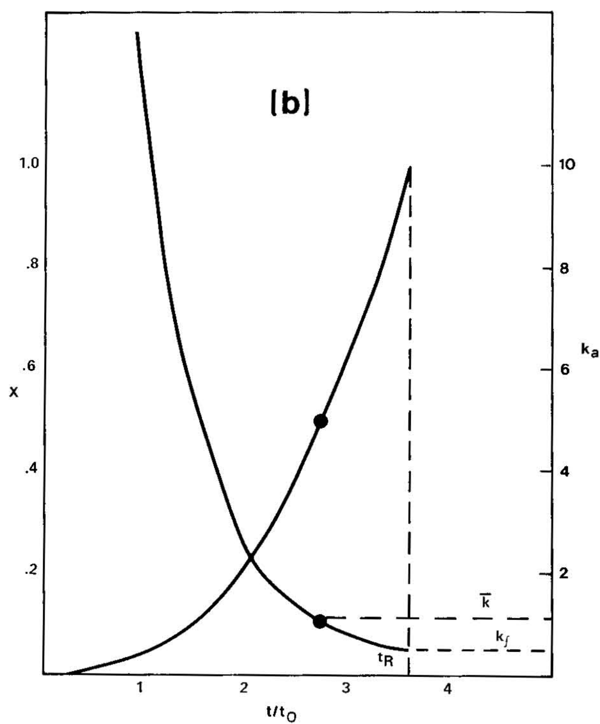


Fig. 4. Migration of band 8 along column in separation of Fig. 3. (a) Conditions as in Figs. 2 and 3; (b) same, except steeper gradient ($b = 0.8$).

plate number. Note in Fig. 4a that the value of $\bar{k}$, comparable to k' in isocratic elution, is in the optimal range of 2–5 (*cf.*, discussion in ref. 11).

Band width and detection sensitivity in gradient elution are determined by the value of N and by the k_a value of the band at the time of elution (k_f), just as in isocratic elution. In Fig. 4a we see that k_f for band 8 is 2.1, which is small enough to yield a fairly narrow, easily detected band. In contrast, band 8 in the isocratic separation in Fig. 1 has $k' = 64$, yielding a barely detectable band which is about 32-fold wider than in Fig. 3a.

For later eluting bands in RP-GE, the progression of the band along the column proceeds very much as for band 8 in Fig. 4a. The only difference is either an earlier or later beginning of migration; *i.e.*, at t/t_0 values less than or greater than 3. Because the shape of the x or k_a *versus* t/t_0 plots for later eluting bands are essentially similar to that for band 8 in Fig. 4a (for a properly designed solvent program, as in Fig. 2), similar values of $\bar{k}$ and k_f result for every band. This means that resolution for all later eluted bands is comparable when their isocratic α values are similar, and band widths for all later eluting compounds will be essentially constant.

A. Linear solvent strength separations

The most basic and important experimental variable in gradient elution separation is the solvent program: the varying composition of the mobile phase during the elution of sample from the column (as in Fig. 2). The optimal solvent program in turn depends on the relationship of sample k' values (isocratic separation) to the composition of the mobile phase. In a later section we shall consider this latter relationship in more detail. For the moment we shall concentrate instead on gradient programs which are fundamentally optimal from the standpoint of separation; as discussed elsewhere[2–5,12] such solvent programs are of the so-called linear solvent strength (LSS) form. With an LSS solvent program, the inlet k_i values for individual sample compounds decrease during gradient elution according to

$$\log k_i = \log k_0 - b(t/t_0) \qquad (2)$$

As previously, k_0 refers to the k' value for the band in question at the beginning of gradient elution; *i.e.*, for isocratic elution with the mobile phase composition corresponding to the beginning of the solvent program (20% methanol–water in Figs. 2 and 3a). The parameter b should remain constant throughout the solvent program, and ideally b will have the same value for all compounds in the sample (in principle, this is never exactly possible; see later discussion).

Among the advantages of properly designed LSS programs in gradient elution separations are the following:

(1) approximately constant band widths for all bands in the chromatogram;

(2) comparable resolution or effective plate number NQ^2 (see ref. 4) for both early- and late-eluting bands (*i.e.*, equal values of NQ^2 for all bands in the chromatogram);

(3) a regular spacing of bands throughout the chromatogram, without bunching of peaks at the beginning or end (if values of $\alpha - 1$ for all adjacent bands are reasonably large);

(4) a conceptual simplicity which makes it possible to understand easily how separation varies as different experimental variables are changed.

These features of LSS separation will be further illustrated and discussed below.

It has been argued that "custom" (non-LSS) gradients are more appropriate for some samples. With such solvent programs it is possible to tailor the separation at individual points within the chromatogram to provide maximal resolution for difficult to separate band pairs. However, few samples require this approach in practice.

B. Retention time, band width and resolution in LSS gradient elution

Previous papers[2,5,6] have derived relationships for these separation parameters in LSS systems, with particular emphasis on liquid–solid (adsorption) LC. Here we shall generalize this treatment for all forms of LC, and in a following section focus on the special case of RP-GE.

(a) Retention time

As derived in Appendix I, the retention volume, V_g (ml), of a given band in an LSS separation is

$$V_g = (V_m/b) \log(2.3\, k_0 b + 1) + V_m \tag{3}$$

where V_m is the total volume of mobile phase contained within the column. Eqn. 3 has been verified experimentally for the special case of liquid–solid LC[13]. There is no reason to doubt its validity for other forms of LC, including reversed-phase separations.

The retention time, t_g (sec), is then given as V_g/F, where F is the flow-rate (ml/sec) of mobile phase through the column:

$$t_g = (t_0/b) \log(2.3\, k_0 b + 1) + t_0 \tag{3a}$$

Here, t_0 is the column "dead-time", equal to V_m/F.

It can be seen in eqn. 3a that the retention time, t_g, decreases as the gradient steepness, b, increases. This is similar to the case of isocratic elution, where retention times, t_R, decrease with increase in solvent strength. This analogy between b in gradient elution and solvent strength in isocratic elution becomes more quantitative if we rearrange eqn. 3a as follows:

$$(t_g - t_0)/t_0 = (1/b) \log(2.3\, k_0 b + 1)$$

For the case of later eluting bands (large k_0), we then have

$$(t_g - t_0)/t_0 \approx (1/b)\,[\log 2.3 + \log k_0 + \log b] \approx (\log k_0)/b \tag{3b}$$

The term on the left is analogous to k' in isocratic elution, and it is seen to be approximately proportional to $1/b$, for a given band (*i.e.* given value of k_0). The time

of separation, t_s, is equal to t_g for the last eluted compound, or to the time required to complete the gradient. In either case, as $t_s \approx (t_g - t_0)$, the separation time is proportional to $1/b$. Note that in isocratic elution the time of separation is proportional to $1 + k'$ for the last-eluted compound in the sample (*e.g.*, ref. 11), or approximately to k'. Thus, so far as the separation time, t_s, is concerned, $1/b$ in gradient elution corresponds to k' in isocratic elution.

Consider next the effect of beginning the gradient with a mobile phase of higher strength. This is illustrated in Fig. 3b and c, where the starting mobile phase consists of 30% and 40% methanol–water, respectively (compared with 20% in Fig. 3a). We have noted that the initial bands in a gradient elution separation are eluted more or less isocratically, so that bands 1–3 in these examples elute in a stronger solvent in going from Fig. 3a to 3c. The resulting changes in the separation of these bands reflect this increase in solvent strength, the retention times becoming shorter, bands narrower and taller, and resolution poorer. The elution of later bands, however, is less sensitive to the starting composition of the solvent program. While the retention times for these bands (6–8 in Figs. 3) are decreased by the time saved in starting the solvent program at a later point, these later eluting bands still elute at about the same mobile phase composition; see top scale of Fig. 3, *i.e.*:

Starting mobile phase (methanol–water) composition (% methanol)	*Inlet mobile phase (methanol–water) composition at t_g (% methanol)*		
	Band 6	*Band 7*	*Band 8*
20	58	67	76
30	59	68	77
40	62	69	77

The widths, detectability and resolution of these later bands are also essentially constant in Fig. 3a–c.

(b) Band width

The band width in gradient elution separation is the result of three more-or-less independent processes:

(1) the normal broadening of sample bands as they move through a column[11];

(2) a "band compression" phenomenon, which arises from the faster migration of the tail of bands in gradient elution, *versus* the equal migration of all parts of the band in isocratic elution[4];

(3) the instantaneous k' value of the band (k_f) as it leaves the column[14].

If we consider the width, σ_x, of sample bands on the column bed, at the time each band leaves the column, where σ_x is the standard deviation of the Gaussian distribution in length units (cm), then the plate number N of the column can be expressed as[15]

$$N = (L/\sigma_x)^2 \tag{4}$$

where L is the length of the column. The value of N in isocratic elution is generally assumed to be independent of k' (see discussion in refs. 4 and 5), which means that

σ_x will be constant for different bands, and for different mobile phases of similar viscosity. This in turn implies that σ_x will be constant for different bands in a given gradient elution separation, which is indeed the case. The value of σ_x is then given by eqn. 4.

Band compression in gradient elution has been discussed earlier[4]. The band width, σ_x, that would be obtained in isocratic elution is reduced in gradient elution by the factor G, where G can be calculated by numerical integration[4]. For LSS gradients, G is solely a function of the gradient parameter b, as shown in Fig. 5. Note that for intermediate values of b ($0.2 < b < 0.5$), G is roughly constant and equal to 0.8, which means that bands in gradient elution are compressed by about 20% in the usual case. The possibility of further compression of gradient elution bands at higher values of b, as for increasing detection sensitivity in trace analysis, is considered in a following section.

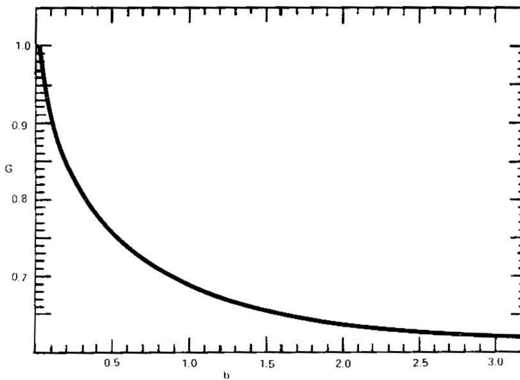


Fig. 5. Band compression factor, G, as a function of gradient steepness, b. Calculated by numerical integration of eqn. i-1.

The final width of a gradient elution band in time units, σ_t, is determined by the value of σ_x (the width of the band on the bed, just prior to elution), and the instantaneous k' value of the band as it leaves the column (k_f) (see the discussion in ref. 14). This final value of σ_t is then given[4,14] as

$$\sigma_t = (1 + k_f)\, \sigma_x\, G(t_0/L) \tag{5}$$

Similarly, as shown in Appendix I for LSS separations,

$$k_f = 1/(2.3b + 1/k_0) \tag{6}$$

As k_0 for most bands is large, eqn. 6 can be approximated by

$$k_f = 1/2.3b \tag{6a}$$

Finally, eqns. 4 and 5 can be combined and re-stated in time units:

$$N = G(1 + k_f)t_0/\sigma_t^2 \tag{7}$$

Combination of eqns. 6a and 7 then yields

$$\sigma_t = (2.3b + 1)Gt_0/2.3bN^{\frac{1}{2}} \tag{7a}$$

Experimental verification of eqn. 7a is afforded by one study involving liquid–solid gradient elution LC[4], as summarized in Table 1. The agreement found between calculated and experimental σ_t values is adequate, considering the various approximations that enter into eqn. 6a, and uncertainty in the values of b that can be estimated in ref. 4.

TABLE 1

COMPARISON OF EXPERIMENTAL AND CALCULATED VALUES OF AVERAGE BAND WIDTH IN GRADIENT ELUTION LIQUID–SOLID CHROMATOGRAPHY (WITH LSS PROGRAMS)

b*	N	G	σ_t/t_0	
			*Experimental***	*Calculated****
0.15	1090	0.93	0.10	0.11
0.27	920	0.90	0.09	0.08
0.52	1600	0.86	0.06	0.04

* Re-calculation of b (on basis of average $A_s = 10$ for the compounds studied; see ref. 4) gave 30% lower values than those reported in ref. 4.

** Data from ref. 4.

*** Eqn. 7a.

According to eqn. 7a, for LSS gradient programs the widths of eluted bands are predicted to be constant throughout the chromatogram. Using the more exact eqn. 6 as opposed to eqn. 6a, early eluting (small k_0) bands are predicted to have slightly reduced band widths compared with later bands. This pattern is apparent in the various calculated chromatograms in Fig. 3. For wider gradients (*e.g.*, 0–100% methanol–water) than shown in Fig. 3, most of the bands in the chromatogram would appear to have equal band widths.

In isocratic elution, band widths increase approximately in proportion to k' (more exactly, $1 + k'$), or in inverse proportion to solvent strength. In gradient elution (eqn. 7a), band width varies inversely as b. Thus, again we see an analogy between gradient steepness b in gradient elution and solvent strength or $1/k'$ in isocratic elution.

(c) Resolution

Resolution in LSS gradient elution has already been discussed in some detail[4,5] and only a practical summary will be repeated here, plus some updating for more recent developments in column technology. The analogies we have already drawn between b in gradient elution and $1/k'$ in isocratic elution will be found to apply to various aspects of resolution. Resolution, R_s, in gradient elution can be defined in much the same way as for isocratic elution (*e.g.*, ref. 11):

$$R_s = (t_2 - t_1)/2(\sigma_1 + \sigma_2) \tag{8}$$

Here, t_1 and t_2 refer to retention times, t_g, in gradient elution for adjacent bands 1 and 2, respectively; σ_1 and σ_2 are the corresponding band widths (σ_t values).

Resolution in gradient elution can also be described in terms of eqn. 1, which is analogous to the corresponding expression for isocratic elution (*e.g.*, ref. 11):

$$R_s = \frac{1}{4}(\alpha - 1)\, N^{\frac{1}{2}}\, [k'/(1 + k')]^2 = \frac{1}{4}(\alpha - 1)\,(NQ^2)^{\frac{1}{2}} \quad (9)$$

The quantity NQ^2 in eqn. 9 is referred to as the effective plate number of the column, and a similar definition is applicable for gradient elution; *i.e.*, in eqn. 1, $NQ^2 = N[\bar{k}/(1 + \bar{k})]^2$. Larger values of NQ^2 provide generally better resolution, other factors (*e.g.*, α) equal. The value of Q (and NQ^2) in LSS gradient elution tends to a constant, limiting value for later eluting bands[4], and this limiting value of Q is determined by the value of b for the solvent program. Q^2 as a function of b is summarized in Table 2, as calculated by numerical integration in ref. 4 of the fundamental gradient elution equation (eqn. i-1 in Appendix I).

TABLE 2

EFFECTIVE PLATES AND DETECTION SENSITIVITY IN LSS GRADIENT ELUTION AS A FUNCTION OF b

b	$\bar{k} = 1/1.3b$	Q^2*	$[\bar{k}/(1+\bar{k})]^2$**	s_g***	s_k§
0.1	7.7	0.79	0.78	0.2	0.1
0.2	3.8	0.63	0.63	0.4	0.2
0.4	1.9	0.45	0.43	0.6	0.3
0.6	1.3	0.33	0.32	0.8	0.4
1.0	0.8	0.20	0.19	1.0	0.6
1.5	0.5	0.12	0.12	1.2	0.7
2.5	0.3	0.06	0.06	1.4	0.8

* Calculated from ref. 4 by numerical integration of eqn. i-1.
** Calculated from the value of b and eqn. 10.
*** Eqns. 11a and 6a.
§ Eqn. iii-6.

If the quantity Q^2 from Table 2 is compared with $[\bar{k}/(1 + \bar{k})]^2$ (*cf.*, eqn. 9 and third column in Table 2), it is found that these two functions are approximately related if we assume

$$\bar{k} = 1/1.3b \quad (10)$$

That is, substitution of $\bar{k}$ from eqn. 10 for various values of b leads to values of $[\bar{k}/(1 + \bar{k})]^2$ in Table 2 which are almost identical with gradient elution Q^2 values for the same value of b. Thus, so far as resolution is concerned, we can regard the term $1/1.3b = \bar{k}$ as an average or effective value of k' for gradient elution.

As in isocratic elution there exists an optimal value of k', so in LSS gradient elution there exists an optimal value of $\bar{k}$ and b. In isocratic elution three experimental cases can be defined, for each of which the optimal k' value is different[11]:

(1) constant mobile phase velocity u, with column length L and pressure drop P varying; k' (optimal) = 2;

(2) constant L, with u and P variable; k' (optimal) = 3–6;

(3) constant P, with L and u variable; k' (optimal) = 2.5–3.

For cases (2) and (3) above, the optimal value of k' varies mainly with the particle size, d_p, of the column packing and the resulting value of the column parameter n ($0.3 \leq n \leq 0.6$, see ref. 11).

Case (1) is of limited interest in gradient elution. Case (2) is the commonest situation, and is discussed in detail in Appendix II. We can summarize by saying that there is an optimal value of b in gradient elution for this case, equal variously to 0.1–0.3 depending on the experimental conditions. An average, optimal value of b in gradient elution of 0.2 can generally be assumed. Note that this range in b values (0.1–0.3) is the same as can be calculated from eqn. 10 and the isocratic (optimal) k' values: $3 < k' < 6$. Again, we see a rather precise analogy between b in gradient elution and k' in isocratic separation. Extending the analogy, although we have not pursued this theoretically, the optimal value of b for case (3) should be 0.25–0.30.

Effective plates (NQ^2) and resolution in gradient elution can always be increased by a decrease in b, as shown in Table 2 and illustrated in Fig. 3a ($b = 0.2$) *vs.* Fig. 6 ($b = 0.8$). However, this results in an increase in separation time, which must be weighed against the alternative of simply slowing the flow-rate through the column while holding b constant (*i.e.*, increasing N rather than Q). The significance of the above optimal values of b is that for an increase in separation time t_s, it is preferable to hold b constant at about 0.2, and to decrease the flow-rate for an increase in N. This approach will generally yield maximum resolution.

(d) Detection sensitivity

Because the bands in gradient elution are generally narrower than those in isocratic elution, gradient elution offers a means of increasing the detection sensitivity

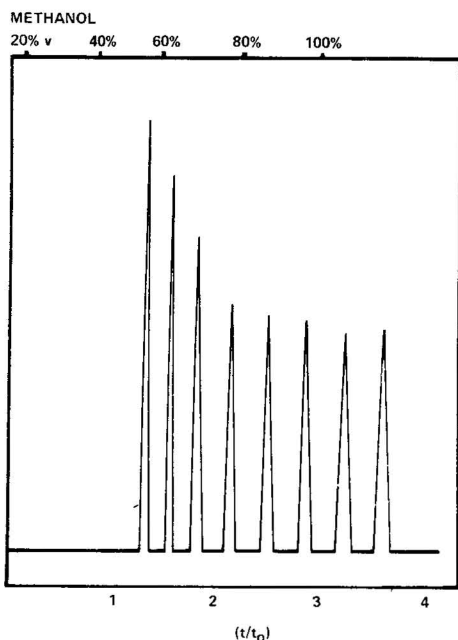


Fig. 6. Calculated gradient elution separation for sample and column of Fig. 1. Same conditions as for Fig. 3a, except $b = 0.8$. Chromatogram attenuated by a factor of 2×.

in applications such as trace analysis. In isocratic elution, the band width decreases and the detection sensitivity increases as k' is decreased (see discussion in ref. 16). We can define a sensitivity function s_k for isocratic elution, equal to the height of some band with $k' \neq 0$, relative to the height of a band eluted at t_0. As discussed in Appendix III,

$$s_k = 1/(1 + k') \tag{11}$$

Similarly, a sensitivity function s_g can be defined, equal to the height of a band in gradient elution, relative to the height of a band eluted isocratically from the same column at t_0. A comparison of s_g with s_k values then permits the increased detection sensitivity in gradient elution to be assessed.

As derived in Appendix III, s_g in gradient elution is given as

$$s_g = 1/G\,(1 + k_f) \tag{11a}$$

By comparing eqns. 11a and 11, we again see the analogy between k' values in isocratic elution and b values in gradient elution, as $k_f = 1/2.3b$ (eqn. 6a). A further comparison of s_g and s_k values is shown in Table 2, where s_g as a function of b is compared with s_k as a function of $b = 1/2.3\ k_f$. Thus the s_g values are for $0.3 < k_f < 8$. Because, for a given value of b in Table 2, s_g is generally larger than s_k, this means that the detection sensitivity in gradient elution is always better than the corresponding isocratic case, when the resolution or NQ^2 is the same ($k' = 1/1.3b$) for both separations.

In view of the band compression effect, it might be assumed that gradient elution bands can be "squeezed" down to any width desired, using a sufficiently steep gradient (large enough value of b). In practice, this is not the case, as seen in Table 2. Because G (Fig. 1) decreases slowly with b*, impractically large b values are required for significant reduction in band widths, relative to bands eluted isocratically at t_0. Thus, in Table 2, it is seen that a value $b = 2.5$ results in only a 40% increase in band height *versus* a band eluted at t_0 (*i.e.*, $s_g = 1.4$), while the entire gradient is compressed into a time 0.7 t_0 (*i.e.*, 1.7–1).

Gradients this steep (or steeper) are not practical with presently available equipment, and it should also be noted that resolution suffers greatly for b values greater than 1 ($Q^2 < 0.2$). Nevertheless, from Table 2 it appears that b values as large as 1.0 allow about a 1.3-fold increase in detection sensitivity in gradient elution, *versus* the case of $b = 0.2$ for optimal resolution. However, an increase of b to 1.0 also results in a 3-fold reduction in column efficiency, NQ^2.

Much larger increases in detection sensitivity can be achieved by gradient elution[16] in other ways. For example, very large sample volumes can be charged if the k_0 values of compounds of interest are fairly large. In this case, the bands of interest will be held initially at the column inlet, and the large sample volume will not appreciably widen the bands for these compounds when they are eventually eluted.

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* *E.g.*, for $b = 3.0$, $G = 0.6$ (only a 40% "squeezing" of the band).

(e) Separation selectivity

By separation selectivity we mean differences in the retention times of two compounds in gradient elution, which in turn implies an average value of α in eqn. 1 which is different from 1. By reference to eqn. 3a, it is apparent that differences in retention time can result from differences in k_0 or b for the two compounds in question. As discussed in the following section, b values for different compounds are usually of similar magnitude in a given gradient elution system, which means that we rely mainly on differences in k_0 to achieve separation selectivity. Differences in k_0 for two compounds can in turn only be achieved by changing the nature of the organic solvent in the RP-GE system, and this is the commonest approach where $\alpha - 1$ must be increased. Several studies have shown (*e.g.*, refs. 17–19 and especially 20) that significant changes in α can be achieved in this fashion. One example is shown below, for a change in α for isocratic RP separation[18] of various glycosides.

Band pair	α	
	37% acetonitrile–water	*45% dioxane–water*
Gitaloxigenin, gitoxin	1.07	2.7
Lanatoside B, lanatoside E	1.00	1.04
Gitaloxin, lanatoside A	1.02	1.25

Alternatively, it is possible to change α by changing the gradient steepness b or the rate of change of the volume fraction of the organic solvent, when the b values for two compounds in a given RP-GE system differ. This is discussed in greater detail in Appendix IV, which is based on the further discussion of mobile phase composition in RP-GE in the following section.

4. SOLVENT STRENGTH *VERSUS* COMPOSITION IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

Several studies have been reported on the variation of sample k' values in isocratic, reversed-phase LC, as a function of mobile phase composition (*e.g.*, refs. 10 and 17–34). For mobile phases consisting usually of water–methanol or water–acetonitrile mixtures, and a wide range in sample compounds, it is usually observed for a given system (a specific column and organic component of the binary solvent, *e.g.*, methanol) that sample k' values are related to the volume fraction, Φ_b, of organic solvent B in the mobile phase as

$$\log k' = \log k_w - S\,\Phi_b \qquad (12)$$

Here k_w refers to the isocratic k' value for pure water as mobile phase, and is usually an extrapolated value. Several studies (*e.g.*, refs. 21–25 and 27–32) have shown that eqn. 12 is valid within experimental error over wide limits in both k' and Φ_b. The coefficient S in eqn. 12 is seen to be related to the strength of pure solvent B as mobile phase, as larger values of S lead to a faster decrease in k' with increase in Φ_b. For a given reversed-phase system, *e.g.*, different mixtures of methanol–water as mobile

phase, and a given column, the parameter S is often observed to be roughly constant, even for solutes of varying molecular size and structure. The value of S when B is either methanol or acetonitrile is usually about 3, but varies (in different studies) from 2 to 4 for reasons that are not yet clear. As discussed below, S varies further as the solvent B is changed for less polar solvents such as ethanol or tetrahydrofuran, with S increasing as the polarity of B decreases.

A. Optimal gradients for reversed-phase LC

It will next be shown that LSS gradients in reversed-phase systems correspond to linear solvent composition gradients; *i.e.*, where the volume fraction Φ_b of the organic solvent B increases linearly with time:

$$\Phi_b = \Phi_0 + \Phi' t \quad (13)$$

Here, Φ_0 is the value of Φ_b at the beginning of the separation, and $\Phi' = d\Phi_b/dt$ is the rate of change of Φ_b with time (and is constant for a given separation). If eqns. 12 and 13 are combined, we obtain

$$\log k' = (\log k_w - S\Phi_0) - S\Phi' t \quad (13a)$$

This is of the same form as eqn. 2 (LSS gradient), provided that $\log k_w - S\Phi_0$ is set equal to $\log k_0$, and the factor $S\Phi'$ is equated to b/t_0. As we have argued that LSS gradients are generally optimal, it follows that linear gradients as in eqn. 13 are likewise optimal for reversed-phase gradient elution in LC.

(a) Exceptions to eqns. 12 and 13

Schoenmakers *et al.*[10] have noted that eqn. 12 is not strictly obeyed in reversed-phase systems studied by them, but instead plots of $\log k'$ *versus* Φ_b are slightly concave. Other studies[33,34] show a similar relationship between k' and Φ_b, and in extreme cases these plots actually pass through a minimum in the region of $\Phi_b \approx 0.9$. A more careful analysis of these effects and their impact on our preceding analysis is presented in Appendix V and also Part II[1]. To summarize that discussion, it appears that these deviations of experimental data from eqn. 12 have essentially no effect on the conclusions so far presented in this paper.

The data of ref. 31 clearly show that S increases regularly with molecular size in a homologous series of solutes. A similar increase in S with increasing size of the solute molecule is suggested for oligomeric series of polymers (*e.g.*, ref. 35). For homologous or oligomeric samples, use of a linear gradient (eqn. 13) is expected to provide poorer resolution of later bands, together with progressive narrowing of these bands. This can be corrected by maintaining an LSS gradient. As b and $S\Phi'$ must remain constant throughout an LSS gradient, if S increases (for polymeric samples), Φ' must decrease with time; *i.e.* a convex gradient. Poile[9] has argued that this is also true for the elution of polycyclic aromatic hydrocarbons, as their S and t_g values increase regularly with molecular size.

B. The strength, S, of other solvents B in reversed-phase LC

A number of studies have been reported which allow values of S to be derived for methanol as the organic solvent B in water–organic mixtures as the mobile phase. While S is usually fairly constant for a given study (and column), its values vary from one study to another by ± 1 unit, *i.e.*, $2 < S < 4$. It thus appears that S is not a constant which is characteristic of a given solvent B, but varies somewhat with other separation parameters. A few studies have compared k' values in reversed-phase systems for more than one solvent B, which in turn allows estimates of the variation of S as a function of the solvent B. Data from several such studies are summarized in Table 3. The resulting S values are in rough agreement for different solvents, and are averaged at the bottom of Table 3. Because of the as yet unexplained variability of S, for a given solvent B (as in Table 3), these "best" values of S in Table 3 must be considered to be approximate at most. There is a definite need for a definitive experimental study of S as a function of all probable variables that might affect it such as sample molecular structure, the solvent B, variations in the column packing (coverage, alkyl chain length, etc.) and separation temperature.

TABLE 3

SUMMARY OF SOLVENT STRENGTH (S) VALUES FOR DIFFERENT ORGANIC SOLVENTS B IN REVERSED-PHASE LC (25°)

Reference	*Solvent B*						
	Methanol	*Acetonitrile*	*Ethanol*	*Acetone*	*Dioxane*	*Isopropanol*	*Tetrahydrofuran*
18–29	2–4						
1	3.5	2.9					4.2
10	2.7		3.4			4.1	
17	(3)*	4.1					4.7
36		(2.5)*		3.4	3.5	4.2	4.4
"Best"	3.0	3.1	3.6	3.4	3.5	4.2	4.4

* Assumed value for calculation of S for other solvents (k' *versus* Φ_b data not provided).

(a) Optimal value of Φ' in reversed-phase LC systems

We have argued in an earlier section that the optimal value of b in reversed-phase LC should generally be about 0.2. In terms of eqn. 13a, we have seen that

$$\Phi' = b/S\,t_0 \qquad (14)$$

Values of $b = 0.2$ and S from Table 3 (for various solvents B) can now be inserted into eqn. 14 for calculation of the optimal gradient steepness (value of Φ') for maximum resolution (or NQ^2) per unit separation time. For example, with methanol–water solutions as mobile phase ($S = 3$), and a value of $t_0 = 1$ min, the optimal value of Φ' is 0.067, or a 6.7% increase in methanol concentration per minute for the gradient. Optimal values of Φ' for various values of t_0 and different solvents B are summarized in Table 4.

TABLE 4

OPTIMAL GRADIENT STEEPNESS (FOR MAXIMAL RESOLUTION) IN REVERSED-PHASE LC ($b = 0.2$)

Solvent B	Φ' (%v/min change in B)			
	$t_0 = 10$ sec	$t_0 = 30$ sec	$t_0 = 1$ min	$t_0 = 2$ min
Methanol	40	13	6.7	3.3
Acetonitrile	39	13	6.5	3.2
Acetone	35	12	5.9	3.0
Dioxane	34	11	5.7	2.8
Ethanol	33	11	5.6	2.8
Isopropanol	29	10	4.8	2.4
Tetrahydrofuran	27	9	4.5	2.3

5. MISCELLANEOUS OTHER CONSIDERATIONS

A. Design of isocratic separations on the basis of initial gradient elution separation

In some cases gradient elution is used as a "scouting" technique for unknown samples. An initial separation by reversed-phase gradient elution provides an immediate picture of the sample, with often adequate separation in the first attempt. However, it may then be desired to repeat the separation isocratically, for any of several reasons. In this case, it is useful to be able to estimate the correct solvent strength for the isocratic separation from the results of the initial gradient elution separation.

For isocratic elution, we require appropriate k' values for bands of interest. In this connection, eqn. 6a is useful, as it defines the k' value (k_f) of a band as it leaves the column (in the mobile phase leaving the column at the same time). For the optimal b value of 0.2, $k' = k_f$ in an isocratic separation is then $1/2.3 \cdot 0.2 = 2.2$. For a column of fixed length, an optimal value of k' in an isocratic separation is about 4 (see preceding section), so that a somewhat weaker solvent is required in isocratic elution than actually elutes a band of interest in gradient elution. In fact, we require k' (isocratic elution) to be increased about 2-fold (over gradient elution). From eqn. 2, for $b = 0.2$, this corresponds to mobile phase leaving the column at 1.5 t_0 prior to the elution of the band of interest (gradient elution). As it is generally more convenient to consider the composition of mobile phase entering the column, the isocratic separation will require a solvent corresponding to that entering the gradient column at a time $t_g - 2.5\ t_0$, where t_g is the retention time of the band of interest in gradient elution.

B. Calculation of column plate number in gradient elution

It is apparent to most workers that the column plate number, N, cannot be determined from a gradient separation by means of the usual relationship for isocratic elution:

$$N = (t_R/\sigma_t)^2 \tag{15}$$

Application of eqn. 15 to a gradient chromatogram grossly overestimates N in most cases, because of the lower value of k_f at the time of elution of each band. Nevertheless, eqn. 15 is periodically used in the literature (*e.g.*, refs. 37–40) for this purpose. In many of these cases, the shortcomings of the resulting N values are acknowledged, but then conclusions based on these "apparent" N values are drawn. The use of such N values (eqn. 15) in gradient elution is not recommended, as even relative N values can vary with t_g by large factors. An alternative is to use the correct expression for N for gradient elution, which is derivable from eqn. 7a:

$$N = \left[\frac{(2.3\,b + 1)\,G\,t_0}{2.3\,b\,\sigma_t}\right]^2 \quad (15a)$$

Eqn. 15a allows the calculation of the plate number from a gradient elution separation. The gradient steepness parameter, b, must be known, but it can be calculated from eqn. 14. The compression factor, G, can in turn be estimated from Fig. 5, where G is plotted as a function of b. Finally, the experimental quantities t_0 and σ_t are determined from the chromatogram.

6. CONCLUSIONS

The reciprocal of the gradient steepness parameter, b, increases for shallower gradients (smaller values of Φ'). The quantity $1/b$ plays an almost identical role in gradient elution as the parameter k' does in isocratic separation. Thus separation in gradient elution can be understood and controlled very much as in isocratic elution. Part II[1] summarizes a number of specific rules in this connection, and provides experimental illustration and verification of various conclusions presented in preceding sections of this paper. The various similarities that exist between isocratic and gradient elution when we substitute $1/b$ from the latter for k' in the former are summarized in Table 5.

For reversed-phase gradient elution, the parameter b is defined by the experimental variables Φ' (%/min change in concentration of B in the mobile phase), the solvent strength S of the pure solvent B and the column dead-time t_0 (min) as

$$b = 100\,\Phi'\,S\,t_0 \quad (16)$$

TABLE 5

ANALOGIES BETWEEN ISOCRATIC AND GRADIENT ELUTION WHEN k' (ISOCRATIC) IS SUBSTITUTED FOR $1/b$ (GRADIENT)

Variable	*Depends on*		*Equation*
	isocratic elution	*gradient elution*	
Calculated capacity factor	$(t_R - t_0)/t_0 = k'$	$(t_g - t_0)t_0 \approx (\log k_0)/b$	Eqn. 3b
Separation time, t_s	$1 + k'$	$(\log k_0)/b$	Eqn. 3b
Resolution, R_s	$k/(1 + k')$	$\bar{k}/(1 + \bar{k})$, where $\bar{k} = 1/1.3b$	Eqn. 10
Detection sensitivity	$1/(1 + k')$	$1/G(1 + k_f)$, where $k_f = 1/2.3b$	Eqn. 6a
Maximal resolution	Optimal k' varies with column, but is usually $3 < k' < 6$	Optimal b varies with column, but is usually given by $3 < \bar{k} < 6$, where $\bar{k} = 1/1.3b$	Appendix II

Values of S for various organic solvents are summarized in Table 3. Resolution increases as b decreases, but so does the separation time. For a given separation time, there is an optimal value of b which is generally close to 0.2, but which can vary by ± 0.1 with little effect on resolution. Resolution can be increased, while holding b constant, by either decreasing F or increasing L (see Part II[1] for a detailed discussion). Table 4 summarizes optimal gradient rates, Φ', for varying t_0 and different organic solvents B that are used in the water–B gradient. The parameter Φ' should be held constant during a gradient elution run, which means that the solvent gradient in reversed-phase gradient elution should be linear.

The detection sensitivity increases as b is increased. For a 5-fold increase in b (from the normally optimal value of 0.2–1.0), the detection sensitivity will increase 3-fold, but with a 3-fold loss in resolution (see Table 2).

7. SYMBOLS

A, B	refers to solvents A (water) and B (organic) used in the gradient program.
b	coefficient in eqn. 2; larger values of b correspond to steeper gradients (eqn. 14).
F	flow-rate of mobile phase through column (ml/sec).
G	band compression factor[4]; corresponds to fractional reduction in width of band as a result of compression; see Fig. 5 for G as a function of b.
k'	capacity factor[11].
k_a	actual value of k' for a band in gradient elution at some time t during elution; determined by the composition of the mobile phase at the same point in the column where the band is located.
k_f	value of k' for a band at the moment it leaves the column in gradient elution; equal to k_a at time $t = t_g$.
k_i	value of k' for a given compound if injected at the column inlet at any time t after a gradient elution separation begins; equal to k' in an isocratic separation, using mobile phase of the same composition as that entering the column at time t in gradient elution.
k_0	value of k' for a compound at the beginning of gradient elution ($k_0 = k_a$ or k_i at $t = 0$); equal to k' in an isocratic separation with same mobile phase used to begin gradient elution.
k_w	a value of k' for a given compound, with water as mobile phase; an extrapolated value based on eqn. 12.
$\bar{k}$	a (roughly) average value of k_a during gradient elution (Fig. 3); $\bar{k}$ determines R_s as a function of b.
L	column length (cm).
LSS	linear solvent strength.
n	column parameter as defined by eqn. ii-3 (Appendix II).
N	column plate number, defined by eqn. 15 for isocratic elution or eqn. 15a for gradient elution.
P	pressure drop across column (p.s.i.).
Q	column efficiency factor, equal to $k'/(1 + k')$ for isocratic elution and $\bar{k}/(1 + \bar{k})$ for gradient elution.
RP-GE	reversed-phase gradient elution.

R_s resolution factor; eqns. 1, 8 and 9 for gradient elution. (see also ref. 11 for analogous expression in isocratic elution).

S solvent strength parameter; see eqn. 12.

s_g, s_k sensitivity parameters, equal to peak height in gradient (g) or isocratic (k) elution relative to height of t_0 band in isocratic elution (see eqns. iii-6 and iii-7, Appendix III.

t time after sample injection and start of gradient (sec).

t_g retention time in gradient elution; time (sec) from sample injection to elution of band maximum from column.

t_0 column dead time[11] (sec).

t_1, t_2 values of t_g for adjacent bands 1 and 2 (eqn. 8).

t_R retention time (sec) in isocratic elution[11].

t_s time to complete separation after sample injection; equal variously to time from beginning to end of gradient program, or time to elute last sample band (sec).

u velocity (cm/sec) of mobile phase in column.

V volume of mobile phase eluted from column after sample injection and start of gradient (ml).

V_g retention volume of band in gradient elution (ml); analogous to retention volume in isocratic separation[11].

V_m total volume of mobile phase contained within column (ml).

V_t instantaneous, corrected retention volume for a band at some time during gradient elution; equal to k' V_m (ml).

x fractional distance a band has migrated along column at some time t (see Fig. 4).

α separation factor for two adjacent bands; defined for isocratic elution as in ref. 11.

σ_0 σ_t value in isocratic elution for $k' = 0$ (sec).

σ_t width of eluted band in either isocratic or gradient elution; standard deviation of Gaussian band (sec).

σ_1, σ_2 σ_t values for adjacent bands (1) and (2).

σ_x width of band on column, just prior to elution (cm).

Φ_b volume fraction of organic solvent B in water–organic mixture.

Φ' rate of change of Φ_b with time: $d\Phi_b/dt$ (sec^{-1}) (can also be expressed as %/min).

Φ_0 initial value of Φ at $t = 0$.

Φ_f final value of Φ at end of gradient.

8. APPENDIX I

Derivation of retention time, t_g, and capacity factor at time of elution, k_f, in LSS gradient elution

From the fundamental equation of gradient elution (*e.g.*, ref. 2), we have for the retention volume, V_g

$$\int_0^{V_a} (dV/V_a) = 1 \qquad \text{(i-1)}$$

Here dV refers to the passage of a differential volume of mobile phase through the band center, and V_a is the instantaneous, corrected retention volume (corresponding to k_a):

$$V_a = k_a V_m \tag{i-2}$$

The quantity V_m refers to the dead-volume of the column. Eqn. 2 can be re-stated as

$$\log k_i = \log k_0 - b(V/V_m) \tag{i-3}$$

and k_i and k_a then become equivalent in terms of eqn. i-1. Substitution of k_i from eqn. i-3 for k_a in eqn. i-2, followed by substitution of V_a from eqn. i-2 into eqn. i-1, then gives

$$\int_0^{V_g} \frac{10^{bV/V_m}\, dV}{k_0 V_m} = 1 \tag{i-4}$$

Integration of eqn. i-4 then gives eqn. 3.

The value of k' for a band at the time of elution (k_f) is obtained by substituting the corrected retention volume ($V_g - V_m$) from eqn. 2 into eqn. 1a:

$$\log k_f = \log k_0 - \log (2.3k_0 b + 1) \tag{i-5}$$

Eqn. i-5 can then be rearranged into eqn. 6.

9. APPENDIX II

Optimal value of b in gradient elution for column length L, fixed and variable separation time

We desire to maximize the effective plate number, NQ^2, for a column of fixed length L, for various separation times t_s (and corresponding variation in mobile phase flow-rate, F, and velocity, u) by optimizing the gradient parameter b. For a given value of t_s, the k_i value of the last eluted band (k_z) is given from eqn. 2 as

$$\log k_z = \log k_0 - b(t_s/t_0)$$

which rearranges to

$$b = (\log k_0/k_z)\,(t_0/t_s)$$

As $u = L/t_0$,

$$b = [(\log k_0/k_z)\, L/t_s]u \tag{ii-1}$$

As the bracketed factor on the right is constant for a given separation, b is seen to vary inversely as u, *i.e.*,

$$b = C/u \tag{ii-2}$$

Now we can approximate the plate height H in most cases[11,16,17] by

$$H = D\,u^n \tag{ii-3}$$

Combining eqns. ii-2 and ii-3, we obtain

$$H = (DC^n)/b^n \tag{ii-4}$$

As the effective plate number of the column can be represented as

$$NQ^2 = (L/H)\,[\bar{k}/(1 + \bar{k})]^2$$

H from eqn. ii-4 and $\bar{k}$ from eqn. 10 can be substituted into the latter relationship to yield

$$NQ^2 = (L/DC^n)\,b^n\,[1/(1.3b + 1)]^2 \tag{ii-5}$$

The optimal value of b, for maximal NQ^2 in eqn. ii-5, is obtained in the usual fashion by differentiation to give

$$b\,(\text{optimal}) = n/(2.6 - 1.3n) \tag{ii-6}$$

The column parameter n for pellicular packings is 0.4, which yields an optimal value of $b = 0.24$. For large-particle separations with porous packings, n is usually 0.4–0.6, corresponding to $b = 0.24$–0.33. For small-particle (*e.g.*, $d_p \leqslant 10\ \mu m$) separations, n is usually smaller, as discussed in ref. 17. However, eqn. ii-3 is also less reliable, so a different approach to discussing the optimal value of b is indicated.

For the case of small particles, we can describe $H = hd_p$ as function of the reduced plate height, h and reduced velocity, $\nu = u\,d_p/D_m$ (see discussion in ref. 17), where D_m is the sample diffusion coefficient in the mobile phase. h is then given as a function of ν for well packed columns of porous particles[17]:

$$h = 2/\nu + \nu^{0.33} + 0.05\,\nu \tag{ii-7}$$

Finally, NQ^2 can be calculated as above, substituting the latter expression for H for eqn. ii-3:

$$NQ^2 = (Lh/d_p)\,[\bar{k}/(1 + \bar{k})]^2 \tag{ii-8}$$

As an example, consider the case discussed in Part II[1] of a 25-cm column of 5-μm particles, a 5–95% gradient of methanol–water and a separation time of 20 min. Assume the calculation for the case of $b = 0.1$. Let the initial value of Φ_b (0.05) be given as Φ_0, and the final value ($\Phi_b = 0.95$) be Φ_f. Combination of eqns. 2 and 12 then yields

$$b = S\,(\Phi_f - \Phi_0)/(t_s/t_0) \tag{ii-9}$$

Inserting the above experimental conditions, we have

$$0.1 = 3.0\,(0.95 - 0.05)/(20 \cdot 60/t_0)$$

from which $t_0 = 44$ sec. The velocity, u, of the mobile phase is then $L/t_0 = 25/44 = 0.57$ cm/sec. We can calculate the reduced velocity, ν, as

$$\nu = u\, d_p / D_m \tag{ii-10}$$

assuming that the solute diffusion coefficient is $3 \cdot 10^{-5}$ (see ref. 17): $\nu = 0.57 \cdot 0.0005 / 0.00003 = 9.5$. From eqn. ii-7, this yields a value of $h = 2.78$. Similarly, from eqn. 10, $\bar{k} = 1/1.3 \cdot 0.1 = 7.7$. Inserting the latter values into eqn. ii-8, we obtain

$$NQ^2 = (25 \cdot 2.78/0.0005)\,(7.7/8.7)^2 = 10{,}900$$

It is found for a particular set of conditions that characteristic plots of NQ^2 *versus* ν result, as illustrated for a 10-μm particle, reversed-phase separation at room temperature, shown in Fig. 7 for a 25-cm column. In this case, as the separation time increases the optimal value of b shifts from a value of about 0.2 to lower values, *e.g.*, 0.08 for $t_s = 75$ min.

The curves in Fig. 7 do not change as L is varied, other than to give a proportionate increase in NQ^2 and t_s for an increase in L. For smaller particles, *e.g.*, 5-μm diameter packings, the same basic curves of Fig. 7 apply, except that the NQ^2 values are proportionately greater and t_s proportionately smaller, *e.g.*, 3.7, 11.2 and 37.5 min in Fig. 7.

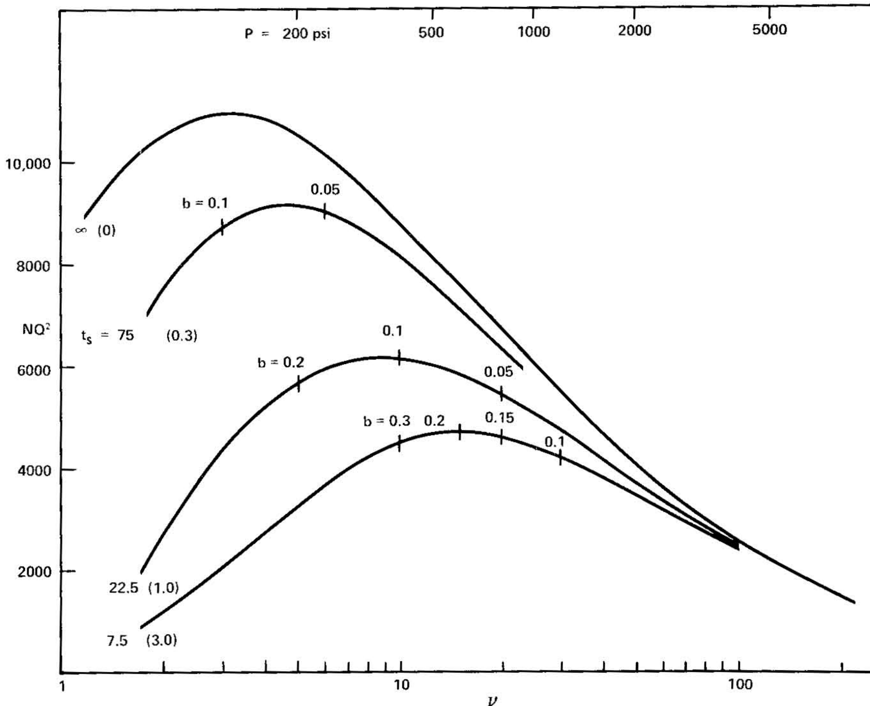

Fig. 7. Variation of NQ^2 with reduced velocity, ν, and b in gradient elution. Assumes 0–100% methanol–water gradient, 25-cm column of 10-μm porous particles for reversed-phase separation of molecular weight 300 sample at toom temperature. Values in parentheses refer to the product $b\nu$.

10. APPENDIX III

Derivations of sensitivity equations for isocratic and gradient elution

The column plate number, N, can be defined in terms of retention time, t_R, and band width, σ_t (standard deviation of Gaussian curve, in time units):

$$N = (t_R/\sigma_t)^2 \quad \text{(iii-1)}$$

The retention time is in turn given as

$$t_R = (1 + k')\, t_0 \quad \text{(iii-2)}$$

From eqns. iii-1 and iii-2, we obtain

$$\sigma_t = (1 + k')\, t_0/N^{\frac{1}{2}} \quad \text{(iii-3)}$$

and the band width, σ_0, of a non-retained band ($k' = 0$) is then

$$\sigma_0 = t_0/N^{\frac{1}{2}} \quad \text{(iii-4)}$$

We can define a sensitivity function, s_k:

$$s_k = \sigma_0/\sigma_t \quad \text{(iii-5)}$$

As peak height and sensitivity are inversely proportional to band width, s_k represents the relative height (and sensitivity) of a band eluted with some value of k', relative to a band eluted at t_0. From eqns. iii-3–iii-5, we have

$$s_k = 1/(1 + k') \quad \text{(iii-6)}$$

In a similar manner, we can define a sensitivity function, s_g, for gradient elution bands from eqn. 7a:

$$\begin{aligned} s_g &= \sigma_0/\sigma_{t,t} \\ &= 2.3\, b/(2.3\, b + 1)G \\ &= 1/G(1 + k_f) \end{aligned} \quad \text{(iii-7)}$$

11. APPENDIX IV

Changes in separation selectivity with change in Φ' (reversed-phase LC)

Workers who have used gradient elution have often observed that for a given mobile phase A–B, a change in the gradient steepness, Φ', can lead to changes in band position within the final chromatogram. We shall show that this can occur for any two bands whose b or S values are different for that LC system.

Assume two compounds i and j with k_0 values of 100 (i) and 465 (j), S values of 3 (i) and 4.5 (j), and $t_0 = 1$ min. The parameter b is defined by eqn. 14 ($= \Phi'\, S\, t_0$),

and can be calculated for various values of Φ'. If we assume Φ' varies as below, we can then calculate t_g for each band (i and j) from eqn. 3a:

Band	t_g *(min)*		
	$\Phi' = 0.033$	$\Phi' = 0.067$	$\Phi' = 0.133$
i	14.8	9.4	5.9
j	15.7	9.4	5.7

It can be seen that the two bands have equal t_g values (no separation) for $\Phi' = 0.067$, whereas the bands are separable at either higher or lower values of Φ' (steeper or shallower gradients). Or, as the gradient steepness is increased from $\Phi' = 0.033$, band i is eluted first, but then band j overtakes band i for $\Phi' > 0.067$, and the band positions are reversed.

12. APPENDIX V

Deviations from eqn. 12 and their effect on separations in reversed-phase gradient elution

Schoenmakers *et al.*[10] carried out a detailed study of the variation of k' with Φ_b for 16 solutes and three different solvents B (methanol, ethanol and propanol). On the basis of these data they suggest that eqn. 12 is generally invalid, and k' as a function of Φ_b is instead given by a fitting function of the form

$$\log k' = A\,\Phi_b^2 + B\,\Phi_b + C \qquad \text{(v-1)}$$

Examples of resulting plots of log k' *versus* Φ_b for two such solutes from ref. 10 are redrawn in Fig. 8, and the curvature of these data is readily apparent. While the actual experimental data obtained by Schoenmakers *et al.*[10] are not reported for verification of these curves, other data[1,32–34] show similar non-linearity of these log k' *versus* Φ_b plots. However, the effect of this curvature of the plots in Fig. 8 (and elsewhere) on the resulting gradient elution separation is less pronounced than might be expected. The reason is that for an optimal gradient (LSS program with $b \approx 0.2$), migration of a band along the column occurs mainly when $2 < k_a < 8$ (*e.g.*, Fig. 4a, for $x \geqslant 0.2$). If we compare plots such as those in Fig. 8 with corresponding "best fit" linear curves over this range $^{2-8}$ in k' values, we find resulting deviations of experimental k' values from the linear curve of no more than about 3%. We can show this better by taking the average A, B and C values from ref. 10 for methanol–water as mobile phase and the 16 solutes studied. This average k' *versus* Φ_b plot is then given as

$$\log k' = 1.88\,\Phi_b^2 - 5.24\,\Phi_b + 3.06 \qquad \text{(v-2)}$$

The best linear fit to this curve can be calculated from the tangent at $x \approx 0.5$, or $k' \approx 4$. This yields a value of $S = 3.02$ ($\Phi_b = 0.59$). We can now calculate the various separation parameters for a model case, first assuming that eqn. 12 is correct ($S = 3.02$), then repeating the calculation using the true curve (eqn. v-2). The latter cal-

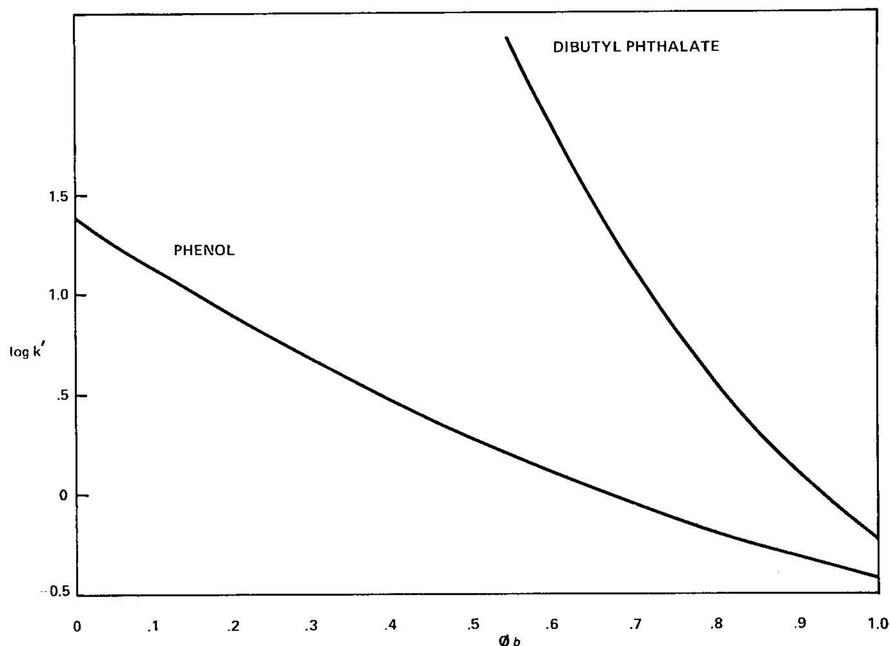

Fig. 8. Calculated plots of log k' *versus* Φ_b for phenol and dibutyl phthalate from data in ref. 10. Mobile phase, methanol-water.

culation requires numerical integration of eqn. i-1, while the former calculations are summarized in the text (eqns. 3a, 6a and 10). Calculated data for these two cases are as follows [assuming $b = 0.2$ at $k' = 4$, $k_0 = 64$ (eqn. 12) or 135 (eqn. v-2)]:

Parameter	*Value*	
	Eqn. 12	*Eqn. v-2*
$\frac{t_g - t_0}{t_0}$	7.4	7.5
k_f	2.1	2.0
$\bar{k}$	3.7	3.4

These deviations in absolute separation parameters due to the failure of eqn. 12 are even less significant in practical applications of the theory described in the main text. There we are concerned with relative, rather than absolute, changes as a function of separation parameters. Considerable cancellation of errors introduced by using eqn. 12 then results.

13. ACKNOWLEDGEMENTS

We express our appreciation for useful discussions and modifications of this manuscript provided by Dr. P. J. Schoenmakers and associates, Dr. J. J. Kirkland, Dr. B. L. Karger, Dr. D. L. Saunders and Dr. H. Engelhardt.

14. SUMMARY

A general theory of separation is presented for gradient elution with reversed-phase systems. Expressions for retention, resolution, band width and other separation parameters are presented as a function of experimental variables. So-called "linear solvent strength" gradients are assumed.

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GRADIENT ELUTION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

II. PRACTICAL APPLICATION TO REVERSED-PHASE SYSTEMS

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1. INTRODUCTION

In Part I[1] we presented a practical theory of gradient elution separation, with emphasis on reversed-phase systems and high-performance liquid chromatography (LC). In this paper we continue this examination of reversed-phase gradient elution (RP-GE) liquid chromatography. Here we shall focus on three separate areas: (1) the nature of the relationship between isocratic capacity factor (k') values and mobile phase composition in reversed-phase LC, (2) an experimental verification of the various conclusions reached in the theoretical study[1] and (3) a practical summary of preferred separation conditions for achieving various goals in RP-GE applications.

2. EXPERIMENTAL

A. Equipment

The LC system consisted of two Waters Model 6000A LC pumps and a Model 660 solvent programmer (Waters Assoc., Milford, Mass., U.S.A.). Samples were injected using an injection valve fitted with a 10-μl sample loop (Model 7120, Rheodyne, Berkeley, Calif., U.S.A.). A 2.0-μm pre-filter (Model 7302, Rheodyne) was placed between the injection valve and the chromatographic column. Unless stated otherwise, separations were performed at ambient temperature with a 23 $\times$ 0.46 cm column with 6-μm C_{18} packing (Zorbax ODS, DuPont, Wilmington, Del., U.S.A.). A DuPont Model 901 254-nm fixed-wavelength detector was used with an *x–y* recorder (Model 2000, Houston Instruments, Austin, Texas, U.S.A.).

Additional columns were used for the study shown in Table 3: Merck C_8 (25 $\times$ 0.46 cm, 10-μm particles, EM Labs., Elmsford, N.Y., U.S.A.), Waters C_{18} (30 $\times$ 0.39 cm, 10-μm particles, Waters Assoc.), Hypersil C_{18} (16 $\times$ 0.5 cm, 5–7-μm particles, Shandon Southern Instruments, Selwickley, Pa., U.S.A.), DuPont C_{18} (23 $\times$ 0.46 cm, 6-μm particles; one column prepared with octadecyldimethylchlorosilane and one with octadecyltrichlorosilane, DuPont), DuPont C_8 (23 $\times$ 0.46 cm, 6-μm particles).

B. Chemicals

Mobile phases consisted of HPLC-grade methanol (MeOH), acetonitrile (AN) or tetrahydrofuran (THF) (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.) mixed with high-purity water from a Milli-Q unit (Millipore, Bedford, Mass., U.S.A.). For gradient elution, the organic–water mobile phases were mixed 5% organic–95% water for initial solvent A and 95% organic–5% water for final solvent B. This premixing plus helium sparging eliminated solvent de-gassing upon mixing during gradient formation. Thus 0–100% gradients were really 5–95% organic; actual mobile phase compositions are referred to throughout this paper.

C. Procedure

(a) Isocratic

Isocratic data were gathered using either the gradient former to mix the isocratic mobile phase or, for log k' *versus* Φ_b data, precise-composition mobile phases were mixed independently of the gradient device, and one pump was used in the isocratic mode in order to eliminate any bias introduced by the gradient system.

(b) Gradient

All gradients were 0–100% B in 20 min except as noted; b values were changed by changing the mobile phase flow-rates in convenient increments (0.5–1.0 ml/min). The column was regenerated after a gradient run to the initial mobile phase conditions by running a 10-min reverse gradient at 2.0 ml/min followed by at least 10 min of isocratic operation at initial mobile phase conditions before injection of the next sample. All separations were performed in duplicate.

3. SOLVENT EFFECTS IN ISOCRATIC REVERSED-PHASE LC

A brief review and discussion of this topic was presented in Part I[1]. There we concluded, to a first approximation, that solute k' values in reversed-phase systems can be represented by the general equation

$$\log k' = \log k_w - S\,\Phi_b \quad (1)$$

Here, for a given sample component or solute X, and a given organic solvent B (*e.g.*, methanol), k' is the isocratic capacity factor for some volume fraction Φ_b of B in the water–organic mobile phase. The quantity k_w is an extrapolated value of k' for $\Phi_b = 0$. Thus, if eqn. 1 holds exactly over the range $0 \leqslant \Phi_b \leqslant 1$, k_w is the k' value of the compound X in pure water as mobile phase. The solvent-strength parameter S is determined by the organic solvent B; *e.g.*, $S \approx 3$ for methanol and $S \approx 4$ for tetrahydrofuran as solvent. S is known to vary somewhat (for a given organic solvent B) for different reversed-phase columns. It was assumed in Part I[1] that S does not vary significantly with solute molecular structure in the case of most samples. However, it was noted that there is a general increase in S with increasing solute molecular weight for samples composed of either a homologous series or certain oligomers.

The validity of eqn. 1 as discussed above forms the basis of:

(1) the general treatment of Part I[1] for RP-GE separation;

(2) the experimental test of that general treatment presented in a later section of this paper;

(3) the practical summary of RP-GE separation found in the final section of this paper.

We feel that eqn. 1 can be accepted as a reliable first approximation for reversed phase systems, without serious reservation. Nevertheless, there is value in further examining this relationship, for two reasons: firstly, to allay any questions concerning the value of eqn. 1 for interpreting RP-GE systems, and secondly, to gain insight into the importance of second-order effects (deviations from eqn. 1, variation of S with solute structure, etc.) in special cases. The present study does not allow final answers to the questions we shall raise, but is intended in part as a stimulus to further experimental investigation.

There are four main points of discussion with respect to the validity of eqn. 1:

(1) deviations from linearity of log k' *versus* Φ_b plots in reversed-phase systems;

(2) variation of S (other variables fixed) with change in solute structure;

(3) variation of S with different reversed-phase packings;

(4) variation of S for different solvents B.

A. Linearity of log k' versus Φ_b

As reviewed in Part I[1], most previous experimental studies have shown essentially linear plots of log k' *versus* Φ_b in reversed-phase systems. A few studies suggest curvature of such plots, particularly in the region of $\Phi_b \approx 1$. The most detailed of these studies is that of Schoenmakers *et al.*[2], who summarized data on a large number of solutes and three organic solvents B (methanol, ethanol and propanol). They found that their plots of log k' *versus* Φ_b are better represented by the quadratic expression

$$\log k' = A\,\Phi_b^2 + B\,\Phi_b + C \tag{2}$$

If data are averaged for the various solutes studied by Schoenmakers *et al.*[2], for methanol and propanol as organic solvents, the resulting plots of log k' *versus* Φ_b shown in Fig. 1 are obtained. The curvature of these plots is readily apparent, with the data for propanol showing a distinct minimum in k' in the region of $\Phi_b = 0.9$. If the curves are extrapolated according to eqn. 2 beyond $\Phi_b = 1$ (dashed lines), it is seen that a minimum in k' results for methanol also (for $\Phi_b \approx 1.4$).

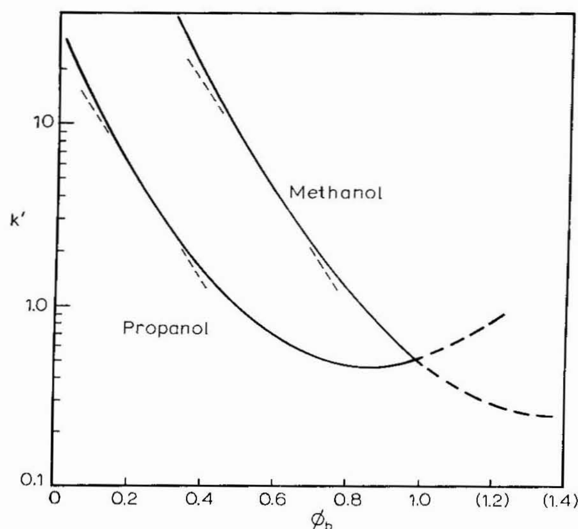

Fig. 1. Averaged data of ref. 2 for variation of log k' *versus* Φ_b for *n*-propanol and methanol as organic solvents B. *n*-Propanol, calculated from eqn. 2 with $A = 2.42$, $B = -4.19$ and $C = 1.50$; methanol, same, with $A = 1.88$, $B = -5.24$ and $C = 3.06$.

In Part I[1] we noted that migration of bands in RP-GE separation occurs mainly during the time (or mobile phase composition) when k' is between 2 and 10. It can be seen in Fig. 1 that plots of log k' over this region (light, dashed lines) hardly differ from the experimental plots (heavy lines). In particular, the minimum in k' found near $\Phi_b = 0.9$ for propanol (and other less polar solvents) is of little practical significance in RP-GE separation.

For $k' > 1$, it is not obvious that significant non-linearity of log k' *versus* Φ_b plots ever occurs. When a limited number of data points are collected (for different values of Φ_b), small errors in one or more points can easily suggest curvature in log k' *versus* Φ_b plots, even where such curvature is non-existent. That this may be true to some extent in the study of Schoenmakers *et al.*[2] is suggested by examination of values of C from eqn. 2 for the same solute and different solvents B (methanol, ethanol, propanol). If eqn. 2 were a reliable fitting function, values of C for a given solute should be constant, as C is then the value of k' for the solute in question in pure water as the mobile phase, regardless of the solvent B considered. In fact, the data of Schoenmakers *et al.*[2] show differences in C for a given solute, in some cases by as much as 1.74 units (corresponding to differences in k' of 55-fold for that solute with water as mobile phase).

We feel that a better test of eqns. 1 or 2 for a given set of reversed-phase data is provided by superimposing plots of log k' *versus* Φ_b for different solutes. This can be achieved by shifting such plots horizontally until they roughly coincide, then examining the resulting plot for possible curvature. An example is provided in Fig. 2, for the reversed-phase system water (A)–methanol (B) studied by us (data of Table 1). The solid straight line through these data suggests no curvature of these plots (within experimental error). The average plot from ref. 2, based on eqn. 2, is superimposed on these same data as the dashed curve. While the similarity of the two plots is apparent, the slight curvature noted in ref. 2 appears to be absent in our own data for the same reversed-phase system.

TABLE 1

ISOCRATIC k' VALUES FOR DIFFERENT METHANOL–WATER MIXTURES AS MOBILE PHASE USING A DUPONT ZORBAX-ODS COLUMN

*Solute**	*Methanol (%) in methanol–water*			
	70	*60*	*50*	*45*
Phenol (◒) (0.175)		0.74	1.38	1.94
p-Nitrophenol (▼) (0.146)		0.72	1.97	2.86
p-Cresol (●) (0.083)	0.67	1.45	2.87	4.49
2,5-Xylenol (■) (−0.004)	1.18	2.66	6.14	9.65
Methyl benzoate (✹) (−0.037)	1.75	3.54		11.1
Anisole (○) (−0.053)	1.87	3.88		12.1
Benzene (▽) (−0.073)	1.90	5.26		
Phenetole (□) (−0.108)	2.87	6.08		
Toluene (○) (−0.135)	3.36	7.94		

* Symbols refer to experimental points in Fig. 2; numbers in parentheses refer to shift in Φ_b of plots in Fig. 2; *e.g.*, data for phenol (0.175) are plotted at Φ_b values of 0.775, 0.675 and 0.625, respectively.

Finally, even if log k' *versus* Φ_b plots are actually curvilinear for some reversed-phase systems (*e.g.*, as in ref. 2), the effect of such non-linearity on RP-GE separation is minor (see Appendix V in Part I[1]).

Further study of the validity of eqn. 1 in reversed-phase systems is needed, with particular reference to the linearity of log k' *versus* Φ_b plots. Apart from the

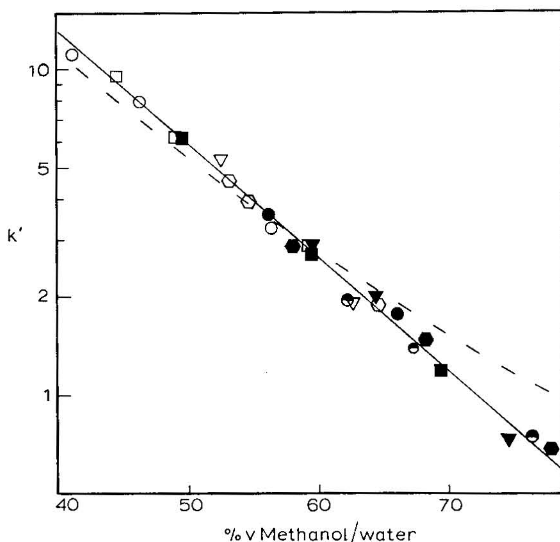

Fig. 2. Dependence of log k' on Φ_b for methanol-water as mobile phase; data of Table 1. Data shifted horizontally to obtain best fit to solid (linear) curve. Methanol curve of Fig. 1 (from ref. 2) similarly shifted and plotted as dashed curve. Experimental points defined in Table 1.

superposition technique described in Fig. 1, emphasis should be given to certain experimental considerations when collecting k' data for such purposes:

(a) complete equilibration of column and mobile phase before collecting data;

(b) verification that k' is not a function of solute concentration, expecially when $k' > 5$;

(c) constancy of the temperature of the column and incoming mobile phase during collection of k' data;

(d) use of column packings that exhibit full coverage of the silica surface by the bonded-phase.

(e) determination of the possible error in t_0 and its effect on reported k' values.

B. Variation of S with solute structure

Few studies have been concerned with the dependence of S on the molecular structure of the solute. A total of 17 solutes were investigated by Schoenmakers *et al.*[2], with the resulting S values (methanol as solvent B) shown in Table 2. Average S values from several columns (see the following section) and a number of different solutes studied by us are also summarized in Table 2. There is no obvious correlation of S with solute structure that appears from these data. Furthermore, for these representative solutes the average variation of S for a given column (and organic solvent B) is only of the order of $\pm$ 10–20%. That is, for typical samples little variation in S among the constituents of the sample is to be expected.

The situation is somewhat different in the case of solutes that form part of a homologous series. S values derived from the study of Tanaka and Thornton[3] are plotted for various homologous series of solutes (methanol–water as mobile phase) in Fig. 3. Here, a strong dependence of S on the alkyl carbon number, n, of the solute is

TABLE 2

VALUES OF S AS A FUNCTION OF SOLUTE STRUCTURE

Methanol–water solutions as mobile phase, ambient temperature.

Solute	S	
	*Ref. 2**	*Data in Table 3***
Phenol	1.7	2.6
Acetophenone	2.0	3.2
Benzene	2.1	2.7
Toluene	2.6	3.4
Ethyl benzene	3.2	
Diethyl phthalate	2.6	
Dibutyl phthalate	4.0	
Benzophenone	2.7	
Aniline	1.8	
N-Methylaniline	2.2	
N,N-Dimethylaniline	2.4	
Quinoline	2.2	
Benzyl alcohol	1.8	
2,4-Xylenol	2.3	
2-Cresol	2.1	
3-Cresol	2.1	
Benzaldehyde		2.9
Nitrobenzene		2.9
Methyl benzoate		3.6
Anisole		3.0
Fluorobenzene		3.0
Average	2.4 ± 0.6	3.0 ± 0.3

* Calculated from ref. 2 for $k' = 1.4$.
** Average values.

clearly evident. The slopes of these various plots for different homologous series are seen to be roughly constant (0.4 unit per methylene group). Extrapolation of the plots in Fig. 3 to $n = 0$ for the n-alkane and alkylbenzene series suggests that the addition of a phenyl group to a solute molecule increases S by about 0.8 unit, or much less per aromatic carbon (0.1 unit) than per aliphatic carbon (0.4 unit).

C. Variation of S among different reversed-phase packings

We have earlier expressed concern over the variability of S values among different reversed-phase packings (and columns). Table 3 summarizes data collected by us for nine different solutes and five different columns. The absolute values of S in Table 3 are found to vary as much for a given solute among the five columns as for a given column among the nine solutes. The effect of the column on S could be corrected for, however, by normalizing S values for each column. This was accomplished by dividing each S value by the average value of S for a given column. The resulting normalized S values for a given solute were then found to remain relatively constant among the five columns (average coefficient of variation in $S = 4\%$).

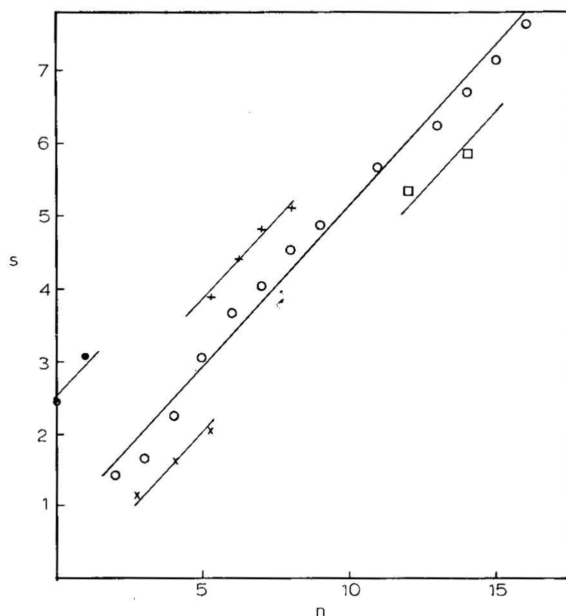

Fig. 3. Dependence of S on alkyl carbon number, n, in homologous series of solutes (from ref. 3). ●, Alkyl benzenes; +, n-alkanes; ○, carboxylic acids; ×, n-alkanols; □, dicarboxylic acids.

TABLE 3

VALUES OF S FOR SELECTED SOLUTES ON FIVE DIFFERENT REVERSED-PHASE COLUMNS

Water–methanol as mobile phase, ambient temperature.

Solute	*S for indicated columns*				*Relative S value*§§
	Waters C_{18}*	*Shandon* C_{18}**	*DuPont* C_{18}***	*DuPont* C_8§	
Phenol	2.21	2.52	2.35, 2.97	3.13	0.87 ± 0.06
Benzaldehyde	2.52	2.72	2.92, 3.07	3.08	0.95 ± 0.03
Acetophenone	2.82	3.04	3.08, 3.63	3.39	1.06 ± 0.03
Nitrobenzene	2.61	2.78	2.79, 3.18	3.16	0.96 ± 0.01
Methyl benzoate	3.17	3.46	3.44, 3.82	3.78	1.18 ± 0.02
Anisole	2.61	2.93	2.90, 3.29	3.28	1.00 ± 0.01
Fluorobenzene	2.70	3.07	2.90, 3.27	3.28	1.01 ± 0.02
Benzene	2.32	2.66	2.58, 2.94	3.02	0.90 ± 0.02
Toluene	2.90	3.24	3.13, 3.52	3.56	1.13 ± 0.06
Average	2.65	2.94	2.90, 3.29	3.29	(1.00)

* Monochlorosilane plus additional silanization ("capping").

** Trichlorosilane plus additional silanization.

*** Trichlorosilane (first column), monochlorosilane (second column), no additional silanization.

§ Monochlorosilane, no additional silanization.

§§ Average S value for given solute, relative to S for all columns.

D. Variation of S for different organic solvents B

Tables 4 and 5 summarize isocratic k' values as a function of mobile phase composition for two additional binary mixtures: acetonitrile–water and tetrahydrofuran–water. Values of S for these various solutes are also tabulated. Apart from a general increase in S for tetrahydrofuran, and a decrease in S for acetonitrile, these data follow the same pattern as for the methanol data in Table 1. They add little to our general understanding of the dependence of S on solute structure.

TABLE 4

ISOCRATIC k' VALUES FOR DIFFERENT TETRAHYDROFURAN–WATER MIXTURES AS MOBILE PHASE USING A DUPONT ZORBAX-ODS COLUMN AT AMBIENT TEMPERATURE

Solute	k'				S
	55% THF	*50% THF*	*45% THF*	*40% THF*	
Phenol			0.79	1.27	4.1
p-Nitrophenol			0.99	1.80	5.2
p-Cresol			1.00	1.76	4.9
2,5-Xylenol	0.69	1.05	1.55	2.79	4.0
Methyl benzoate	0.76	0.93	1.31	2.11	3.0
Anisole	0.92	1.35	1.87	3.09	3.4
Benzene	1.17	1.76	2.41	4.00	3.5
Phenetole	1.17	1.80	2.61	4.69	3.9
Toluene	1.49	2.33	3.39	6.08	4.0
Butyl benzoate	1.45	2.53	3.84		4.2
Anthracene	1.65	2.87	4.76		4.6
Benzanthracene	1.87	3.49	6.19		5.2
Average					4.2 ± 0.6

TABLE 5

ISOCRATIC k' VALUES FOR DIFFERENT ACETONITRILE–WATER MIXTURES AS MOBILE PHASE USING A DUPONT ZORBAX-ODS COLUMN AT AMBIENT TEMPERATURE

Solute	k'						S
	80% AN	*70% AN*	*60% AN*	*50% AN*	*40% AN*	*30% AN*	
p-Nitrophenol				0.61	1.39	4.12	3.6
Phenol				0.64	1.18	3.24	2.7
p-Cresol				0.98	1.99	10.0	3.1
2,5-Xylenol			0.95	1.68	3.71	14.5	3.0
Methyl benzoate			1.57	2.66	5.68		2.8
Anisole			1.64	2.80	6.00		2.8
Benzene			1.78	3.10	6.42		2.2
Phenetole			2.37	4.46	10.4		3.1
Toluene	0.95	1.57	2.78	5.13			2.5
Butyl benzoate	1.57	2.85	5.58				3.0
Anthracene	2.71	5.03	10.4				2.9
Average							2.9 ± 0.4

4. RETENTION, BAND WIDTH AND RESOLUTION IN LSS-GE

A. Retention time

As was discussed in Part I[1], eqn. 3*, and thus eqn. 3a*, has been experimentally verified in another study[4]. The present study provides further confirmation of eqn. 3* for RP-GE. For convenience in calculation, eqn. 3a* is modified to read

$$t_g = (t_0/b) \log (2.3\ k_0 b + 1) + t_0 + t_d \tag{3}$$

Here, t_d is the delay time of the system corresponding to the time from initiation of the gradient until a change in mobile phase composition is observed at the head of the column. In our case, $t_d = 2.0$ ml/F (F = flow-rate), and is accounted for by the volumes of the pulse dampener, pre-column connecting tubing, injection valve and filter. Eqn. 3 assumes that solutes do not move along the column during t_d. There is, in fact, little or no migration during t_d except for compounds which elute close to t_0 (*e.g.*, uracil).

The compounds listed in Table 6 elute over the range of the AN–water gradient. The b values were calculated for each compound (eqn. 14*) and the k_0 values are extrapolated from individual log k' *versus* Φ_b curves. The experimental and calculated retention times agree well (coefficient of variation = 0.6%), confirming the validity of eqn. 3. Here, the importance of using individual b or S values is shown from the last column in Table 6, where use of average b values for the retention calculation results in a coefficient of variation significantly greater than for the individual compounds and, in this case, a prediction of retention order which is incorrect.

TABLE 6

PREDICTION OF RETENTION IN RP-GE SEPARATION

5–95% AN–water; t_0 = 2.15 min, t_d = 2.0 min, t_s = 20 min, F = 1.0 ml/min.

Solute	b*	k_0**	t_g *(min)*		
			Exptl.	*Calc.****	*Calc.*§
p-Cresol	0.30	24	13.2	13.1	13.5
Benzene	0.27	59	16.5	16.7	16.4
Phenetole	0.31	134	17.7	17.9	19.1
Toluene	0.24	63	18.1	18.1	16.6
Butyl benzoate	0.27	180	20.5	20.5	20.00

* b value calculated for each solute.

** k_0 extrapolated to 5% water from log k' *versus* Φ_b curve.

*** Calculated from eqn. 3 using individual b values; coefficient of variation for deviation from experimental values = 0.6%.

§ Calculated from eqn. 3 using average b = 0.28 for AN; coefficient of variation = 4.3%.

B. Initial mobile phase concentration

The effect of varying the initial mobile phase composition, Φ_0 (value of Φ for mobile phase entering the column at time $t = 0$), is illustrated in Fig. 4A–E and in

* All Figure, Table and equation numbers followed by an asterisk are taken from Part I[1].

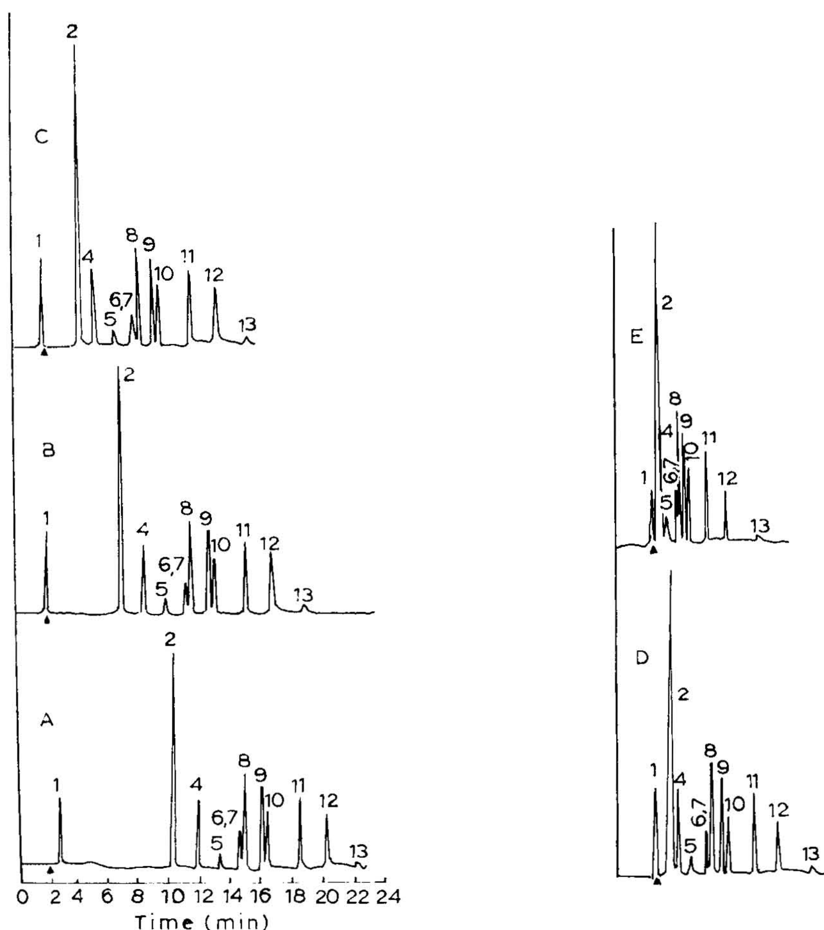

Fig. 4. Influence of initial mobile phase composition on gradient chromatogram. Solutes: 1, uracil; 2, phenol; 3, *p*-nitrophenol; 4, *p*-cresol; 5, 2,5-xylenol; 6, anisole; 7, methyl benzoate; 8, benzene; 9, phenetole; 10, toluene; 11, anthracene; 12, butyl benzoate; 13, benzanthracene. All gradients: $\Phi_f = 95\%$ AN–water, $b = 0.28$, $F = 1$ ml/min. ▲ = start of gradient at head of column (t_d). (A) $\Phi_0 = 5\%$ AN, $t_s = 20$ min; (B) $\Phi_0 = 26\%$ AN, $t_s = 16$ min; (C) $\Phi_0 = 46\%$ AN, $t_s = 12$ min; (D) $\Phi_0 = 64\%$ AN, $t_s = 8$ min; (E) $\Phi_0 = 79\%$ AN, $t_s = 4$ min.

Table 7. As was discussed in Part I[1], only the initial part of a gradient elution chromatogram is affected by a change in Φ_0. An increase in Φ_0 generally leads to poorer resolution and higher bands for initially eluted compounds that elute near t_0. This effect is obvious in Fig. 4 and is quantified in Table 7. In the latter, we have tabulated values of Φ_g for each solute in each separation in Fig. 4, where Φ_g is the value of Φ at the column inlet at the time t_g of elution of the given band, *i.e.*,

$$\Phi_g = \Phi_0 + \Phi' t_g \tag{4}$$

It can be seen that, in most cases, a given band elutes at a characteristic value of Φ_g, until Φ_0 is increased to the point where it is similar in value to Φ_g. As Φ' is constant

TABLE 7

EFFECT OF INITIAL MOBILE PHASE COMPOSITION ON RETENTION AND DETECTABILITY

All gradients: Φ_f = 95% AN–water, b = 0.28.

Solute	Φ_0									
	0.05		*0.26*		*0.46*		*0.64*		*0.79*	
	Φ_g*	*Detectability***	Φ_g*	*Detectability***	Φ_g*	*Detectability***	Φ_g*	*Detectability***	Φ_g*	*Detectability***
Phenol	0.54	127	0.56	148	0.60		0.71	—	0.83	—
p-Nitrophenol	0.63	39	0.64	40	0.63	43	0.73	51	0.84	—
Phenetole	0.86	48	0.87	48	0.88	49	0.89	55	0.92	66
Toluene	0.89	30	0.89	30	0.90	30	0.91	34	0.94	42
Anthracene	0.95	44	0.95	42	0.95	43	0.95	46	0.95	52

* From eqn. 4.

** Peak height, arbitrary units.

for the various separations in Fig. 4, this effectively means that a given solute band is eluted by the same composition of mobile phase, provided that $\Phi_0 \ll \Phi_g$.

As Φ_0 has no effect on the separation or resolution of later eluting bands, provided that $\Phi_0 \leqslant \Phi_g$, in practice the largest possible value of Φ_0 should be selected. This in turn minimizes the separation time. For example, t_s for the separation in Fig. 4A can be reduced significantly by changing Φ_0 to the conditions shown in Fig. 4C, while maintaining adequate resolution.

C. Band width

The band width in RP-GE is predictable by eqn. 7a* of ref. 1. The validity of this equation for RP-GE is shown in Table 8 for several compounds in an AN–water gradient. One can see that it makes little difference whether individual or average b values are used to calculate σ_t, with either method giving predictions in agreement with experimental values. The data in Table 8 show that the band width is relatively constant throughout the RP chromatogram (coefficient of variation = 10%), whereas under isocratic conditions the band width increases in proportion to $k' + 1$.

D. Resolution

For maximal resolution R_s, the discussion in Part I[1] predicts that b = 0.2 is roughly optimal. More precisely, for t_s = 20 min and the 5-μm particles as used in this study, Appendix II in Part I[1] predicts that b = 0.1 is optimal. The chromatograms in Figs. 5–7 show the effect of varying b while holding the separation time (and column length, L) constant. These examples provide a rough confirmation for an intermediate value of $b \approx$ 0.1–0.2 being preferred, so far as resolution is concerned.

Another (more precise) measure of R_s as a function of b (or $\bar{k}$) is provided by the peak capacity, PC, equal here to the difference in retention times for the first- and

TABLE 8

COMPARISON OF EXPERIMENTAL AND THEORETICAL BAND WIDTHS IN REVERSED-PHASE GRADIENT ELUTION

5–95% AN, t_0 = 129 sec, F = 1 ml/min.

Solute	σ_t *(sec)*				b[§]	G[§§]	N $(\times 10^{-4})$[§§§]
	Gradient elution			*Isocratic elution*[***]			
	Exptl.	*Calc.*[*]	*Calc.*[**]				
p-Cresol	2.5	3.5	3.6	2.4	0.30	0.81	0.79
Benzene	2.4	2.5	2.4	3.0	0.27	0.82	1.77
Phenetole	2.4	2.3	2.4	3.8	0.31	0.81	1.67
Toluene	2.4	2.6	2.4	4.1	0.24	0.83	1.79
Butyl benzoate	3.0	2.7	2.6	8.6	0.27	0.82	1.46

[*] From eqn. 7a* in Part. I[1], using individual b values; coefficient of variation = 12%, calc. *vs.* exptl.

[**] From eqn. 7a* in Part I[1], using average value of b = 0.28.

[***] Isocratic value, 64% AN, F = 1 ml/min.

[§] Calculated for each compound from eqn. 7a* in Part I[1].

[§§] From Fig. 5* in Part I[1].

[§§§] Isocratic N value.

last-eluted compounds in a given sample, divided by average band widths. As N and b are changed (*e.g.*, Figs. 5–7), PC should vary as NQ^2. In Fig. 8 experimental values of PC are plotted against b valves from Figs. 5–7, and the theoretical plot of NQ^2 *versus* b is superimposed on these data (calculated as described in Appendix II in Part I[1]). The data follow the theoretical plot reasonably closely, and confirm a maximal resolution in the range of $0.05 < b < 0.2$. Within this range of b values, there is little change in PC or NQ^2 with b.

Visual examination of the chromatograms in Fig. 5 suggests a maximal resolution of this sample for b = 0.28 (Fig. 5B), rather than for lower values of b. This is the result of selectivity changes which accompany variation in b, and is not an atypical result (*i.e.*, better separation for a slightly non-optimal value of b). Similar observations concerning the separation of Figs. 6 and 7 can also be drawn.

The separations in Figs. 5–7 and the data plot in Fig. 8 provide general confirmation of an optimal value of b in these cases of about 0.1. However, even more important is the finding that (as predicted) NQ^2 is not very sensitive to changes in b (with parallel changes in F, as in Figs. 5–7), when the separation time is held constant. Similarly, small differences in NQ^2 can be overshadowed by changes in α with variation in b. Finally, it should not be overlooked that larger values of b give greater detection sensitivity (see next section).

E. Detection sensitivity

Eqn. 11a* predicts an increased detection sensitivity as b increases. The data in Table 9 illustrate this effect. To increase the detection sensitivity in the case of a fixed $t_$ (as in this study), b is increased by lowering the flow-rate. Table 9 indicates that s_g and peak height increase by about 3-fold from b = 0.07 to b = 0.56. This is

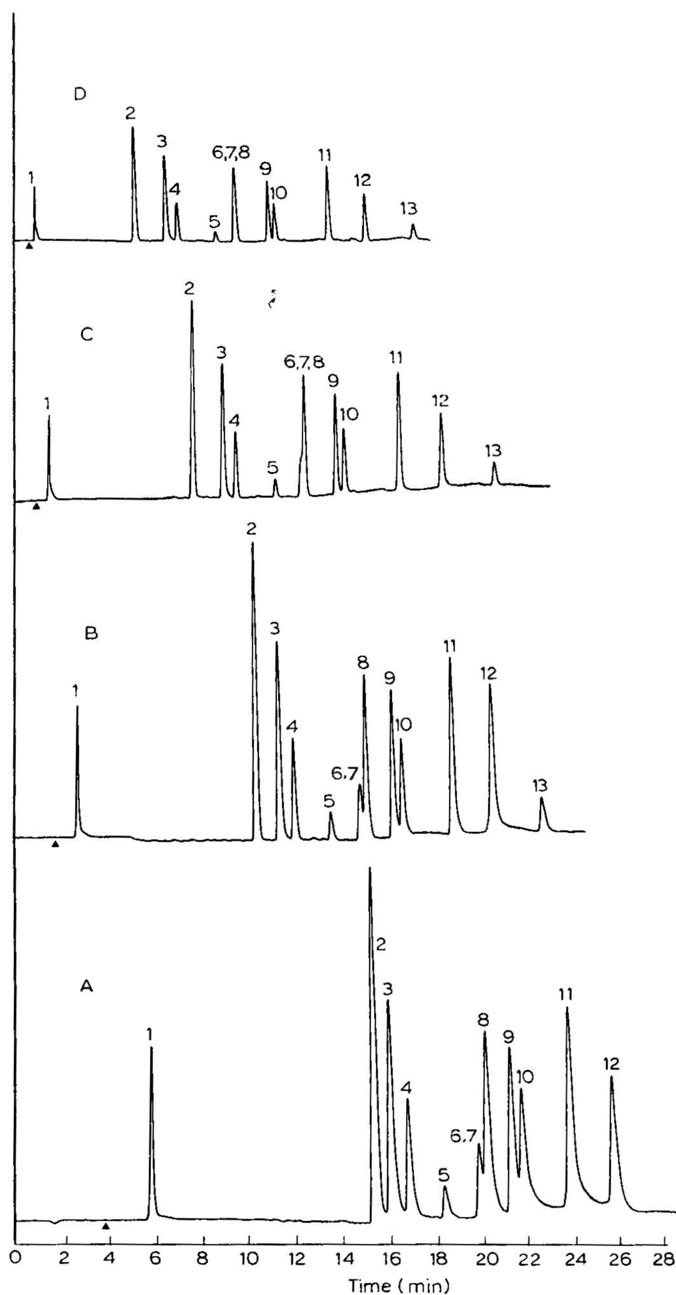

Fig. 5. Influence of b on chromatographic parameters with AN mobile phase. Solutes as in Fig. 4. All gradients: 5–95% AN–water; t_s = 20 min; ▲ = t_d. (A) b = 0.56, F = 0.5 ml/min; (B) b = 0.28, F = 1.0 ml/min; (C) b = 0.14, F = 2.0 ml/min; (D) b = 0.07, F = 4.0 ml/min.

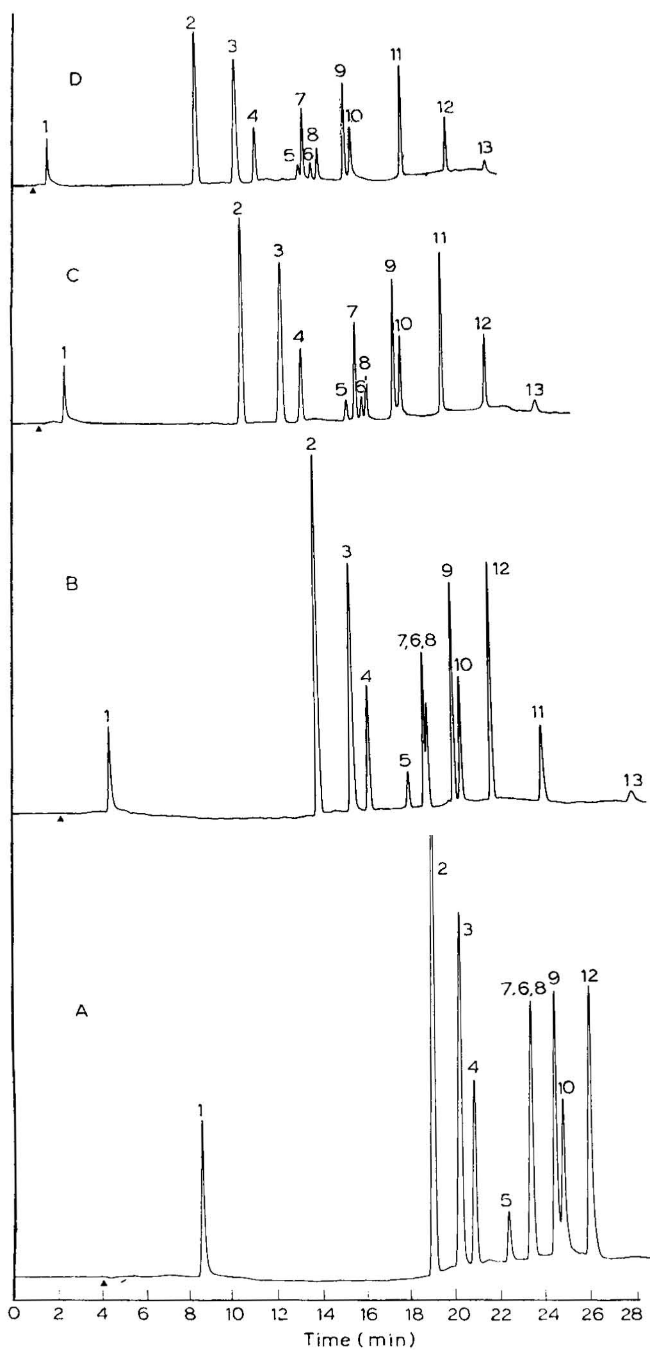

Fig. 6. Influence of b on chromatographic parameters with MeOH mobile phase. Solutes as in Fig. 4. All gradients: 5–95% MeOH–water; t_s = 20 min; ▲, t_a. (A) b = 0.68, F = 0.5 ml/min; (B) b = 0.34, F = 1.0 ml/min; (C) b = 0.17, F = 2.0 ml/min; (D) b = 0.11, F = 3.0 ml/min.

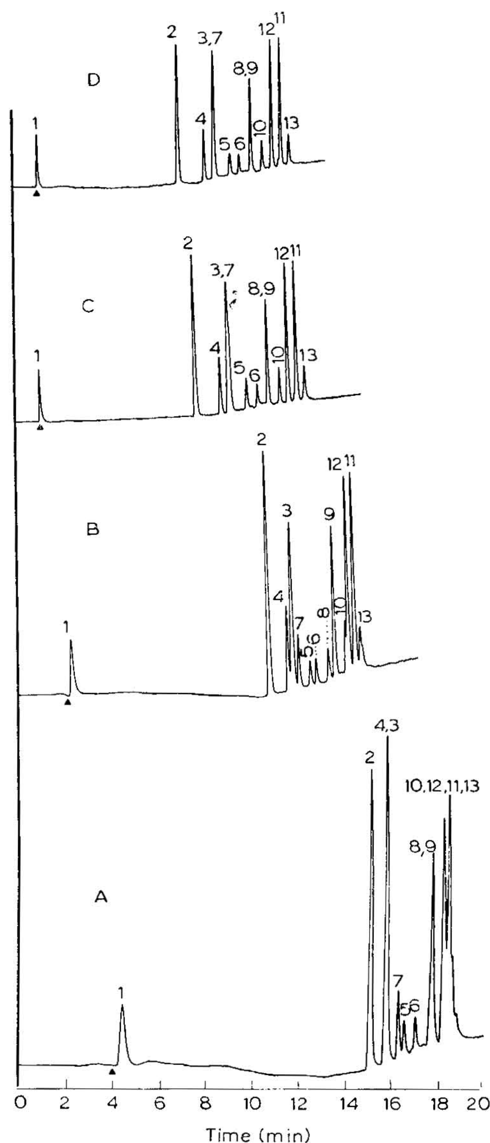

Fig. 7. Influence of b on chromatographic parameters with THF mobile phase. Solutes as in Fig. 4. All gradients: 5–95% THF–water; t_s = 20 min; ▲ = t_d. (A) b = 0.81, F = 0.5 ml/min; (B) b = 0.41, F = 1.0 ml/min; (C) b = 0.20, F = 2.0 ml/min; (D) b = 0.17, F = 2.5 ml/min.

visually apparent for AN as solvent by comparing Fig. 5A (b = 0.56) with Fig. 5D (b = 0.07). Similarly, we can compare Table 9 with Fig. 6A and D for MeOH or Fig. 7A and D for THF. Thus, as predicted in eqn. 11a*, we achieve a predictable increase in detection sensitivity in GE by increasing b. We must, of course, keep in mind that this simultaneously decreases R_s.

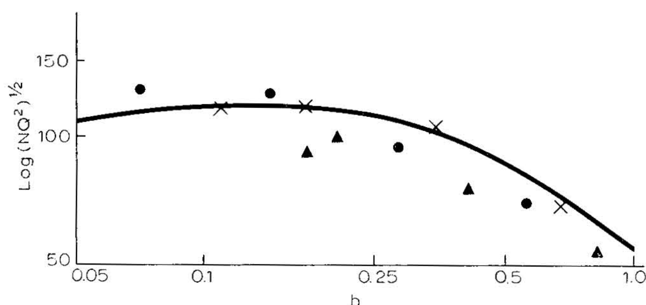

Fig. 8. Resolution as a function of b. Solid line, theoretical curve; individual points, experimental data for AN (●), MeOH (×) and THF (▲) shifted vertically for superposition onto theoretical curve.

F. Separation selectivity

As was discussed in Part I[1], we can change the selectivity (or resolution) of a given solute pair by changing α. The two options for GE are to change the type of mobile phase or the slope (b value) for the gradient. Let us take as an illustration the solute pair benzene–phenetole. Assume we chose for our initial separation the optimal THF–water gradient shown in Fig. 7C ($b = 0.2$). Under these conditions (THF as mobile phase) we see that the two solutes are completely merged into one peak. In a case such as this where we have essentially no separation under "ideal" conditions, it is preferable to try a mobile phase with different solvent properties. If we chose either MeOH or AN and ran an optimal ($b = 0.2$) LSS gradient we observe (Figs. 5C and 6C) baseline resolution for benzene and phenetole.

If, on the other hand, because of some other restriction, we need to use THF–water as the mobile phase, we might try to separate the two solutes by changing the b value of the gradient. This possibility is based on the (normally) small

TABLE 9

COMPARISON OF EXPERIMENTAL AND THEORETICAL DETECTION SENSITIVITY IN REVERSED-PHASE GRADIENT ELUTION

Mobile phase	*b*	S_g*	
		*Exptl.***	*Calc.****
AN–water	0.07	0.15 ± 0.03	0.15
	0.14	0.23 ± 0.05	0.28
	0.28	0.35 ± 0.07	0.48
	0.56	0.41 ± 0.10	0.76
MeOH–water	0.11	–	0.23
	0.68	–	0.85
THF–water	0.18	–	0.34
	0.81	–	0.92

* Average value for *p*-cresol, phenetole, toluene and butyl benzoate.
** s_g (exptl.) = h_2/h_1, where h_1 = peak height at t_0 and h_2 = GE peak height, ± 1σ.
*** s_g (calc.) = $2.3b/(1 + 2.3b)G$.

differences in S values for various solutes in a given mobile phase. The net result of these small variations in S is that b can be changed slightly in order to improve the separation of the two solutes. In the case of benzene–phenetole, we can increase b (or reduce $\bar{k}$) by reducing the flow-rate to 1.0 ml/min (with t_s constant). Observe that benzene and phenetole now have baseline resolution (Fig. 7B) (in this case reducing F also increases N). However, it is usually much easier to obtain the necessary resolution by changing the type of mobile phase (*e.g.*, from THF to MeOH) than by adjusting the b value with the same mobile phase. Tanaka *et al.*[5] have recently investigated the influence of organic modifiers on solvent selectivity in isocratic RP-LC. Their findings emphasize the possibility of improving separation in RP by change in organic solvent.

5. MISCELLANEOUS CONSIDERATIONS

A. Design of isocratic separations

In the discussion in Part I[1], it was predicted that preferred isocratic elution conditions ($k' = 4$) can be obtained by using the mobile phase composition corresponding to the gradient mobile phase at the head of the column at $t_g - 2.5\, t_0$. Taking t_d into account, we then require the mobile phase at $t_g - 2.5t_0 - t_d$. The data in Table 10 show that the experimental values obtained from the log k' *versus* Φ_b curves are in agreement with predicted values for $k' = 4$ (coefficient of variation = 2%).

TABLE 10

PREDICTION OF ISOCRATIC CONDITIONS FROM REVERSED-PHASE GRADIENT ELUTION

Mobile phase, acetonitrile–water.

Solute	t_g* *(min)*	*Isocratic mobile phase***	
		*Exptl.****	*Calc.*§
p-Cresol	13.2	0.30	0.31
Benzene	16.5	0.47	0.46
Phenetole	17.7	0.52	0.52
Toluene	18.1	0.54	0.53
Butyl benzoate	20.5	0.65	0.64

* 5–95% AN, $b = 0.28$, $t_0 = 2.15$ min, $t_d = 2.0$ min.
** Φ_b for $k' = 4$.
*** From log k' *vs.* Φ_b plots.
§ Mobile phase at head of column at $t_g - 2.5\, t_0 - t_d$, coefficient of variation = 2%, calc. *vs.* exptl.

Thus GE greatly simplifies the optimization of isocratic conditions. Instead of an "educated guess" of isocratic conditions followed by trial-and-error optimization, we can run a single LSS-GE separation at optimal b and predict the desired isocratic elution conditions within a few percent.

B. Calculation of column plate number in GE

Eqn. 15a* was derived in Part I[1] to allow for correct estimates of N with GE. Table 11 compares N values under isocratic conditions with N values for GE for five

TABLE 11

CALCULATION OF COLUMN EFFICIENCY IN REVERSED-PHASE GRADIENT ELUTION

Isocratic: 64% AN, $F = 1$ ml/min. Gradient: 5–95% AN, $F = 1$ ml/min, $b = 0.28$.

Solute	*N* ($\times 10^{-3}$)			
	*Isocratic**	*Gradient*		
		*Correct***	*Correct****	*Incorrect*§
p-Cresol	8	13	14	100
Benzene	18	16	15	170
Phenetole	17	13	15	190
Toluene	18	19	15	200
Butyl benzoate	15	10	10	260
Average	15	14	14	180

* Proper application of eqn. 15* in Part I[1].
** Proper application of eqn. 15a* using individual *b* values, coefficient of variation = 25%.
*** Proper application of eqn. 15a* using average $b = 0.28$, coefficient of variation = 28%.
§ Improper application of eqn. 15* to compute *N* for gradient elution.

solutes. The two sets of data correlate well when one considers that manual measurements of *N* are only precise to about 10%. We also see that an average *b* value predicts approximately the same plate count as the use of *b* values for individual compounds. The last column indicates the large discrepancy in *N* values when they are calculated improperly, using eqn. 15* of ref. 1.

C. Summary

In this section we have experimentally verified the theoretical predictions of Part I[1]. This greatly increases the practical utility of GE-LC, by allowing systematic and predictable optimization of gradients, as well as the use of gradient data to predict reliably isocratic separation conditions.

In the next section we shall discuss the actual measurement of the gradient parameters discussed above, plus some practical "rules-of-thumb" for successful use of GE-LC.

6. APPLICATION OF THEORY TO PRACTICE IN REVERSED-PHASE GRADIENT ELUTION: INITIAL SEPARATION

In this section, a simple procedure for designing "general, optimal" RP gradients will be presented. Evaluation of the gradient chromatograms by means of the theory verified above enables rapid, logical "tuning" of the gradient, *i.e.*, optimization of resolution, detection sensitivity and gradient time. The so-called "general, optimal" gradient results as a compromise among the latter three goals. Table 12 summarizes the important instrumental and mobile phase parameters in designing the gradient, thus serving as an outline of the following discussion. A typical example will be developed during this discussion, as summarized in Table 13.

TABLE 12

SUMMARY OF PARAMETERS IMPORTANT IN DESIGNING RP-GE SEPARATIONS

Type of parameter	*Important parameters*
Instrument parameters	t_0
	t_d
	Gradient profile
Mobile phase characteristics	Solvent selectivity
	Φ'
	Range
	Gradient blank

TABLE 13

TYPICAL INITIAL GRADIENT CONDITIONS FOR RP-GE

Parameter	*Value*
Gradient profile	Linear
Solvent A	95% water, 5% AN
Solvent B	5% water, 95% AN
Gradient steepness, Φ'	6.5%/min
t_0	1.07 min
Flow-rate	2.0 ml/min
Gradient range	0–100% B
Gradient time, t_s	14 min
Gradient delay, t_d	1.0 min

A. Instrument parameters

It is necessary to determine several parameters characteristic of a given instrument (*i.e.*, the pump, injector, column and connecting tubes) in order to efficiently develop a GE separation.

In order to estimate Φ' (see below), t_0 must be known. A good estimate of t_0 can be obtained easily by isocratic elution of uracil, using a mobile phase with greater than 60% organic modifier. In our present example, t_0 was found to be about 1.07 min (V_m = 2.15 ml; F = 2.0 ml/min).

Another important characteristic of a given instrument is the gradient delay. This is easily determined by using a UV-absorbing solute dissolved in the strong solvent, B, (*e.g.*, uracil in methanol) and the same mobile phase without any solute as A (methanol). The detector is connected where the column is usually attached, and the gradient of interest run. Typical results are illustrated in Fig. 9. The gradient delay time, t_d, is determined from this trace by simply measuring the time between the start of the gradient program and the initial increase in the baseline due to the arrival of solvent B at the detector. A knowledge of delay time is useful in fine tuning the gradient for a particular sample, as will be illustrated shortly.

The gradient profile produced by the instrument is also illustrated in Fig. 9. This nominally linear gradient is in fact seen to be slightly convex, with deviations from the theoretical value as great as 6%. However, the discussions in Part I[1] and above indicate that the results obtained in RP-GE are relatively insensitive to devia-

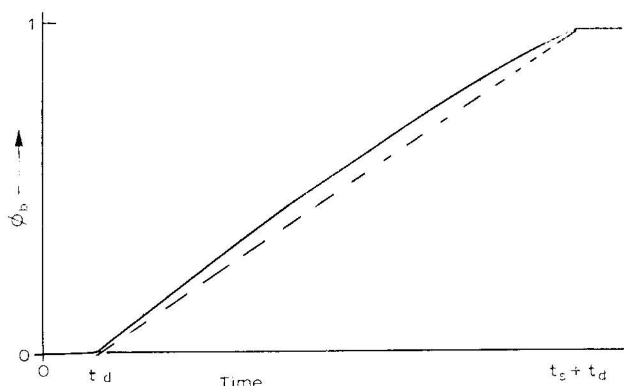

Fig. 9. Gradient profile for pre-set system. Solid line, actual gradient; broken line, theoretical gradient.

tions of this magnitude. It is convenient to determine the gradient delay (and verify gradient profile) after the initial solvent program has been designed (see below). The gradient delay volume (t_d/F) and the relative gradient profile need be checked only once for a given instrumental configuration, as they should remain constant for a properly functioning instrument.

B. Mobile phase characteristics

The first step entails selection of the gradient profile. LSS gradients are preferred, and linear solvent programs generate this type of gradient for most RP-GE separations[1]. Therefore, we assume a linear solvent program.

Next, we select the organic modifier: methanol, acetonitrile and tetrahydrofuran are most commonly used in RP-GE. The choice of a given organic solvent is dictated mainly by the selectivity required for a given sample. This is generally not known in advance of the separation, so the choice of initial solvent is somewhat arbitrary. For this discussion, we shall assume the selection of water–acetonitrile as mobile phase. Gradient steepness is estimated from Table 4*. Using the t_0 value determined above (1.07 min), Φ' is found to be approximately 6.5%/min for acetonitrile.

At this point, the gradient range must be considered. The latter refers to the range in k_i values during the separation; the gradient range is greater for larger S values of the organic solvent and for larger changes in Φ_b (*e.g.*, 0–1.0) during separation. As the gradient range increases, it is more likely that a given compound will be successfully separated, *i.e.*, eluting neither near t_0 nor long after the completion of the gradient. For unknown samples, we recommend an initial gradient of 5% to 95% acetonitrile–water, using pre-mixed solutions of 5% and 95% acetonitrile–water as solvents A and B, and running the gradient from 0 to 100% B. This provides a reasonable gradient range, yet avoids certain practical problems. For example, de-gassing frequently occurs if pure water and organic solvents are mixed on-line. Also, some reversed-phase columns show poor efficiency with mobile phases that contain 90–100% of water, because wetting of the packing is poor. In subsequent

sections we shall discuss possible alteration of the gradient range for various reasons.

The gradient time, t_s, can be calculated by dividing gradient range by Φ'. In our example, this is approximately 14 min (90/6.5). At this point, all of the parameters necessary to run our initial RP gradient have been determined and are summarized in Table 13 for our example. However, before running gradients of actual samples, it is advisable to run a blank gradient at the most sensitive detector attenuation anticipated in the ensuing gradients. This provides valuable information about baseline fluctuations and ghost peaks, which can be a problem with solvents of insufficient purity.

The importance of solvent purity in GE-LC cannot be overemphasized. Solvents which are acceptable for isocratic LC may be useless in GE, as is illustrated in Fig. 10A and B. Fig. 10A shows a MeOH–water gradient using ACS-quality anhydrous methanol, which is often used without any problems in routine isocratic LC. One can see that the impurities are concentrated on the column when the mobile phase is weak and then elute later in the gradient. On the other hand, highly purified HPLC-grade MeOH under the same conditions provides an acceptable blank, as shown in Fig. 10B. Similar results can be shown with water of varying quality; here one must be careful to remove all UV-absorbing impurities before use. A further problem of not using highly purified solvents is that blank runs are not reproducible, as only rarely are the recycle and equilibration times exactly the same from run to run, and varying amounts of impurities can build up on the column prior to each gradient run.

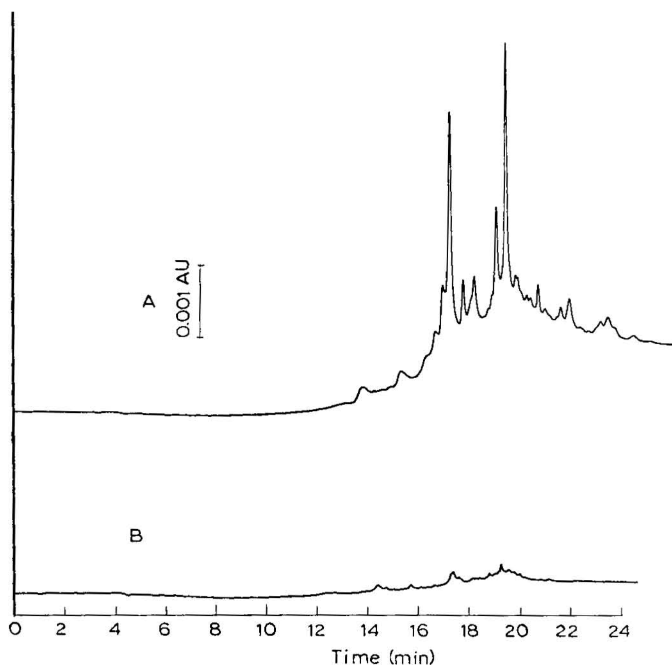

Fig. 10. Influence of solvent purity on gradient. 5–95% MeOH–water, t_s = 20 min. (A) ACS-grade anhydrous MeOH; (B) HPLC-grade high-purity MeOH.

Given an acceptable blank, we are now prepared to run our initial RP-GE separation.

C. Analysis of samples

As in isocratic LC, it is useful first to obtain an acceptable chromatogram of standard compounds of interest if these are available. Thus, the first gradient should be of standards. Next, a gradient of the actual sample is obtained. By using the standard chromatogram as a guide, attention can be focused on the most important parts of the separation. At this point, we have designed and run our initial RP-GE separation. The steps summarizing this development are presented in Table 14, and can be used as a check list in developing a RP-GE separation.

In the following section, we shall discuss our initial solvent program, decide what improvements are necessary, and present straightforward procedures for implementing these improvements.

TABLE 14

STEPS IN DESIGNING A "GENERAL OPTIMAL" RP-GE SEPARATION

No.	*Step*
1	Select a linear gradient profile
2	Choose organic modifier (acetonitrile)
3	Determine Φ' (given t_0); an approximate value is adequate
4	Choose gradient range (0–100% B for unknown sample)
5	Calculate gradient time, t_s
6	Determine gradient delay, t_d
7	Gradient blank
8	Gradient of standards
9	Gradient of sample(s)
10	Modify gradient range
11	Fine tune the system (see Table 15 and accompanying discussion)

D. Column regeneration

The importance of re-equilibration to initial gradient conditions cannot be overemphasized. Too often, irreproducible results are due to inadequate re-equilibration. A generally accepted procedure is to run a 10–15-min reverse gradient followed by 10 min under the initial conditions (with a typical flow-rate of 2.0 ml/min). It should also be noted that column regeneration is dependent on the total volume of liquid passing through the column, not the time of column reconditioning. Therefore, regeneration will be faster using a steep reverse gradient at a high flow-rate.

7. "FINE TUNING"

As our "general optimal" RP gradient represents a compromise among resolution, detection sensitivity and analysis time, it stands to reason that we can optimize each of these parameters individually (at the expense of one or both of the

remaining two). In this section, we shall illustrate procedures for adjusting resolution and improving detection sensitivity, so that satisfactory separations can be obtained. Finally, procedures for minimizing the analysis time will be discussed (see Table 15 for a summary).

TABLE 15

SUMMARY OF FINE-TUNING PROCEDURES

Parameter	*Procedures*
Resolution	Increase gradient range if necessary Increase N Decrease b Change organic modifier
Detection sensitivity	Increase sample size Increase b Increase N
Optimizing analysis time	Increase initial %B (beginning) Decrease final %B (end) Increase b via decreasing t_s (R_s initially "too good")

A. Improving resolution in RP-GE

The principles of resolution in LSS gradient elution have been summarized in Part I[1]. In general, these principles are similar to those associated with isocratic RP-LC, as long as the analogy between k' and b is understood. In isocratic RP-LC, R_s can be improved by making appropriate changes in N, α and/or k'. Likewise, R_s in RP-GE can be improved by appropriate adjustments in N, α and/or b. We shall now discuss how each of these parameters can be adjusted to improve R_s in RP-GE.

(a) Gradient range

Before considering adjustments to N, α or b, we must check for elution of all sample compounds of interest prior to the end of the gradient, with no compounds eluting near t_0. If peaks continue to elute after the end of the gradient (*i.e.*, with gradient "hold", and pure B as mobile phase), a stronger solvent B is required. In this case, it is possible to substitute a solvent of higher S value (*e.g.*, tetrahydrofuran) for the original solvent acetonitrile. Alternatively, for very strongly retained compounds, it may be necessary to consider ternary gradients such as water–tetrahydrofuran–*n*-hexane (which requires a more complex pumping system). When one or more compounds of interest elute at t_0, it is necessary to consider some means for increasing their retention, *e.g.* by changing the pH or by use of ion pairing.

(b) Varying k' or b

Having approximately optimized b as discussed in Part I[1] and above, there is usually little reason to consider further adjustment of b for the purpose of increasing R_s (however, it may be worthwhile increasing b for increased detection sensitivity; see below). However, two minor points should be mentioned in passing as they relate to the question of optimal b in RP-GE.

First, as discussed in Appendix I, the optimal values of k' and of b in isocratic or gradient elution, respectively, do vary somewhat with the particle size of the column packing, and with separation time. For a relatively fast separation (14 min) as assumed in Table 13, and the use of 10-μm particles, a value of b close to 0.2 is indeed correct. However, for longer separation times and/or smaller particles, a value of $b = 0.1$ would have a slight advantage. Similarly, for larger particles (*e.g.*, 50-μm) and/or still shorter separation times (< 10 min), a value of b as large as 0.3 might be preferable. In any of these cases, however, we are talking of an increase in R_s (other factors being equal) of generally no more than 5–10%. At the same time, a change in b can result in small changes in α, which could largely cancel the increase in R_s as a result of change in b.

Secondly, if our initial separation involves a reduced velocity, $\nu \approx 3$ (*i.e.*, at minimal h), and if we do not want to change the solvent B or increase column length L, there is only one option available for increasing R_s: a decrease in b by 2–5-fold can in this case provide an increase in R_s of 10–20%. However, this would be accompanied by a corresponding increase in separation time of 2–5-fold, with a loss in detection sensitivity by the same factor.

(c) Varying N

The same options are available for increasing N in gradient elution as for isocratic elution. The two major approaches are a decrease in F (holding the column length L constant), or an increase in L with a proportionate increase in F (*i.e.*, holding pressure P constant). A predictable change in resolution in either of these two ways can be effected exactly as in the case of isocratic elution[7]. The only requirements during this change in L and/or F is that the gradient steepness be held constant, in terms of the change in $\%B/t_0$ (*i.e.*, b must be held constant). A summary of the necessary changes in the gradient steepness accompanying these two options for increasing N and R_s is given in Table 16, together with the necessary change in other separation variables.

TABLE 16

INCREASING RESOLUTION AND N IN GRADIENT ELUTION

Variable	*Column length constant*	*Column length varied*
Flow-rate, F	Decrease by factor x	Decrease by x
Column length, L	No change	Increase by x
Gradient steepness, %B/min	Decrease by x	Decrease by x^2
Separation time, t	Increase by x	Increase by x^2
Column pressure, P	Decrease by x	No change

It is important to note that if the gradient steepness (measured simply as Φ') is left unchanged when the flow-rate is decreased or the column length is increased, the true steepness in terms of b actually increases, because t_0 is increased in each case. This in turn means a decrease in the effective value of k' during separation, and a loss in resolution in some cases. Another reason for keeping b constant during a change in N is that then (and only then) will the relative elution order of different sample bands remain absolutely the same.

(d) Varying α

As in isocratic separation, α values in gradient elution do not vary as N is varied. Sometimes changes in the α values of adjacent bands result when b is changed. These changes in α are analogous to those occurring in isocratic separation when k' is varied by adjusting Φ_b. This has recently been discussed by Karger *et al.*[5,8]. To change α values in RP-GE deliberately, one must usually change either the mobile phase or the stationary phase, while holding b constant. Normally, the mobile phase composition will be varied in one of two ways. Firstly, another organic modifier can be selected, and the gradient re-optimized for this new solvent. For example, if our initial gradient were 7 %/min methanol–water, 4.5 %/min tetrahydrofuran–water can be substituted (see Table 4*). Hopefully this change in mobile phase will provide some change in α values, but leave average k' and N values at their original optimal levels. This is dramatically illustrated by comparing Figs. 5C and 6C, where the only difference is a change from AN to MeOH as organic modifier. Looking at components 5, 6, 7 and 8, remarkable changes in separation are observed.

In the second approach for changing α values, a third solvent C can be added to both solvents A and B (*e.g.*, ref. 9).

B. Detection sensitivity

If the detection sensitivity must be improved, there are two possible approaches in RP-GE. If R_s is not a problem, b can be increased to improve detection. This is analogous to decreasing k' in isocratic LC. This has been discussed in detail above, and Table 17 summarizes the relationship between b and detection sensitivity (increasing b increases detection sensitivity).

TABLE 17

RELATIONSHIP OF RESOLUTION AND PEAK SENSITIVITY IN GRADIENT ELUTION TO THE GRADIENT STEEPNESS, b

b	Relative R_s	Relative sensitivity***
0.05	0.94	0.1
0.1	0.79	0.2
0.2*	0.63	0.4
0.3**	0.54	0.5
0.5	0.39	0.7
1.0	0.20	1.0
2.5	0.06	1.4

* Optimal value when column length L is held constant.
** Optimal value when column pressure P is held constant.
*** Relative to an isocratic band at t_0.

When resolution is more critical and cannot be attained at higher b values, the detection sensitivity can be improved by charging a larger sample to the column. Again, the analogy with isocratic LC is valid. Large samples can be charged to the column, provided that the solvent in which the sample is dissolved is sufficiently weak (so as not to cause significant migration of the sample bands of interest), and Φ_0 is as small as possible.

In general, the first step in increasing detection sensitivity should be to increase sample size when possible.

C. Minimizing analysis time

Having obtained the desired resolution and detection sensitivity, the final step in fine tuning our RP gradient is to minimize the analysis time. The two most general cases in which analysis time is wasted in RP-GE will now be discussed.

In the first case, the polarity range of the solvent program may be larger than required to elute the sample(s) of interest. This is easily recognized when there is empty space (*i.e.*, no peaks) at the beginning and/or end of the chromatogram. In this case, optimal use of the analysis time results by adjusting the initial and/or final Φ_b with concurrent adjustment of t_s in order to maintain b constant. This procedure effectively eliminates wasted time, while keeping R_s constant. A similar situation exists when early eluting peaks are present, but are of no interest for the particular sample(s). In this case the initial Φ_b is increased to the point where the sample component(s) of interest are resolved, but the early eluting peaks elute close to t_0. Fig. 4 provides an illustration of time wasted at the beginning of the gradient. Fig. 4B–E illustrates the decrease in t_s resulting from increasing the initial Φ_b at constant b.

In the second case, R_s is larger than required at optimal values of b, but the full gradient range is required (*i.e.*, 0–100% B). The most direct solution in this case is to increase Φ' (and hence b) by decreasing t_s, while keeping F constant. As t_s determines the analysis time, the improvement here is obvious. Alternatively, N can be offset against t_s by decreasing L and/or increasing F. Finally, simultaneously increasing b and F can result in significant time savings, when we have a higher N than is required.

8. SYMBOLS*

- Φ_0 — value of Φ for mobile phase entering column at $t = 0$.
- Φ_g — value of Φ for mobile phase entering column at $t = t_g$.
- t_d — delay time between initiation of gradient and actual change in Φ at head of column.
- V_0 — void volume of chromatographic column.

9. APPENDIX I

Optimal values of k' and b for isocratic and gradient elution

In isocratic separations on large-particle (> 20-μm) columns, it has been shown[6] that the optimal value of k' is related to the slope, n, of the log k' *versus* log u plot for that column. If the column length L is held constant,

$$k' \text{ (optimal)} = 2/n \tag{i-1}$$

If the column pressure is held constant (L allowed to vary), then the optimal value of k' is

$$k' \text{ (optimal)} = 4/(1 + n) \tag{i-2}$$

* See also the symbols in Part I[1] (Section 7).

The values of n for large-particle columns generally range from 0.4 to 0.6, so that optimal values of k' vary between 2.5 and 5. As resolution is relatively insensitive to k' in this range, while separation time and detection sensitivity are adversely affected by an increase in k' beyond 5, there is little reason to consider adjustment in k' for most cases.

The situation is somewhat more complex in the case of separations on small-particle columns. In a preceding paper[7] values of n were derived for various values of the reduced velocity v, and values of v are in turn roughly related to particle size, d_p (for typical separation conditions). We can summarize these preceding treatments for porous particles as follows:

Reduced velocity v	n	*Typical* d_p *(μm)*	*Optimal* k' *(eqns. i-1 and i-2)*	
			Fixed L	*Fixed P*
3	0.02	–	100	3.9
10	0.35	5	5.7	3.0
30	0.53	15	3.8	2.6
100	0.68	45	2.9	2.4

Again, the above optimal values of k' refer to the maximization of R_s, without regard to possible loss in detection sensitivity. The main conclusion to draw is that the optimal k' tends to increase somewhat as the particle size becomes smaller, and this effect is more pronounced when the column length is fixed.

The situation is precisely analogous in the case of gradient elution. While a value of $b = 0.2$ is a good general compromise for most separations, resolution can be increased somewhat by using lower values of b in the case of separations on small-particle columns. This trend is apparent in Fig. 7* in Part I[1], where a 10-μm column shows optimal values of b that are generally closer to 0.1. Similarly, in Fig. 5 in the present paper, it is apparent that maximal resolution occurs at $b \approx 0.1$ for the 5-μm column used. Also apparent in Fig. 7* in Part I[1] is the fact that longer separation times generally favor smaller values of b, and this is true also of small-particle separations by isocratic elution.

10. SUMMARY

The theory developed in Part I is verified experimentally in gradient separations with C_{18} columns and solvent systems consisting of water–methanol, water–acetonitrile and water–tetrahydrofuran. Linear solvent strength separations correspond to gradients that vary linearly with time. Some practical rules for optimizing such separations are presented.

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TWENTY YEARS OF THIN-LAYER CHROMATOGRAPHY

A REPORT ON WORK WITH OBSERVATIONS AND FUTURE PROSPECTS

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1. INTRODUCTION

The first work with the title "thin-layer chromatography" (TLC) appeared in 1956[1]. It described a simple procedure for preparing very thin layers and reported the influence of layer thicknesses from 20 to 340 μm. It was found, using silica gel, that layers permitting fast flow and yielding good separation could be obtained only when the grain size lay mainly between 1 and 5 μm. Thus was the decisive step taken in chromatography for the transition from the relatively coarse-grained aluminium oxides used hitherto in the Tswett column chromatography to the very fine adsorbents with a narrow range of grain size (Fig. 1).

The real breakthrough in the method came with a second publication in 1958, in which standardization, detection, documentation and applications were described[2]. In the same year, the basic kits for thin-layer chromatography (Desaga, Heidelberg, G.F.R.) and silica gel G according to Stahl for TLC (Merck, Darmstadt, G.F.R.) were presented at the ACHEMA exhibition of chemical equipment at Frankfurt. It was already clear at that time that the method involved essentially adsorption chromatography on "open columns". The first applications were therefore principally in the domain of lipophilic compounds (Table 1). The existing rules of thumb of adsorption chromatography could be taken over and the relationships displayed in a "triangular scheme" (Fig. 2)[5].

The chromatography of polar, hydrophilic substance mixtures remained the preserve of paper chromatography (PC) for some time. In 1961, we were able to

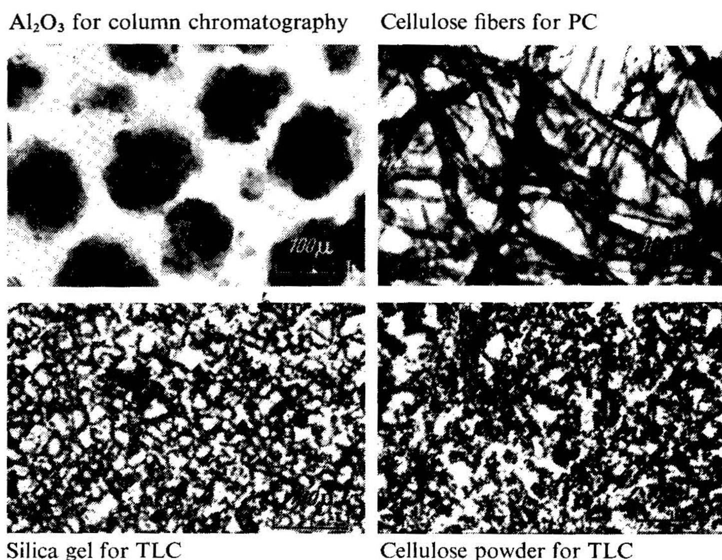

Fig. 1. Comparison of differences between the adsorbents for classical column chromatography and paper chromatography (above) and the fine adsorbents with a narrow range of grain size introduced for TLC (below).

extend the use of TLC to hydrophilic materials, as described in four publications. It could be shown that mixtures of sugars[8], heart glycosides[11] and numerous other hydrophilic plant components[10] were better and more rapidly separable by TLC than by PC. These publications recommended the use of TLC in trace analysis and demonstrated that amounts down to 0.005 μg, *i.e.*, 5 ng of, for example, certain indole derivatives such as heteroauxin, could be detected[9]. Nanogram-scale TLC had thus already come into existence at that time and, further, only short runs were required for trace analysis. This and much else is often forgotten today, or at least overlooked as a result of a lack of awareness of the literature. So many publications had appeared by 1962 that a team of specialists was able to bring out the first laboratory handbook on TLC[13]. The number of yearly publications increased exponentially, and 5 years later, the second, greatly expanded edition of the handbook, with *ca.* 1000 pages, appeared[24].

2. SPECIAL WORKING TECHNIQUES

In the first decade of TLC, we extended its application to virtually all types of mixtures and also devoted special attention to working techniques. Thus, the circular and wedged tip techniques and simple equipment for carrying them out were described in 1958[3]. This method, known from PC, is of particular interest through the apparent elimination of longitudinal diffusion of the spots which are formed into small bands as a result of the radial movement of the solvent. We recommended this "ring chromatography" for rapidly establishing the best solvent, for instance. Stepwise development was described for separating mixtures of substances with widely differing polarities[4]. Another study was of the influence of the degree of saturation of the separating chamber, with subsequent introduction of the so-called chamber saturation. Along with this, the sandwich chamber (S-chamber) was developed with the tubular

TABLE 1

LIPOPHILIC SUBSTANCE CLASSES CHROMATOGRAPHED BY STAHL AND CO-WORKERS (1957–1977)

Class of substance	*References*
Alkaloids	1, 18, 25, 27, 51, 53
Acids, organic	48
Anthraquinone derivatives	62
Azulene derivatives	1
Balsams	3
Calamus oils	7
Cannabinoids	59
Capsaicin	36
Carotinoids	4, 14
Chamomile oils	32
Colour indicators	18
Coumarin derivatives	54
Daucus oils	17
Drugs, hallucinogenic	46
Dyes, lipophilic	4
Ergot alkaloids	4
Essential oils	2, 3, 7, 17, 20, 28, 32, 37
Flavonoids	52
Flavours	37
Glechoma constituents	39
Glycols	48
Iodine compounds, organic	16
Lipids, ointments, etc.	60, 61
Morphactines	34
Opium alkaloids	27
Peroxides, organic	2
Pesticides	35
Phenols and derivatives	4, 42, 43, 49, 59
Phloroglucinolbutanones	12, 15
Phthalids	23
Picrocrocin	33
Polyamines	48
Preservatives	35
Pyrethrins	6, 21
α-Pyrone derivatives	45
Resins	3
Safranal	33
Saponins	41
Steroids	5
Tars	3
Terpene alcohols	20
Tropa alkaloids	25
Tyrosine derivatives	16
Valepotriates	31
X-ray contrast agents	16

trough. In 1960, the two-dimensional SRS (separation–reaction–separation) technique was described for investigating photooxidation[6]. This is a simple but very useful method, later termed "reaction chromatography", in my opinion a somewhat unfortunate description.

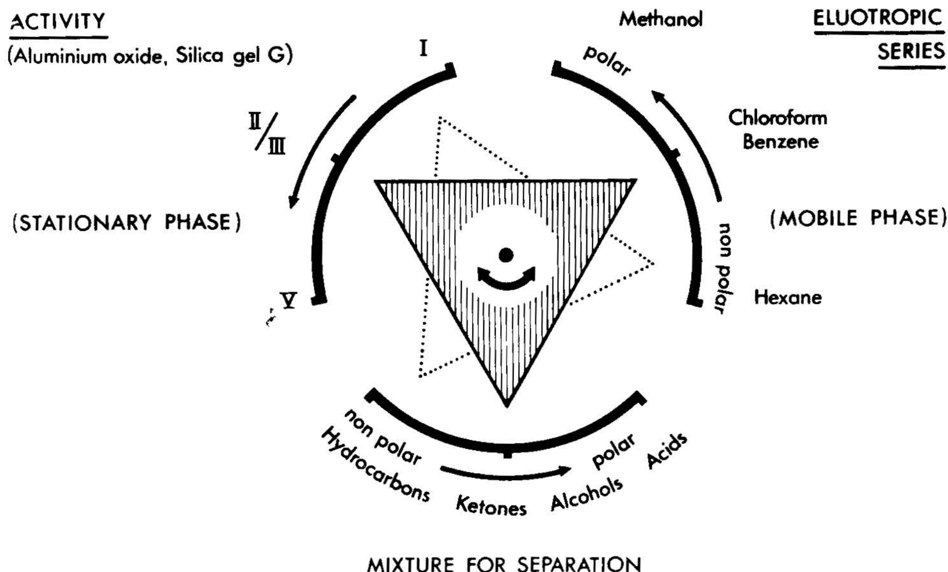

Fig. 2. Triangular scheme showing the close relation of the three variable quantities in chromatography, demonstrated on the example of adsorption chromatography. One has to imagine that the central triangle is turnable with one tip directed towards the mixture.

The so-called gradient-TLC[19] brought a climax and genuine step forward in 1964. Gradient layers can be easily and rapidly prepared using a special gradient spreader. Three different separating surfaces are available on a gradient layer, in contrast to the one type on the usual uniform layers (Fig. 3). Development can be carried out at right-angles to the gradient (T-gradient technique) or in two different directions along the line of the gradient. A few years later we succeeded in preparing defined pH-gradient layers. Chromatography on these of, *e.g.*, basic, amphoteric or acidic substances, perpendicular to the gradient, furnishes typical curves which are sometimes even substance-specific (Fig. 4). These can serve for the identification of individual compounds in an unknown mixture. The possibilities of application of such gradient layers have by no means been exhausted. It does seem, however, that most workers are still content with the uniform layers or avoid preparing gradient layers, simple though that is.

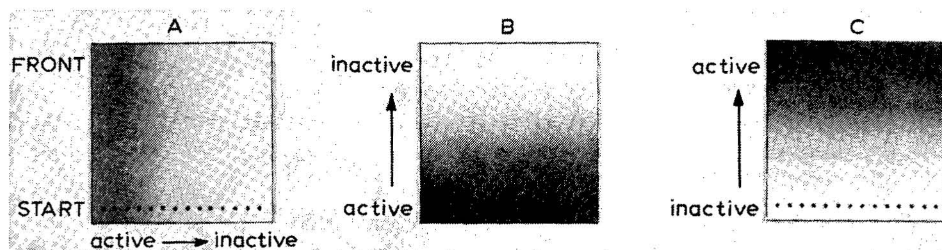

Fig. 3. Three different separating surfaces available on a gradient layer. Development is possible (A) at right-angles to the gradient and (B) and (C) along the line of the gradient, in the example from active to inactive or from inactive to active.

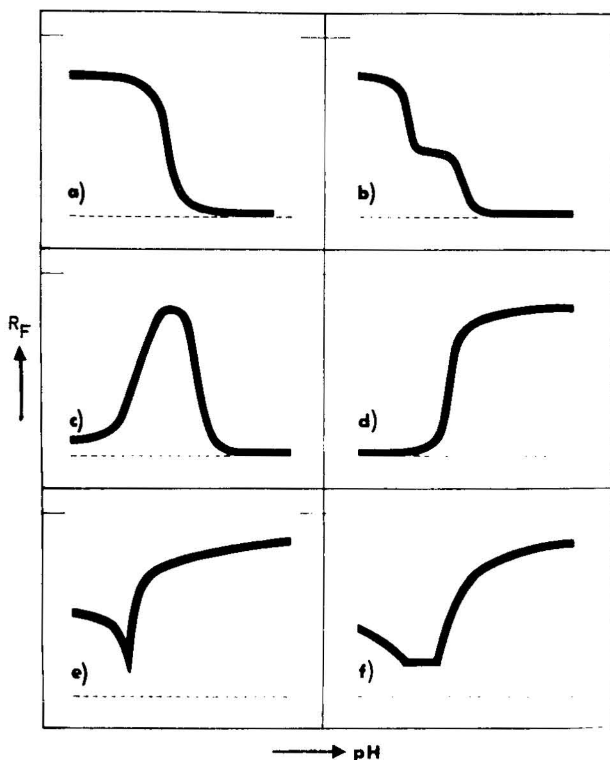

Fig. 4. Typical curve shapes obtained in pH–T-gradient chromatography. (a) Monobasic acid; (b) dibasic acid; (c) amphoteric compounds, such as anthranilic acid; (d) monoacidic amine; (e) opium alkaloids; (f) Tropa alkaloids.

We regard R_F values in TLC only as guide values and therefore showed an early interest in methods for identifying substances present in only microgram amounts. The techniques for transferring such small amounts from one method to another raised problems here. The possibilities were summarized in 1966 in a scheme (Fig. 5) and a "strategy for substance identification" was laid down[22]. At this time our experiments in collaboration with the firm of Zeiss for the direct quantitative evaluation of thin-layer chromatograms were concluded and production of the chromatogram–spectrophotometer began. A former pupil, Professor H. Jork, has continued detailed studies of quantitative evaluation.

3. STANDARDIZATION AND TERMINOLOGY

I recognised early the necessity for standardization of the TLC method. This began with the plate size (20 × 20 cm), the length of run, the position of the starting points, chamber saturation and, not to be forgotten, the standardization of adsorbents with the help of test mixtures. This enabled work to be performed everywhere under the same conditions, in contrast to that with other chromatographic and electrophoretic methods; the results obtained were therefore comparable. This yielded great

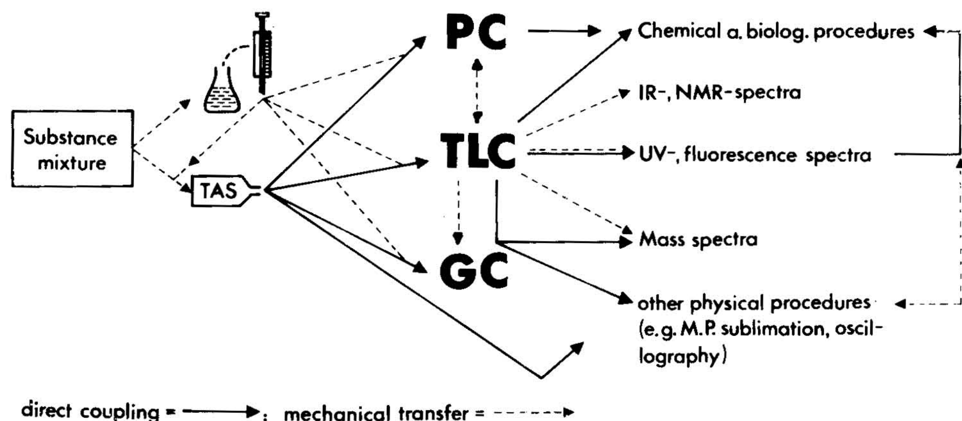

Fig. 5. Possibilities of coupling and combination of TLC and other methods.

advantages for the manufacturer of TLC equipment and especially for the user. International understanding demanded also that we concerned ourselves with terminology and definitions[26]. As a result, TLC rapidly found a place in legally binding test procedures, *e.g.*, in pharmacopoeias. Suggestions were made for preparing such monographs and numerous procedures were devised for drug characterization in the European and German Pharmacopoeias[25].

4. THE SECOND DECADE OF THIN-LAYER CHROMATOGRAPHY

In the second decade of TLC, after 1967, the emphasis of our work lay at first on the micro-extraction of substances from complex samples and the direct transfer to the thin layer. Experience had already shown that the preparation of the sample, *i.e.*, extraction with liquid, filtration, evaporation, redissolution and application often took much longer than the TLC itself and, moreover, usually led to considerable substance losses. Hence, we sought coupling procedures that would avoid this preliminary work and permit largely quantitative transfer to TLC.

In addition, we were interested in the problem of characterization, *i.e.*, the analysis of natural and synthetic polymeric material with the help of TLC. At the time, it was not possible to detect cellulose, lignin, polyphenols or proteins in plant drugs; nor could analyses of synthetic polymers, a vast domain, be performed by TLC.

5. TAS PROCEDURE

As already stated, the preliminary preparation of plant material for subjection to TLC is often the most time-consuming part of the whole procedure. Consequently, we made many attempts to develop a direct extraction and transfer method. Stimulated by the work on microsublimation by Kofler and Fischer in the 1930s, we first tested out thermal extraction procedures. Step by step this led to the TAS procedure, patented in 1967.

The sample, usually 1–10 mg, is placed in a glass cartridge, which is closed at

one end, the other end being drawn out to form a capillary. The cartridge is then placed in the TAS oven (Fig. 6) which has been heated to a given temperature (up to 350°). The volatile substances pass as a jet through the capillary directly on to a thin layer positioned 0.1–0.5 mm from the capillary exit. There they form the starting points on the thin-layer plate and can be submitted to the usual development.

The possibilities of application in various fields were demonstrated in twelve publications in 1968 and subsequent years. Special studies were devoted to the procedures taking place during extraction, to optimization and to quantitative aspects. The TAS method was quickly adopted in many laboratories and is used widely both in industry and in pharmacognosy teaching for rapid extraction coupled with TLC.

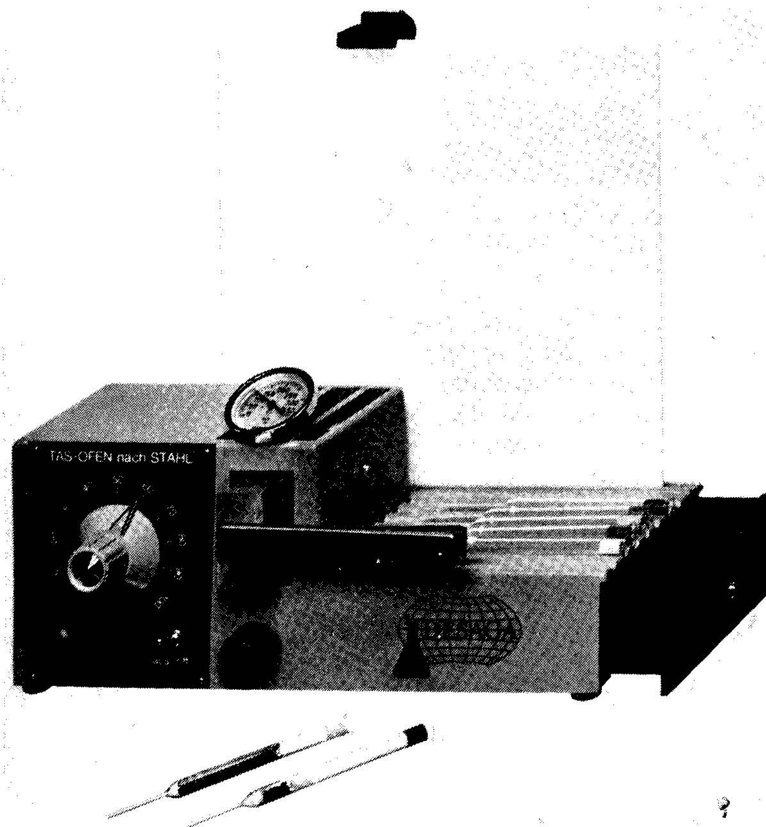

Fig. 6. TAS oven for thermal extraction and for direct transfer of the sample to the thin layer.

6. THERMOFRACTOGRAPHY[40]

A logical further development of the TAS procedure led first to band condensation of the substances for preparative recovery and later to the so-called thermofractography (TFG). The apparatus for the latter was called the TASOMAT (Fig. 7). A suitable controlling mechanism was developed for regulating and controlling the temperature, the rate of heating and the final temperature. In the TASOMAT, the temperature of a few milligrams of sample is raised linearly from room temperature

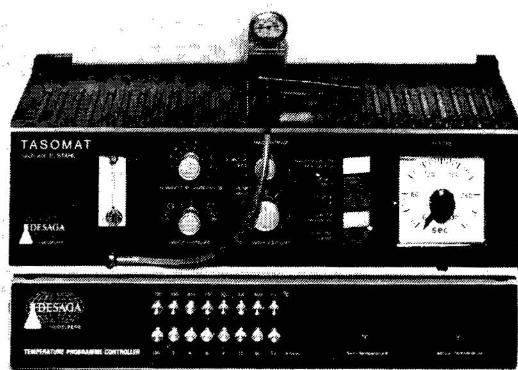

Fig. 7. TASOMAT, an apparatus for thermal extraction and thermal pyrolysis in the temperature gradient 50–450°. The lower part serves for the control of the TAS oven.

to 450° at a pre-selected rate. The volatile products are then collected as a starting band on a thin layer. This is effected by moving the thin layer at a definite speed across the exit capillary of the TAS cartridge. The band is then chromatographed in the customary way. The chromatogram, the so-called thermofractogram, then yields the substances separated along the abscissa according to their volatility (boiling or sublimation temperatures) and along the ordinate according to their chromatographic behaviour. A typical example is shown in Fig. 8. This fractional thermal extraction is in principle a distillation or sublimation in a carrier gas. This is the first time that

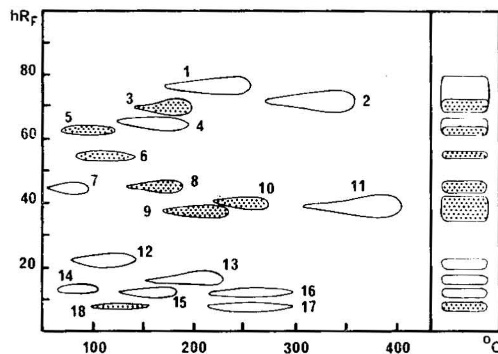

Fig. 8. Typical thermofractogram, with the substances separated along the abscissa according to their boiling temperatures and along the ordinate according to their chromatographic behaviour. On the right: a normal application of samples in fluid. 1 = Squalene; 2 = cholesteryl stearate; 3 = sulphur; 4 = guaiazulene; 5 = biphenyl; 6 = anethole; 7 = 1,8-cineole; 8 = benzyl benzoate; 9 = benzyl cinnamate; 10 = dioctyl phthalate; 11 = triolein; 12 = eugenol; 13 = oleic acid; 14 = menthol; 15 = palmitic alcohol; 16 = wool wax aliphatic alcohols; 17 = wool wax sterols; 18 = vanillin.

microgram amounts of many substances with high boiling or sublimation points were transferred directly on to a thin layer. The separation effect corresponds roughly to that of a distillation under a pressure of 0.1 mmHg. This is shown in particular by our work on the rapid analysis of lipid mixtures, *e.g.*, of ointments, suppositories and cosmetic preparations such as creams and lipsticks[58,60,61]. Such analyses previously used to take several days, whereas one now has a picture of the composition within an hour.

Basically, all substances which can be subjected to gas chromatography are amenable to thermal separation by TFG. Naturally, the procedure does not apply to a number of polar and non-volatile substances. However, during TFG these undergo thermal decomposition within a particular temperature range, that is, pyrolysis or, better, thermolysis, occurs. Definite fragmentation products are thus obtained which can serve for identification on the thermofractogram (fingerprint). For example, we have had good success in distinguishing different lignins[42]. Fig. 9 shows the difference between thermolysates from beech and pine lignins. The characterization of tannins by means of TFG was also valuable. These investigations form the basis for extensive analyses of leather samples[43]. Using only a few milligrams of leather sample, it is possible to show how the tanning was carried out. After these encouraging results we turned to the investigation of plastics and were able to carry out the rapid identification of condensation polymers (nylon and Perlon types), phenol resins, vinyl polymers and also of plasticizers and other additives[48,49,63].

In further studies, we then performed classical thermal reactions in the temperature gradient of the TFG. This had the advantage that such reactions can be

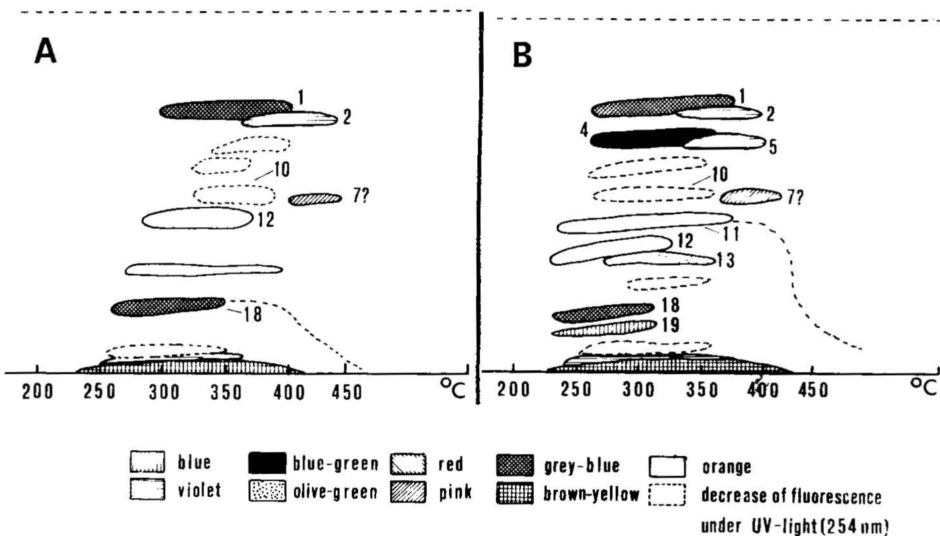

Fig. 9. Thermofractograms of lignins. (A) Spruce lignin. Colours are obtained after spraying with Fast Blue Salt B; only the corresponding guaiacyl derivatives are obtained. 1 = Vinylguaiacol; 2 = guaiacol; 10 = vanillin; 12 = coniferaldehyde; 18 = coniferyl alcohol. (B) beach lignin. Besides the guaiacyl compounds the corresponding syringyl derivatives are obtained. 4 = 4-Vinyl-2,6-dimethoxyphenol; 5 = 4-ethyl(or methyl)-2,6-dimethoxyphenol; 11 = syringaldehyde; 13 = sinapaldehyde; 19 = sinapyl alcohol.

TABLE 2

TYPICAL CONDITIONS FOR THERMAL REACTIONS AND DEHYDROGENATIONS COUPLED WITH TLC

The optimal conditions in the given ranges vary from substance to substance.

Method	*Sample* (*μg*)	*Reaction partner or catalyst*	*Temperature* (*°C*)
Zinc dust distillation	5–50	200–300 mg of Cu-activated zinc dust	350–450
Sulphur dehydrogenation	50–100	10 mg of sulphur dehydrogenation mixture	160–220
Selenium dehydrogenation	100–200	20–30 mg of selenium dehydrogenation mixture	250–320
Catalytic dehydrogenation	20–100	25 mg of Pd–$BaSO_4$ (10%)	250–350

conducted with microgram amounts, as found, for example, in chromatographic zones. All of the substances formed are found on the corresponding thermofractograms and this enables the course of the reaction to be recognized. Work in the ultramicro region was carried out on dehydrogenation with sulphur and with selenium, catalytic dehydrogenation and zinc dust distillation. A summary is given in Table 2 of the necessary amounts, catalysts and temperatures[44,47,50].

These techniques can be regarded also as carbon-skeleton TLC, analogous to that in gas chromatography. A review of thermal work coupled with TLC was prepared for the occasion of the ACS award in chromatography[55].

Labile natural products are, however, not ideal subjects for separation using thermal methods. We thus sought less drastic extraction methods which permitted direct coupling with TLC; this led us to the supercritical gases.

7. FLUID EXTRACTION COUPLED WITH THIN-LAYER CHROMATOGRAPHY

The solubilities of many substances in supercritical gases increase as the pressure is raised. It was therefore of interest to employ such gases under pressure in the compressed state, *i.e.*, in the supercritical region, for extraction. The problems of coupling with TLC could be circumvented by pressure release through very fine capillaries (I.D. 50 μm). The gas stream exits through the fine capillary and impinges directly on to the thin layer. The layer is moved relatively rapidly back and forth and thus collects a starting band of the extracted substances. Normally, 1 Nl* gas is used per pressure stage for extraction. The thin layer is then displaced and a further extraction performed with 1 Nl at a correspondingly higher pressure, etc. We first developed an apparatus for this new type of coupling procedure[56] and then carried out many experiments on model mixtures to test their extractability. We were able to establish rules of thumb which enabled the extractability to be estimated from the structural formulae[57]. At first, we worked in the pressure region up to 500 bar and subsequently extended this to 2500 bar[64]. Supercritical carbon dioxide was used and also, especially for alkaloid extraction, supercritical nitrous oxide[65]. A typical fluid extraction–thin-layer chromatogram (FE–TLC) is shown in Fig. 10.

This work on fluid extraction concluded for the time being our efforts to find suitable coupling procedures with TLC. The scheme in Fig. 11 summarizes our endeavours in this domain during the second decade of TLC. Various means of extracting sample components and transferring them directly to TLC are now available.

* Nl = Normal liter.

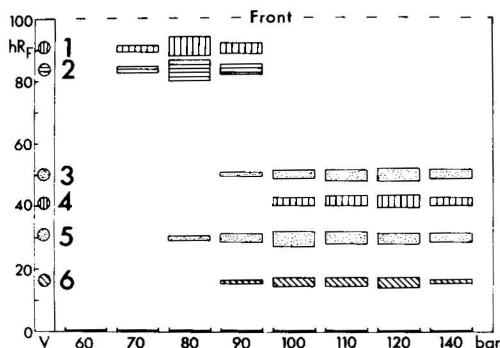

Fig. 10. Thin-layer chromatogram after fluid extraction of fat-soluble dye mixtures at increasing pressures. The amount of flowing carbon dioxide is 1 l in each instance. 1 = Guaiazulene; 2 = azobenzene; 3 = Ceres red; 4 = Ceres blue; 5 = Sudan red; 6 = indophenol.

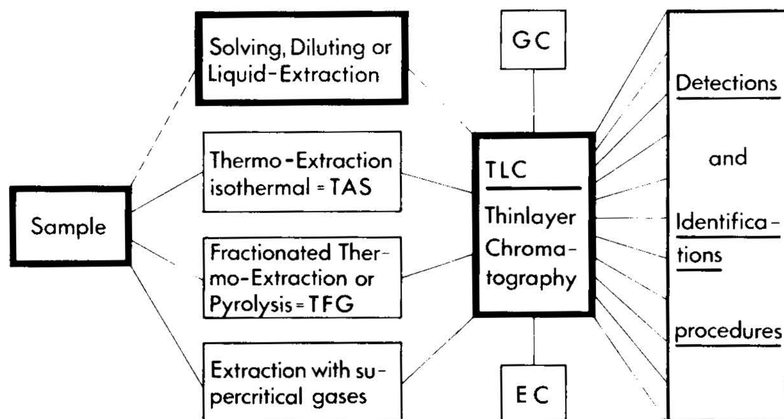

Fig. 11. Summary of the various possibilities of transferring substances from sample to thin-layer starting point.

8. OUTLOOK FOR THE THIRD DECADE

In considering the possibilities for the future, it must be borne in mind that TLC itself is a coupling procedure and consists of three parts (Fig. 12). The question now arises of the part of the procedure in which further progress is still possible and what are the aims. One can assume that a molecule A can be separated from a molecule B by TLC. This separation is, however, not visible. The lower limit of detection by the human eye cannot be significantly improved by either further diminishing the starting

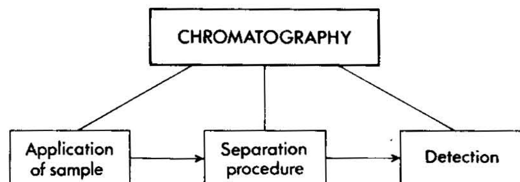

Fig. 12. Illustration that chromatography itself is a coupling procedure and consists of three main parts.

points, by shortening the run or by reducing the grain size of the layer material. Starting points of less than 1 mm and the nanolitre capillaries needed for them are widely considered to be no longer convenient. A much more important objection is, however, the fact that when the amount spotted is reduced to the utmost limit, the subsidiary and trace components usually present in the mixture can no longer be detected. One thus subjects oneself to a deception. It is similar to the use of an insensitive detector in gas chromatography or an insensitive reagent for detection in TLC (Fig. 13). In this way in the microgram range seemingly small zones create the impression of sharp and hence of good separations. The equipment for sample application is sometimes complicated and expensive and further development in the direction of simplification and cost saving would appear desirable. Something new in the sphere of sample application in TLC is, in my opinion, no longer to be expected.

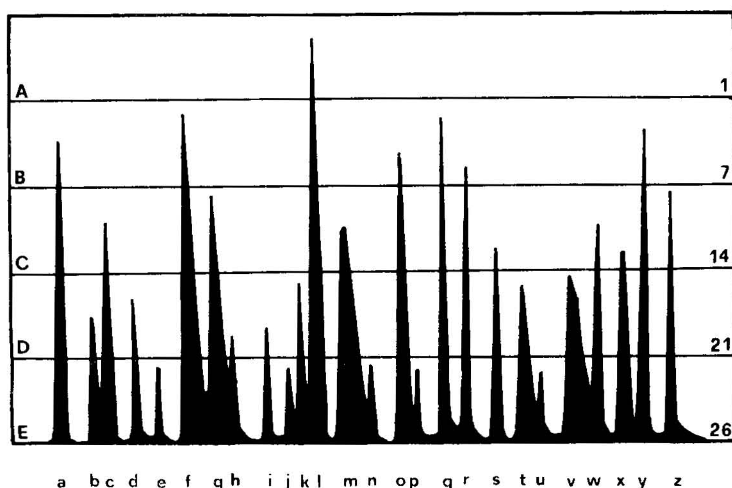

Fig. 13. Peak mountain of 26 substances, arising in various amounts on the chromatogram. In level A the detection is carried out with an insensitive agent, in level B a somewhat better agent allows the identification of 7 components, in level C a sensitive agent identifies 14 components, in level D a still more sensitive agent identifies 21 components and in level E 26 components are identified with a highly sensitive agent. In this range, however, there is a poor separation on account of the overlapping of substances. For a comparison, see the "mock" success of separation in level C.

Genuine progress does seem still possible in the separation systems. This does not mean through an increase or decrease in the plate size or layer thickness but rather through the adsorbents. The possibilities here are not yet exhausted. One should remember how long it took before a definite silica gel product with a narrow range of grain size could be prepared on the technical scale (see Introduction). The best products in this field are used for the industrial preparation of ready-made TLC plates but are still not available to those wishing to prepare layers themselves.

The really high-quality products are often obtainable only as highly expensive small packages for GC or HPLC. Exhaustive scientific investigation and exploration of methods of technical preparation in the vast field of possible adsorbents other than silica gel are also pressing needs. A limit to the solvents used has so far been self-

imposed and materials, other than the usual laboratory solvents and their mixtures have hardly been tried. Many new solvents have become commercially available in the last few years and ought to be tested for their value in solving special separation problems. The properties of a solvent are not determined alone by its dielectric constant.

A broad field, largely unexplored in TLC practice, is that of the gradient techniques. More attention should be paid to these possibilities in future. It is a pity that the relevant manufacturers are content merely to prepare single-stage gradient layers (inactive–active). Gradient layers and gradient elution are as much as ever procedures of future value.

In my opinion, the real emphasis of further development of TLC in the third decade will fall on the detectors (possibilities of detection). The present marked superiority of TLC in detection should be systematically extended. This is relatively easy if one takes into account the tasks and aims of the various organic and biochemical working groups. First, one must be clear that there are necessarily three different "types of detector" (detections):

(1) *Universal detectors:* detection of all compounds.

(2) *Detectors of specific groups:* detection of compounds containing particular functional groups or definite physical or biological properties.

(3) *Detectors of individual substances:* specific detection of individual compounds of a mixture.

A glance at the reagents hitherto used shows that much basic research is necessary here. The development of new biological and pharmacological microdetectors in combination or by coupling with TLC is an attractive and promising field. Another promising and plausible prospect appears to be the development of further specific detection methods for individual substances through suitable fermentation reactions (for example, in clinical diagnosis).

Yet another detection theme is the development of still more sensitive reagents. The conversion of colourless into strongly fluorescing compounds has already provided a good basis for this. One thinks, for example, of the introduction of the fluorescamine reagent in the extremely sensitive fluorescence detection of primary and secondary amines. "Multiplier" reactions, taking place in a similar manner to the sense of the Sandell–Kolthoff reaction, are also conceivable.

Another problem which could be better solved is the completely uniform application of the reagents to the layer, avoiding drops. Promising indications here are from the so-called application via the gas phase. Systematic further study is necessary. The influence of temperature and duration of heating on the optimal development of colour reactions on the thin layer is another study that has been neglected so far. UV lamps of better performance and simpler and cheaper means of documentation of the chromatograms in true colours are also widely desired.

A final question concerns the future of TLC in relation to the other chromatographic procedures. For the following reasons TLC will still remain the most used separation procedure:

(1) the greatest freedom of simple choice of stationary and mobile phases;

(2) the largest number of possibilities of detection (over 250 colour reagents);

(3) the possibility of simultaneous separation and detection of 15 or more samples and reference mixtures on a 20 × 20 cm layer;

(4) the possibility of simple coupling with micro-extraction and identification procedures;

(5) the simplest and cheapest chromatographic procedure for fast qualitative and semi-quantitative separation.

These five advantages justify the statement that TLC will keep its place in laboratories and will develop further. However, despite all possible progress, it must always be remembered that TLC is only one of the possibilities in the domain of separation methods and that research problems are best solved only by the purposeful use of all analytical methods.

9. SUMMARY

In a retrospect of my work on thin-layer chromatography, a summary of results and progress is given. Between 1957 and 1967, emphasis lay on the standardisation of the method, on increasing the number of applications in the hydrophilic field and on the extension of the method to the nanogram range. In the second decade, we developed methods for solvent-free extraction of micro amounts in direct combination with thin-layer chromatography, such as the TAS-procedure, thermofractography and the micro-extraction with supercritical gases.

Finally, the report describes the prospects for the third decade of thin-layer chromatography and the aim of even greater improvement in matters of detection.

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ASSESSMENT OF THE CURRENT STATUS OF REACTION LIQUID CHROMATOGRAPHY

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1. INTRODUCTION

The introduction of chemical reactions in liquid chromatography as an aid to improving the detection properties and selectivities for certain groups of compounds has become well known in recent years, and is demonstrated by the rapidly increasing number of publications and the appearance of reviews and books[1–3] in this area. Pre- and post-chromatographic derivatization techniques can be distinguished, and are treated separately in this paper.

2. PRE-CHROMATOGRAPHIC TECHNIQUES

The major reasons for separating compounds as derivatives in liquid chromatography are to improve the detection properties and to improve the selectivity. The latter can be attained by using the selectivity of the reagent in a clean-up process.

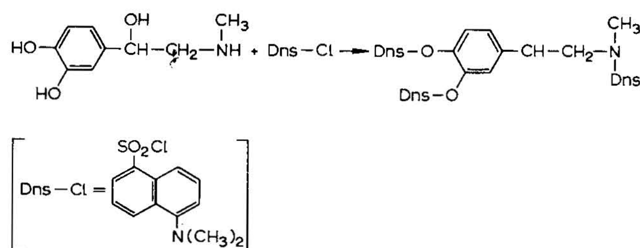
Pre-chromatographic techniques offer several advantages over post-chromatographic methods. One is that the reaction is independent of the mobile phase. The kinetics of the reaction are also not very critical provided that we have a reproducible and relatively simple procedure. The possibility of working with a large excess of reagent and having no difficulty in eliminating such an excess is attractive.

On the negative side we have the high risk of artefact formation, which often necessitates an extensive study of the reaction conditions and of the resulting derivatives.

A typical example which illustrates the problems and limitations of these techniques is the 5-dimethylaminonaphthalene-1-sulphonyl (Dns) derivatization of

adrenaline to form a fluorescent derivative suitable for blood analysis. The possibilities of the fluorescence labelling of amines and phenolic compounds with Dns-Cl and related reagents have recently been discussed[3], and the applications of this technique to biogenic amines, particularly catecholamines, have been studied by Schwedt and Bussemans[4].

The derivatization of the adrenaline proceeds according to the following reaction:



This reaction has been shown by Nachtmann *et al.*[5] to proceed quantitatively by the use of titration procedures.

As can be seen in Fig. 1 (curve A), the reaction proceeds to complete substitution after 10 min, 3 moles of Dns-Cl reacting with 1 mole adrenaline. Some other alkaloids that were also investigated showed ratios of 2:1 and 1:1, respectively. These results were confirmed by nuclear magnetic resonance (NMR) studies on the isolated derivatives. Studies of the fluorescence properties of these derivatives showed that the tri-Dns-adrenaline, in spite of being the most substituted derivative, had the lowest fluorescence yield (Fig. 2) and is unstable in that the fluorescence increases rapidly (*ca.* 50 % in 25 min) upon irradiation with UV light. The other derivatives, in contrast,

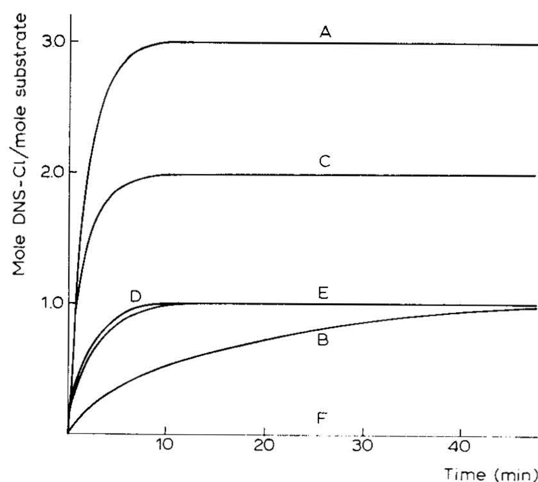


Fig. 1. Corrected molar ratios obtained by continuous titration monitoring of the dansylation reaction. (A) Adrenaline; (B) ephedrine; (C) cephaeline; (D) emetine; (E) morphine; (F) codeine with no reaction occurring[5].

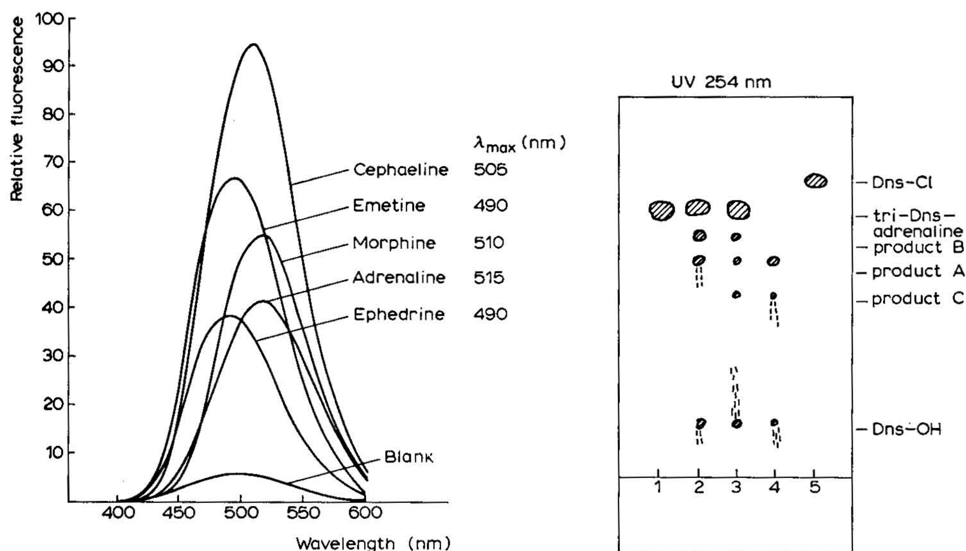
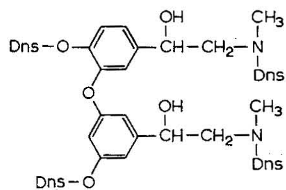


Fig. 2. Fluorescence emission spectra of the Dns derivatives recorded with a Zeiss PMQ 2 instrument equipped with a ZFM4 fluorescence attachment. Excitation at 365 nm[5].

Fig. 3. Thin-layer chromatogram of tri-Dns-adrenaline and its photodegradation products[6]. (1) Tri-Dns-adrenaline (pure); (2) tri-Dns-adrenaline irradiated in benzene solution; (3) tri-Dns-adrenaline irradiated on the plate; (4) product A irradiated in benzene solution; (5) Dns-Cl. Chromatography carried out on Merck SI-60 F_{254} silica gel plates. Mobile phase: benzene–toluene (3:1).

showed a decrease of a few percent in this same irradiation time. This led us to believe that adrenaline is one of the borderline cases for a meaningful application to pre-column derivatization and prompted us to study this system further.

Irradiation of purified tri-Dns-adrenaline (structure confirmed by infrared spectroscopy and NMR) produced several photochemical degradation products, as can be seen in Fig. 3. Tri-Dns-adrenaline and Dns-OH, split off the derivative in the degradation process, were positively identified on the basis of R_F-values. Product A (Fig. 3) was isolated and the structure determined by NMR. Product A seems to correspond to an asymmetrical dimer with the following structure:



There is good evidence that product B corresponds to the symmetrical dimer. Upon irradiation of products A and B, further degradation occurs (Fig. 3), resulting in a rapid increase in fluorescence followed by a decrease at a lower rate.

The following explanation may serve to explain these phenomena. The tri-substituted adrenaline is strained owing to steric hindrance, as can be clearly demon-

strated on a model; this would also explain the relatively low fluorescence yield (Fig. 2). Upon degradation, one Dns moeity splits off in the neighbouring hydroxyl positions of the adrenaline molecule and the dimers are formed. This results in a less strained molecule with a higher fluorescence yield. The fluorescence is further enhanced when the dimers split into the di-substituted monomers (probably product C in Fig. 3). Prolonged irradiation results in a further degradation to the mono-Dns derivative and hence a net decrease in fluorescence.

Practice has shown that with sufficient care it is still possible to use the tri-Dns derivative of adrenaline for analytical purposes. A method has been developed for the determination of Dns-adrenaline and Dns-noradrenaline in blood plasma by high-performance liquid chromatography (HPLC)[6]. On the other hand, this example also draws attention to the general risk of artefact formation in pre-chromatographic derivatization techniques and the need to carry out studies of this nature for critical systems before the development of an analytical technique is perfected.

In some instances UV labelling may be preferred to fluorescence labelling, in spite of losing the inherent sensitivity of fluorescence techniques. The situation can often be considerably simpler and more predictable for UV derivatization and the additivity of extinction values, depending only on the degree of substitution, can be useful in quantitative work, as has been shown in recent studies with sugars and cardiac glycosides[7–9]. For the above system, which involves *p*-nitrobenzoylation of non-aromatic hydroxyl groups, not only a better UV detectability but also a better chromatographic selectivity was obtained, which permitted the analysis of complex mixtures such as that shown in Fig. 4 for an extract from digitalis plants.

The many other possibilities that exist for pre-chromatographic derivatization for fluorescence and UV labelling can be found in detail in two books[2,3].

For molecules that possess no suitable active group for derivatization but have a reasonable basic or acidic activity, there remains the possibility of ion-pair chromatography, the idea again being to choose an ion partner with a good chromophore or fluorophore. Much of the original work in this area was done by Schill and co-workers[3,10], who also treated the theoretical aspects of ion-pair chromatography. Santi *et al.*[11] have demonstrated the application of the technique to tropane alkaloids forming picrate ion pairs. The use of these techniques in routine pharmaceutical analysis in dissolution rate and content uniformity testing has been demonstrated by the same group[12]. An application of this principle to the analysis of active principles in a tablet formulation for the treatment of migraine is shown in Fig. 5.

The problem was to determine small amounts of ergotamine and hyoscyamine in the presence of up to a 1000-fold excess of caffeine and butalbital. By forming picrate ion pairs for the two basic components ergotamine and hyoscyamine, and detection at the UV maximum of the picrate ion (254 nm), it is possible to discriminate partially between the other two components (see Fig. 5). Complete supression of the absorbance of caffeine and butalbital is possible at the second absorption maximum of the picrate ion (345 nm) (see Fig. 6), and a sensitive and reproducible quantitation of the two alkaloids is therefore feasible. The search for other ion partners with still better detection properties is continuing[13].

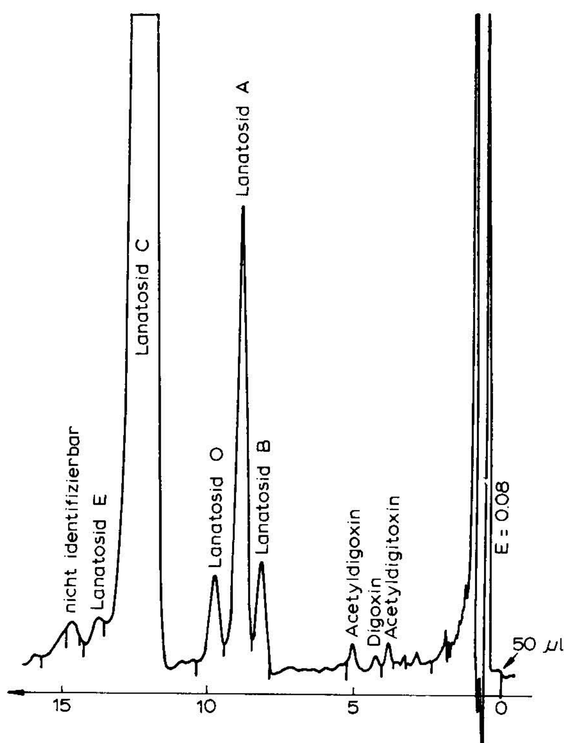


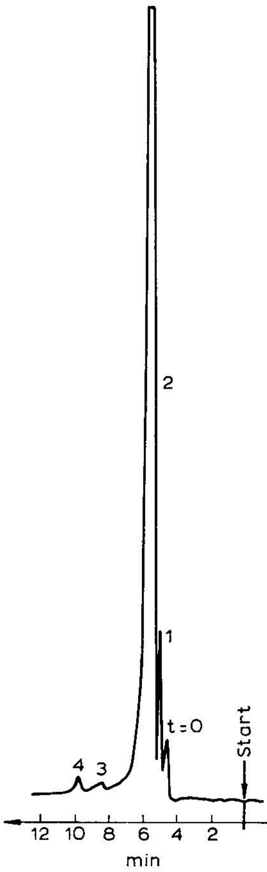
Fig. 4. HPLC of 4-nitrobenzoyl derivatives of some digitalis glycosides in a plant extract. Chromatography: *n*-hexane–methylene chloride–acetonitrile (10:3:3). Column, SI-60 (5 μm), 15 cm × 3 mm I.D.; flow-rate, 1.5 ml/min; Δp, 120 atm. Apparatus: Hewlett-Packard UFC 1000 with DuPont 842 UV detector (254 nm). Rheodyne 7105 injection system; 20-μl injection volume in chloroform.

3. POST-CHROMATOGRAPHIC TECHNIQUES

The developments of post-column reactors in liquid chromatography in recent years can be traced to the lack of detectors with sufficient sensitivity and selectivity for certain analytical problems. The advantages and disadvantages have been elaborated on earlier[1,2], but certainly the major advantages of the reaction detector are that artefact formation is not critical and that the reaction does not have to go to completion or give well defined derivatives, provided that it is reproducible.

The most serious disadvantage, on the other hand, is the interdependence between the mobile phase and the reaction medium, and this has probably been one of the major reasons why the development of post-column techniques in HPLC has not been very widespread until now.

Obviously the kinetics of a reaction are also very important and determines to a large extent the feasibility of a reaction detector and which type should be used. There are three different principles for the construction of such reactors: (1) tubular or capillary reactors, (2) bed reactors and (3) air-segmented streams such as are used in automated analysers.



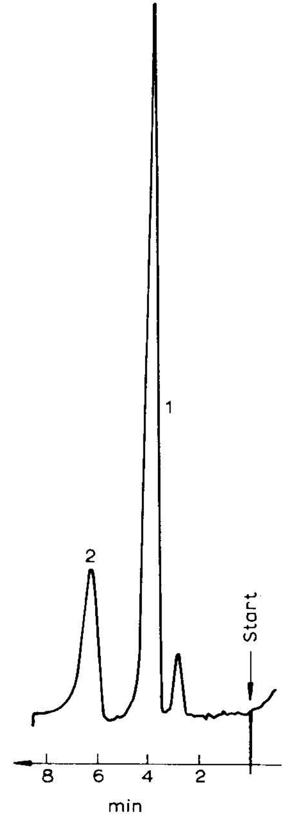


Fig. 5. Separation of components in Cafergot PB®. Column, SI-100 (5 μm), 15 cm × 3 mm I.D. Mobile phase, chloroform saturated with stationary phase, 0.06 *M* picric acid at pH 6. Flow-rate, 0.2 ml/min. Detection at 254 nm (1.0 a.u.f.s.). Peaks: 1 = butalbital; 2 = caffeine; 3 = hyoscyamine; 4 = ergotamine[12] (components 3 and 4 separated as ion pairs).

Fig. 6. Separation of Cafergot PB® components as ion pairs. Detection at 345 nm (0.05 a.u.f.s): 1 = hyoscyamine; 2 = ergotamine[12].

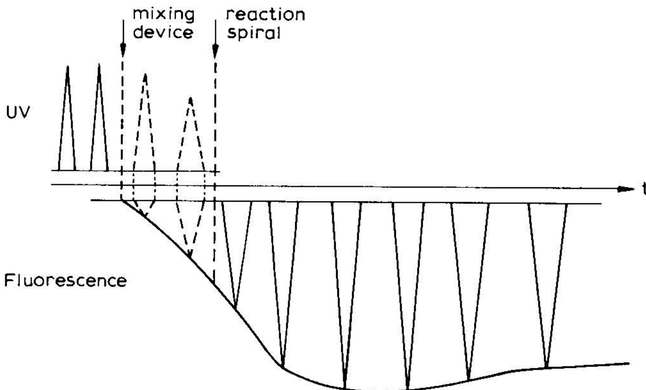


Fig. 7. Schematic representation of peak behaviour in a post-column reactor[16].

3.1. Tubular reactors

As in any other post-column reactor, the major problem is to avoid excessive band broadening during the reaction. As shown schematically in Fig. 7, this band broadening can occur in the mixing device(s) during addition of reagent and in the reaction spiral. An optimal design is therefore essential in order to avoid substantial decreases in the chromatographic resolution. The theoretical aspects of band broadening in tubular reactors have been discussed recently[14]. Based on classical flow dynamic principles, it is relatively simple to predict band broadening in tubular reactors.

A typical example of the use of a tubular reactor is briefly discussed below. It involves the reaction of nonapeptides with Fluram® [15,16].

The rapid kinetics of this fluorigenic reaction are shown in Fig. 8. A plateau for this reaction is reached in *ca.* 50 sec. It is now possible to use a much shorter reaction time, because at 10 sec there is close to 90% of the total fluorescence. Plotting peak heights against signal will result in a maximum (see Fig. 8), which can give an indication of the optimal reaction time as at this point band broadening will offset any further gains in fluorescence.

The design of the mixing unit is also of utmost importance, particularly when solutions of different densities are mixed and when the flow-rates of the mobile phase and the reagent stream differ considerably. In such instances mixing against the eluent stream causes more turbulence and radial mixing and hence less band broadening. Some useful designs are shown in Fig. 9.

An actual application of this principle is shown in Fig. 10, representing a duplicate injection of a peptide mixture, derivatized with Fluram and detected by fluorescence. It can be seen that the reproducibility of the chromatographic pattern is good and the reproducibility of peak areas for major peaks is such that one can truly speak of a quantitative technique. The band broadening measured for the oxytocine peak due to the mixing device and tubular reactor is of the order of 4% for a 10-sec reaction time. Theoretical calculations give a lower value (3.5%), but do not include the influence of the mixing device. Experimental results have shown that with the conditions used (see Fig. 10) one obtains about 7% peak broadening for each 500-μl reaction volume (spiral volume). If we now assume a 1-min reaction time with a total flow-rate (reagent solution + eluent) of, say, 2 ml/min we would have 28% band broadening due to the reaction unit, which for many applications would not be acceptable. Clearly in such a case one might have to consider the use of a bed reactor.

3.2. Bed reactors

The use of bed reactors consisting of columns packed with glass beads of various sizes has been developed primarily by two groups[14,17]. Such a bed reactor can be considered as a chromatographic column essentially used under t_0 conditions (no retention) and the theory for predicting band broadening phenomena in a bed reactor is consequently based on parameters such as packing geometry, average particle size, tortuosity, fluid velocity and diffusion coefficients[14,17]. Taking again the above example in which with a tubular reactor a band broadening of 28% was obtained and calculating the expected band broadening with the equations proposed earlier[14], one would obtain well below 5% band broadening. Hence clearly for reaction times of 1 min or longer one would preferably use bed reactors in spite of the complication introduced.

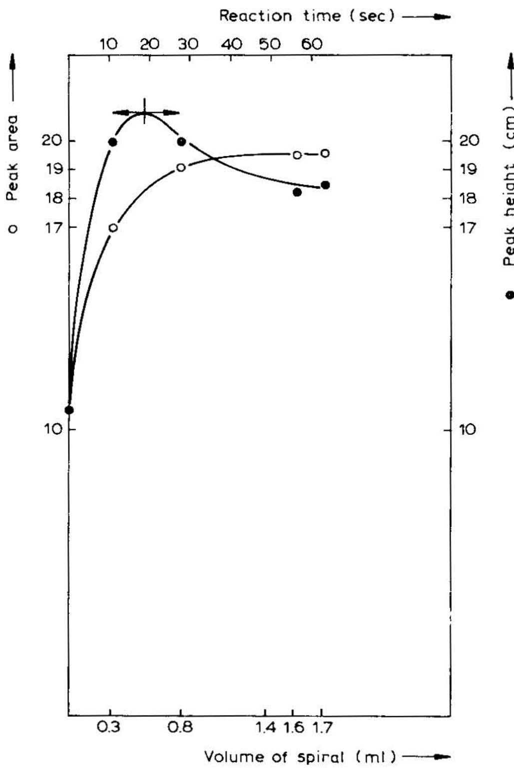


Fig. 8. Influence of length of spiral (reaction volume and time) on the fluorescence signal. Conditions: column, RP-8 (10-μm), 10 cm × 0.4 cm I.D.; eluent, acetonitrile–water (20:80), pH 7; reagent, Fluram (30 mg per 100 ml of acetonitrile). Eluent flow-rate, 1.6 ml/min; reagent flow-rate, 0.15 ml/min; injection of 100 μl of oxytocine (5 I.U./ml) via loop; fluorescence detection with Aminco fluoromonitor[16].

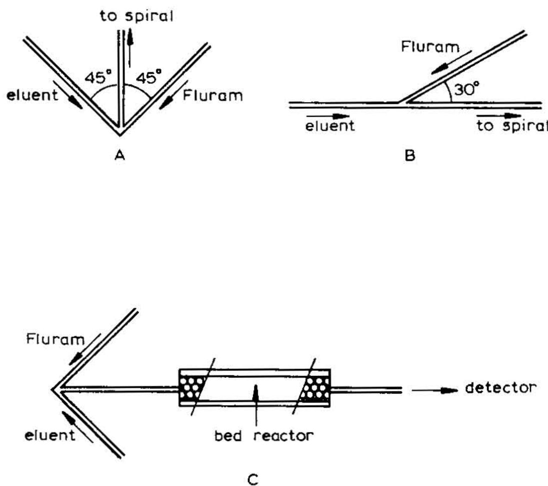


Fig. 9. Different mixing units used for the Fluram–peptide reaction[16].

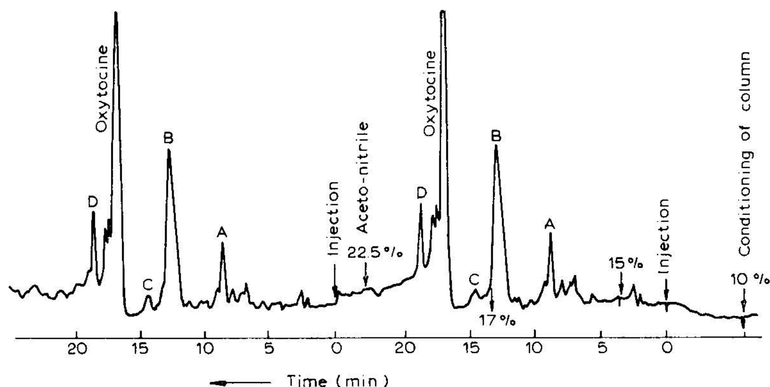


Fig. 10. HPLC of a peptide mixture (purification fraction for oxytocine). Duplicate chromatogram. Step gradient: acetonitrile, 10 to 22.5% (pH 7). Other conditions as in Fig. 8.

3.3. Segmented-flow reactors

For even longer reaction times (*i.e.*, longer than 5 min), one would have to resort to the air-segmentation principle in order to overcome excessive band broadening. The theoretical aspects of air-segmented flows are the most complex and least understood. A semi-empirical approach to achieve a better understanding of the principle was made by Snyder[18].

Band broadening in the reaction unit can be attributed in part to leakage between the segments (spaced by air bubbles) due to wetting of the capillary wall. This effect, however, is small in comparison with the band broadening effects introduced by mixing tees, debubblers or phase separators. Much effort is therefore necessary to achieve better designs of these parts or possibly electronic debubbling techniques.

A typical example of such a longer reaction is given here and involves the dehydration of cardiac glycosides with concentrated hydrochloric acid to form a fluorescent product. This process is enhanced by adding a mixture of hydrogen peroxide and ascorbic acid, the exact mechanism being unknown. The extrapolated kinetic curve for this reaction is shown in Fig. 11. A plateau is reached after about 0.5 h and, following our previous theory, an actual reaction time of 10 min was chosen. Use of the air-segmentation principle is the only feasible approach from the point of view of both reaction time and agressiveness of the reagents. An all-glass–PTFE AutoAnalyzer unit was used.

A schematic diagram of the apparatus is shown in Fig. 12. The system corresponds to the standard Technicon second-generation AutoAnalyzer unit except for the reaction spirals, which where constructed with 1 mm I.D. Portex tubing. It was possible with such a system to keep the band broadening due to the AutoAnalyzer reactor unit below 10%, which is surprising when it is considered that a mechanical debubbler was used. The corresponding decrease in chromatographic resolution compared with the UV signal can be seen for the two pairs of glycosides in Fig. 13. The improvement in detectability, which is about 100 times better than for direct UV detection, and the gain in selectivity compensate fully for this decrease in resolution.

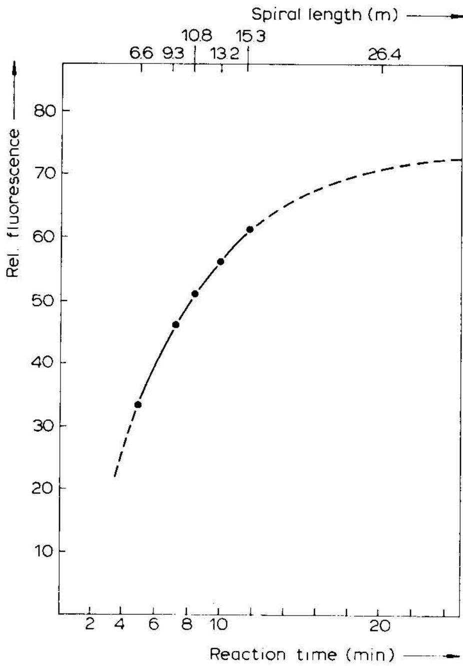


Fig. 11. Extrapolated kinetic curve for the fluorescence reaction of desacetyllanatoside C′ with concentrated hydrochloric acid[19].

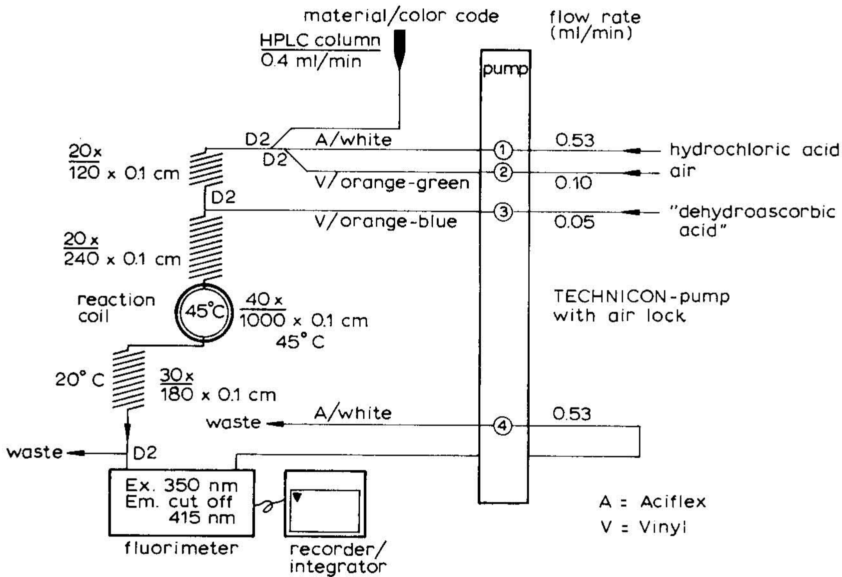


Fig. 12. Schematic diagram of the reaction detector unit for fluorescence detection[19]

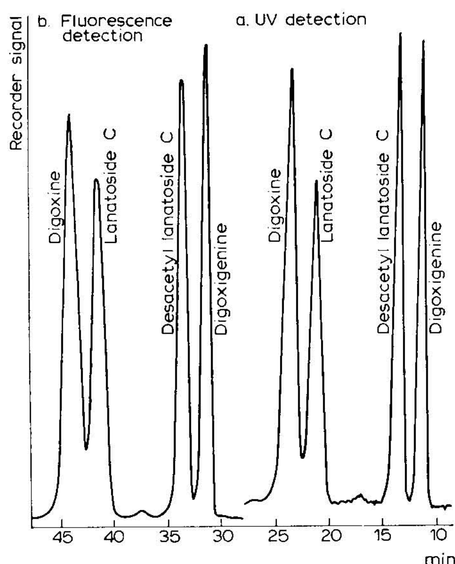


Fig. 13. Comparison of the resolution of the cardiac glycosides obtained with UV detection right after the column and with fluorescence detection after the post-column reaction[19].

4. CONCLUSION AND PROSPECTS

Derivatization techniques can be a very powerful means of enhancing the effectiveness of modern liquid chromatographic techniques and detectors. In order to make full use of them, it is important to recognize the limitations and pitfalls and to make the proper choice of the derivatization mode. As more reagents become known and commercially available for practically all feasible functional groups, it should be possible to adapt these techniques to a wide range of problems.

In post-column techniques, many groups are still working on optimization of technical designs to reduce band broadening further; however, as the theoretical aspects of these reactors become better known one could expect good advances in this area. In 1977 significant advances in this direction could be discerned.

The possibilities of adapting thermal[17], photochemical[20] and catalytic[21] processes to initiate, accelerate and simplify post-column reactions are being actively pursued by many groups. Coupling of reaction detectors with step gradients[16] (see Fig. 10) and with large volume injections[16,22] will greatly enhance their usefulness. Even though the examples discussed in this paper were restricted to UV and fluorescence detection, there is no doubt that this philosophy can be extended to other detection modes. In electroanalytical detection this has in fact already been done[14,23] and work in this direction is also in progress in our laboratories.

The possibilities of using chemiluminescence techniques has been discussed by Neary *et al.*[24]. Metal chelation phenomena have been used to adapt atomic absorption spectrophotometry to HPLC detection[25] and can also be interesting for electroanalytical detectors or flame and plasma emission techniques. The use of flame emission spectroscopy for HPLC has been demonstrated by Kirkland[26] and Freed[27].

Other similar areas of development may be seen in the future for radiochemical[28], mass spectroscopic and phosphorescence detection.

There are many possibilities in this area for the imaginative analytical chemist. The enhanced sensitivity and selectivity that can be gained will often be essential for solving some of the complex trace analytical problems that we are confronted with in modern problem-solving processes.

5. SUMMARY

Some of the advantages and also pitfalls and limitations of chemical derivatization techniques are discussed. For post-column derivatization, different reactor designs are discussed and compared. Finally, prospects are surveyed for further development in this field.

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CAPILLARY ISOTACHOPHORESIS

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1. INTRODUCTION

It is well known that the basic principles underlying many modern analytical techniques were enunciated several decades before their practical applications. In most instances, this was mainly due to the unavailability of materials and equipment necessary for the practical realization of the basic ideas.

This is especially true of electrophoretic techniques. The advent of different kinds of plastic materials, the development of new components and ideas in electronics and the synthesis of new chemicals contributed to bridging the gap between basic principles and daily use. Isotachophoresis is no exception. Although theoretical studies were first published over 50 years ago[1,2] and some use of the principle was made in disc electrophoresis[3], the practical application of isotachophoresis is still in its infancy.

Isotachophoresis is no replacement for the other electrophoretic techniques and its possibilities are not restricted to the biochemical field. In many instances, where rapid and precise analyses of samples with fairly uniform composition must be made, isotachophoresis is becoming the method of choice. Provided that the compounds of interest can be made to move in an electric field, isotachophoresis, with its wide range of operating conditions and detection possibilities, should at least be considered.

Zone electrophoresis, in one or more of its variations depending on the support materials, size and combinations with other techniques, is familiar to most workers.

Isoelectric focusing, although introduced in practice more than a decade ago, is

much less well known. Even if the possibilities of isoelectric focusing are more restricted than those of zone electrophoresis, its superiority in some fields has been proved. The most characteristic feature of this technique is that a true steady-state equilibrium can and must be reached. The method has two outstanding advantages: a very high reproducibility and a useful concentrating effect. Its practical application is restricted almost exclusively to the field of protein chemistry.

Isotachophoresis is the latest of the electrophoretic techniques. The discussion of its theoretical aspects is beyond the scope of this paper and interested readers are referred to a complete treatise on the subject which appeared in 1976 [4].

In zone electrophoresis, the charged molecules constituting the sample are separated under the influence of an electric field into more or less well separated zones in a uniform supporting electrolyte. Each zone moves with a characteristic speed, depending mainly on the charge of the molecules, the ease with which they move through the supporting material and the electric field strength. Each zone is overtaken and diluted continuously by the electrolyte ions, which leads to a continuous broadening of the zones and hence a decrease in the sensitivity.

Isotachophoresis, on the other hand, makes use of a discontinuous system: two different electrolyte solutions are used. The first electrolyte contains an ion (the leading ion) with the same charge sign as that of the sample ions to be separated, but with an effective electrophoretic mobility higher than that of the fastest moving of the sample ions. The second solution contains the terminating ion. It too has the same charge sign, but an effective electrophoretic mobility lower than that of the slowest moving of the sample ions to be separated; a common counter ion, chosen for its buffering capacity at the desired separation pH, is used for both electrolyte solutions.

The mixture of sample ions is brought between the two electrolyte solutions so that the conditions are such that all of the different sample ions always remain sandwiched between the leading and terminating ions. At the same time as the leading and terminating ions move under the influence of the electric field, the sample ions arrange themselves in order of their electrophoretic mobilities. Fig. 1 gives a schematic representation of the different stages of an isotachophoretic separation experiment.

In isoelectric focusing, once the equilibrium has been reached, the different sample components stop and are focused at well defined positions of the stationary pH gradient.

In isotachophoresis, on the other hand, the equilibrium is a dynamic one: the leading and terminating ions move with a constant velocity and the sample ions, also at the same speed, move in an equilibrated manner between them; hence the derivation of the name of this technique (= moving with the same speed).

Several remarks concerning this dynamic equilibrium must be made:

(a) If the different sample ions have sufficiently spaced effective electrophoretic mobilities, then each component will form a separate zone. The different zones follow each other without interruption.

(b) The classification of the different zones is based only on the electrophoretic mobility.

(c) From the Kohlrausch equation[2], it follows that the concentration of the leading ion and the effective mobility of a sample ion are the only factors that determine the concentration of an ion in its zone. It can thus be concluded that for a given set of experimental conditions, the zone length is a direct measure of the amount of a

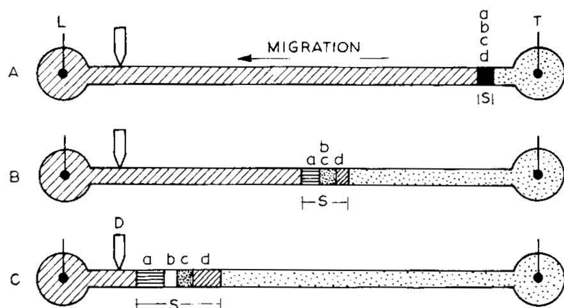

Fig. 1. Different stages of an isotachophoretic separation experiment. L = Leading electrolyte; T = terminating electrolyte; D = detector; S = sample with four components. A, Start condition; B, mid-way, with partial separation of sample ions; C, just prior to detection, all sample ions are separated.

component present in the system. Another important consequence of these properties is the concentrating effect of isotachophoresis. Indeed, if a component is present at very low concentration then, at equilibrium, this component will be concentrated in a very narrow zone. Detection of trace amounts of some components in complex mixtures can often be achieved.

(d) The potential gradient in a given zone is dependent on the mobility of the ion in that zone. The voltage drop per unit length of a zone remains constant over the whole length of a zone. As the current is kept constant, this implies that the heat generated in the different zones can give useful qualitative information about the component in a given zone.

The most important requirements for analytical isotachophoresis can be formulated as follows:

(1) Isotachophoretic separation experiments must be carried out under strictly controlled conditions.

(2) The reliability and accuracy that can be obtained in isotachophoresis are a function of the general operating conditions.

(3) The chemicals used to prepare the electrolyte solutions must be of analytical-reagent grade and be further purified by recrystallization, distillation or other procedures.

(4) The detection systems should be completely without drift and noise.

(5) All of the operating parameters (voltage, current, temperature, electrolyte system and pH) should be recorded precisely.

(6) The duration of an isotachophoretic run depends on the operating conditions, the electrolyte solutions and the sample composition. An average analysis time of between 10 and 20 min is typical.

2. APPARATUS

As isotachophoresis in a capillary tube is by far the most interesting analytical application of this technique, only this system will be considered in detail here.

In Fig. 2, the basic equipment needed for an isotachophoretic experiment is presented. The following components can be considered:

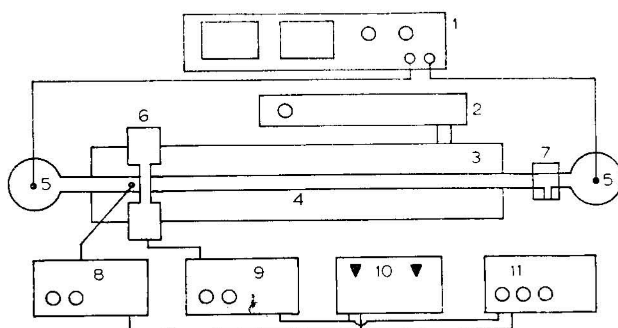

Fig. 2. Equipment for capillary isotachophoresis. 1 = Power supply; 2 = thermostat; 3 = thermostated environment for capillary; 4 = capillary; 5 = electrolyte vessels; 6 = UV detector assembly; 7 = injection port; 8 = thermometric signal amplifier; 9 = UV signal amplifier; 10 = two-pen recorder; 11 = electronic peak surface integrator.

(i) The actual separation takes place in a glass or PTFE capillary tube of less than 0.5 mm I.D.

(ii) The temperature of the capillary is kept constant either by winding it around a metallic cylinder or by immersing it in a liquid. The temperature of the cylinder or liquid must be carefully controlled.

(iii) On each side, the capillary is connected to an electrolyte vessel provided with a platinum electrode.

(iv) On one side of the capillary, provision must be made to allow for sample introduction. This can be done through a septum, as in gas chromatography, or by a multi-port valve system.

(v) A high-voltage power supply capable of delivering 500 μA at up to 20–30 kV is needed. The constant current regulation of the power supply must be extremely well designed. The electrical current through the capillary is very important as it determines not only the time of analysis, but also the temperature and the zone boundary sharpness. Further, as the zone length gives quantitative information about the amount of the components present in the sample, it is extremely important that the actual speed with which all ions are forced through the capillary remains absolutely constant.

(vi) One or more detectors must be provided. The most popular detection systems are thermometric, potentiometric and UV absorption.

We shall not consider the practical details of all of the components here, but the detectors should be considered more closely.

By far the most important detail that distinguishes capillary isotachophoresis from most other electrophoretic techniques is that detection of the separated components forms an integral part of the analytical unit. In zone electrophoretic and isofocusing experiments, detection of the separated components is carried out after the actual separation and often takes more time and effort than the separation stage itself. In isotachophoresis, the results of the separation experiment are immediately available and in most instances not only qualitative but also quantitative information can be obtained.

Temperature measurement on the outside of the capillary tube is the most

universal detection system. As already explained, under a given set of experimental conditions, the heat produced in a zone is dependent on the effective mobility of the ion in that zone. By expressing the heat produced in a certain zone as a percentage of the difference in heat produced in the leading and terminating electrolytes, qualitative information about the ion in a zone is obtained. This value is referred to as the step height (for an example, see Fig. 8). On the other hand, as the different ions form zones with sharp boundaries, it follows that the zone boundaries are characterized by sharp temperature changes. These temperature changes can be used to detect the passage from one zone to the other and hence to obtain information about the zone length. This in its turn is a measure of the amount of a component present in the sample. The practical usefulness of the thermometric detector is hampered by its slow response: heat must pass through the capillary wall. Differentiation of the thermal signal can sometimes be an aid in increasing sensitivity.

Potential gradient detectors consist of electrodes inserted through the capillary wall. The voltage drop between the two electrodes is measured with an independent instrument. As already stated, the potential gradient in a given zone depends on the effective mobility of the ion in that zone. The voltage drop between the electrodes can therefore be used to obtain qualitative information. Also, as with the thermometric detector, the sudden shift in potential gradient from one zone to the next can be used to detect zone boundaries.

Instead of the potential drop between two closely spaced electrodes, the conductivity of the solution can be monitored by resistance measurement, using an alternating current between the electrodes.

In any case, extreme care should be taken to control all leak currents between the electrodes.

A third type of detector makes use of UV absorption. In the dynamic equilibrium stage of an isotachophoretic experiment, all ions move with the same speed in individual zones and only the counter ion is mixed with them. Hence the measurement of the UV absorption can give valuable information about some of the ions and at the same time serve to detect zone boundaries.

In some instances, it is even possible to detect the boundaries between two consecutive non-UV-absorbing zones. Indeed, it is extremely difficult to prepare electrolyte solutions without trace amounts of UV-absorbing material in them. As these impurities are present in trace amounts, they generally form extremely narrow UV-absorbing zones sandwiched between non-UV-absorbing zones of interest. Zone boundaries then show up as spikes on the otherwise flat UV baseline (for an example, see Fig. 3B).

In practice, the UV absorption must be made directly on the capillary tube itself. The total internal volume of the complete capillary is so small (of the order of 40 μl) that no enlargement for a UV-absorbing cell, as in liquid chromatography, can be used. Therefore, stringent conditions are put on the light source, the UV detector and the signal amplifier.

3. CHOICE OF OPERATING CONDITIONS

Several factors must be considered in choosing a suitable electrolyte system for the optimal separation of a given sample. The separation of the different com-

ponents of a sample is primarily influenced by the difference in effective electrophoretic mobility of the ions. All operating conditions influencing the mobilities must be optimized in order to achieve rapid and complete separation of the components of interest.

Mathematical equations governing all the factors that influence an isotachophoretic experiment have been worked out[4]. However, in many instances, not all of the basic data necessary for application of the equations are available. The choice of suitable electrolyte systems will more often than not be a matter of experience and intuition. Positive and negative influences must be weighed against each other.

We cannot go into great detail here, but the most important factors are the following:

(a) The choice of the solvent. The replacement of water by deuterium oxide or a non-aqueous solvent can sometimes improve a separation.

(b) The choice of the buffering counter ion. This must be chosen with consideration of the desired separation pH and offers maximal buffering capacity at that pH.

(c) The choice of the leading ion. A prerequisite for isotachophoresis is that the leading ion must have a mobility higher than that of any of the sample ions of interest. However, its mobility must not be too high otherwise, and this is especially true for large molecules with low mobilities, it may be that insufficient time is allowed for all of the sample ions to reach a true dynamic equilibrium.

(d) The choice of the terminating ion. In most instances, it will have a lower effective mobility than that of the slowest of the sample ions of interest. In some special instances, however, a terminating ion with a mobility such that some of the sample ions are overtaken by it may be of help in simplifying the separation pattern.

(e) Additives, such as mobility spacer ions, stabilizers, surface-active agents and internal standards, can be chosen for addition to the system to suit individual needs.

4. SOME PRACTICAL ASPECTS

As yet there is little choice in the commercial apparatus for analytical isotachophoresis, probably because of the exacting demands put on most of the components needed to form a complete and versatile analytical ensemble.

High-voltage power supplies, d.c. signal amplifiers, thermostating equipment and recorders that satisfy most of the demands of capillary isotachophoresis can be bought separately. However, the construction of the actual analytical sub-unit, consisting of the mounted capillary, the sample injection system and the electrolyte vessels, can only be constructed in advanced workshops with the highest standards.

The different electrolyte solutions must be prepared with the utmost care. Not only can impurities in the chemicals show up as unwanted peaks or zones, but many of the separation parameters can be influenced by them. One of the most important factors, and perhaps the most often overlooked, is the actual surface condition of the inside wall of the capillary. Some of the sample components and/or impurities in the electrolyte solutions can influence this condition. Complete or patchwise coating of the capillary wall with these components can drastically alter the duration of an experiment (electroendosmosis), the voltage reached during detection (loss of bound-

ary sharpness), change in temperature profiles (current leakage) and drift in the UV baseline by slow release of previously absorbed components from the coating. Even analytical-reagent grade chemicals in most instances need one or more additional purification steps. Blank runs without sample should be made regularly, especially after a new batch of electrolyte solutions has been made up.

The composition of the sample, other than the components of interest, must be kept under control. The ionic composition of the sample should be such that the chosen separation pH of the leading electrolyte is not altered appreciably by sample injection.

One of the main advantages of capillary isotachophoresis is the absence of a support material, as in zone electrophoresis. (We compare here capillary isotachophoresis with zone electrophoresis in a support. There is of course also capillary zone electrophoresis which does not have all advantages, and isotachophoresis in supports which has some disadvantages.) This has several advantages: electroendosmosis is reduced to a very low value; irreproducibility of results, due to variations in the physical and chemical properties of a support material, is eliminated; the effective mobility of the charged molecules is not affected by the passage through the support material.

The concentrations of the electrolyte solutions are normally in the range 0.01–0.005 mole/l. The lower limit of detection for a given compound becomes lower as the concentration of the leading electrolyte is lowered. Unfortunately, below 0.001 mole/l, the contribution of the proton and the hydroxyl groups to the migration becomes appreciable. For the same reason, separation pH values outside the range 3–10 are less suitable.

5. PRACTICAL APPLICATIONS

Capillary isotachophoresis has already found a wide range of applications in many different fields of research, production and control. (For literature surveys see Everaerts *et al.*[4] and the LKB reference lists.) It is impossible to give a comprehensive list, but among the most important are the following:

(a) Pollution control: detection and measurement of inorganic and organic compounds.

(b) Process control: metal ions, organic radicals.

(c) Quality control: amino acids, organic acids, antibiotics, organophosphates, etc.

(d) Research: proteins, peptides, amino acids, organic acids, nucleic acids.

The practical applications presented below were selected with the sole aim of stimulating interest in this new analytical technique.

The separation by isotachophoresis of cationic species seems to have attracted the least attention. Everaerts *et al.*[4] described several electrolyte systems that can be used to separate different metal ions and organic compounds with amino groups. Either hydrogen or potassium ions are used as the leading ion with Tris as the terminating ion. Depending on the chosen separation pH, acetic or cacodylic acid can be used as the counter ion. Thermometric detection is most often used. Separations of anionic species have attracted much wider interest.

5.1. Organic acids

Speed, high sensitivity, minimal sample preparation and the combination of direct qualitative and quantitative information are all contributory factors that render capillary isotachophoresis interesting for the study of organic acids, especially in biological solutions.

In Fig. 3A, the application of isotachophoresis in the fermentation industry is presented. Only 2 μl of a 1:500 dilution of crude fermentation broth, obtained during the industrial production of citric acid, was injected. Typical experimental conditions were as follows: leading electrolyte, 5 mM HCl, 0.3% methylcellulose, titrated to pH 3.85 with β-alanine; terminating electrolyte, 5 mM caproic acid; analysis time, 10–20 min, and detection, UV at 254 nm and thermometry.

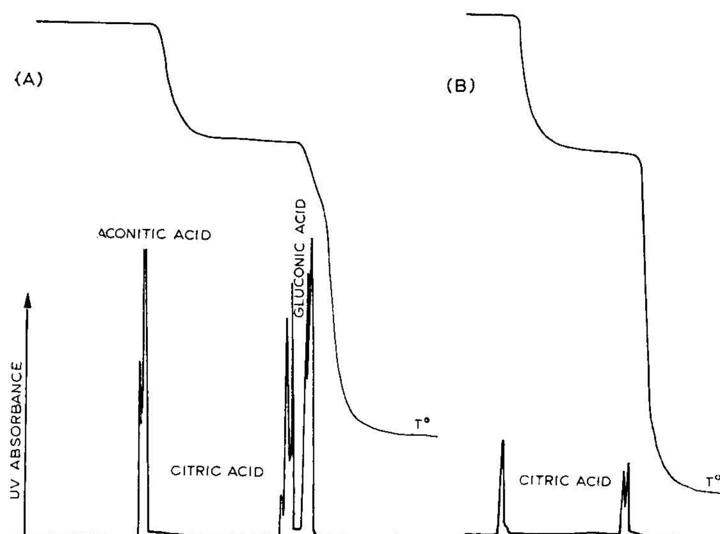

Fig. 3. (A) Isotachophoretic separation of crude fermentation broth in citric acid production. (B) Identical separation of a sample from a batch before the last purification step. T° = thermometric signal (for conditions, see text).

Fig. 3B presents the results obtained under the same conditions by injection of 2 μl of a 1:100 dilution of the concentrate before the last purification step. It is interesting to note that, with a similar electrolyte system at pH 2.45, it is possible to achieve a quantitative separation of citric acid and isocitric acid.

Somewhat similar problems arise in the food industry and Fig. 4A illustrates this[5]. From the injection of a few microlitres of a de-gassed soft drink, the citric acid and ascorbic acid contents can be directly evaluated. The effect of contact with atmospheric air on the ascorbic acid level of the same soft drink is shown in Fig. 4B.

Fig. 5 illustrates the determination of the organic acid content of a fruit yoghourt preparation. The presence or absence of some of the organic acids enables one to judge the fruit content of these commercial preparations.

Recently, problems in human dental hygiene have been succesfully approached by capillary isotachophoretic analysis of the organic acids produced by the growth of microorganisms.

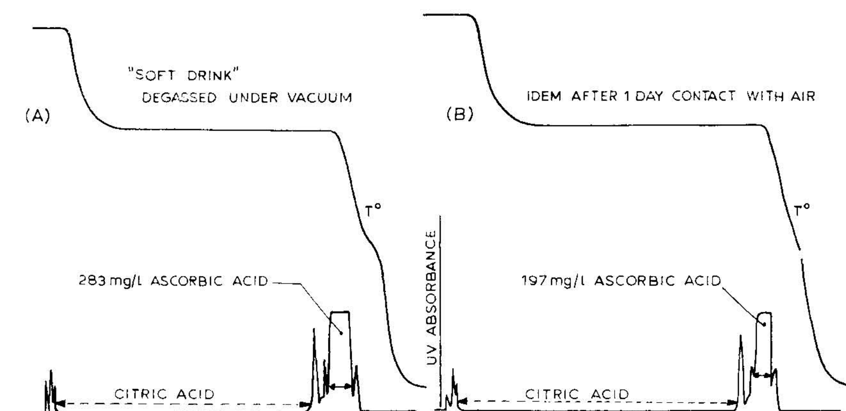

Fig. 4. (A) Isotachophoretic separation experiment in which an untreated sample of a de-gassed commercial soft drink was injected. (B) Separation under the same conditions as in (A), with the same de-gassed soft drink after contact with atmospheric air for 24 h.

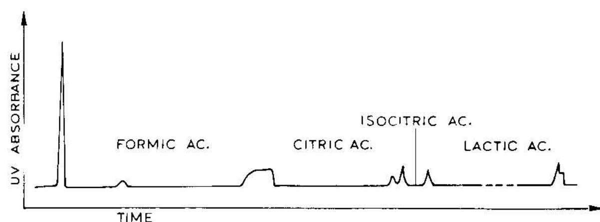

Fig. 5. Result obtained on isotachophoresis of a commercial preparation of fruit yoghourt.

A completely different kind of application can be found in the isotachophoretic determination of phenylglyoxalic, mandelic, hippuric and methylhippuric acids in the urine of human subjects after occupational exposure to styrene, toluene and xylene[6]. The *o*-, *m*- and *p*-isomers of methylhippuric acid can be partially separated. After ether extraction of the urine, all four acids can be measured accurately by isotachophoresis. Even amounts as small as 0.5 nmole can be determined in about 20 min.

5.2. Nucleic acids

Analytical isotachophoresis is an excellent method for the qualitative and quantitative determination of nucleotides[7,8]. The different phosphates of adenosine, cytidine, guanosine and uridine can be easily separated from each other. Measurement of zone lengths gives direct quantitative information about the nucleic acid composition of the sample. Fig. 6 shows the results obtained with a synthetic mixture of 13 nucleic acids when analysed in a 63-cm capillary in about 40 min. Detection was effected by UV absorption at 254 nm. Virtually the same electrolyte system as described above for organic acids was used. The separation pH was 3.89 and about 1.5 nmole of each nucleotide was used (4.5 nmole for c-AMP).

As reported by Gustafsson[13], 5-fluorouracil can be measured in human serum at levels that are of clinical interest. This problem is of interest in human cancer therapy.

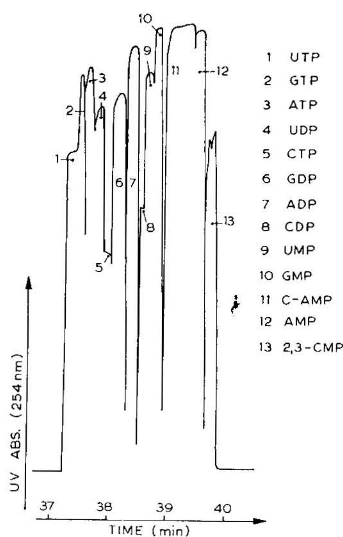

Fig. 6. Isotachophoretic separation of a synthetic mixture of 13 nucleic acids in a 63-cm capillary. Separation pH, 3.89.

5.3. *Antibiotics*

Analytical isotachophoresis is an excellent technique for the quality control of antibiotics such as penicillins and tetracyclines. Despite the small differences in mobility, due to their similarity in structure, they can be separated and quantified. A typical example is the separation of the penicillins carbenicillin, flucloxacillin, ampicillin and amoxicillin. Chloride ion was used for the leading electrolyte, adjusted to pH 7.2 with Tris and with 0.2% methylcellulose as anti-convection additive. The terminating ion was 10 m*M* β-alanine, adjusted to pH 10.3 with barium hydroxide. Other conditions were capillary length, 43 cm; temperature, 11°; total separation time, 32 min; sample, about 2 μg of each antibiotic; detection, UV absorption at 254 nm. The results of such a separation experiment are shown in Fig. 7.

Another example of a similar separation is the analysis of the ingredients of an antibiotic preparation for infusion. As revealed by isotachophoresis, the pharmaceutical mixture contained, in addition to a tetracycline and doxycycline, ascorbic acid, sorbitol and several other UV-absorbing substances in smaller amounts.

5.4. *Amino acids*

The establishment of the amino acid composition is of interest in many fields of research and industry. Nearly all separation techniques have been tried: liquid chromatography, gas chromatography and electrophoresis are among the most important. Despite having received little attention until now, isotachophoresis seems to be an interesting addition to this range of techniques.

To change from one operational system to another in isotachophoresis takes little time (normally not longer than required for rinsing and refilling the capillary and electrode vessels) and the total analysis time is very short (*ca.* 10 min). With this in mind, it can be stated that the application of two different systems to the same sample will lead to more useful information than when one tries to separate all of the

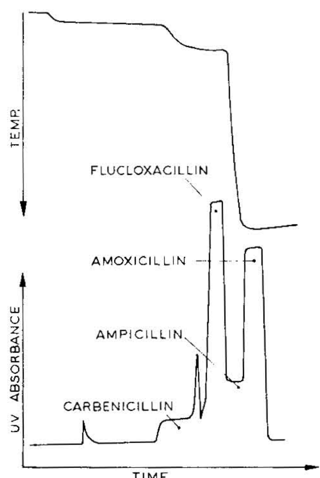

Fig. 7. Isotachophoretic separation of an antibiotic preparation. Lower trace = UV absorption; upper trace = thermometric signal.

amino acids in one run. With a separation pH above 8, most of the amino acids will have an effective mobility for a separation according to the isotachophoretic principle. Most of them will migrate anodically.

Fig. 8 presents the results of an isotachophoretic separation experiment[4] on eight amino acids. The experimental conditions were as follows: leading electrolyte, 0.004 *M* 5-bromo-2,4-dihydroxybenzoic acid adjusted to pH 9 with β-alanine; terminating electrolyte, 0.01 *M* β-alanine adjusted to pH 10.5 with barium hydroxide; electric current, 50–100 μA. It can be seen that both the thermal step height and the UV-absorption trace contribute to the interpretation of the results.

Robinson and Rimpler[9] recently reported the determination by capillary isotachophoresis of aspartic acid, asparagine, glutamic acid and glutamine. An ultrafiltrate of human serum was injected without further treatment.

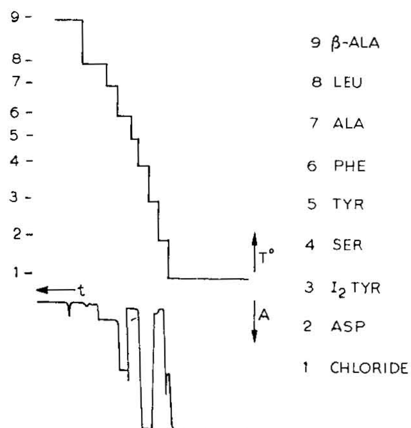

Fig. 8. Isotachophoresis of a mixture of amino acids. Upper trace = thermometric signal; lower trace = UV absorption.

5.5. Peptides

Most of the analytical systems elaborated for amino acid analysis can also be used for the separation of small peptides. The field of biologically active peptides has developed enormously during the last decade. As many of these compounds have potential use in human medicine, their synthesis and purification is important. The same advantages for amino acid analysis by isotachophoresis make this technique ideally suited to the detection and measurement of the small peptides. Capillary isotachophoresis has already been applied to most of the naturally occuring peptides such as oxytocin, vasopressin and somatostatin. Typical results obtained by capillary isotachophoresis during the purification of the vasoactive intestinal peptide (VIP) are shown in Fig. 9. In each instance, about 30–40 μg of material were injected and the total analysis time was about 9 min.

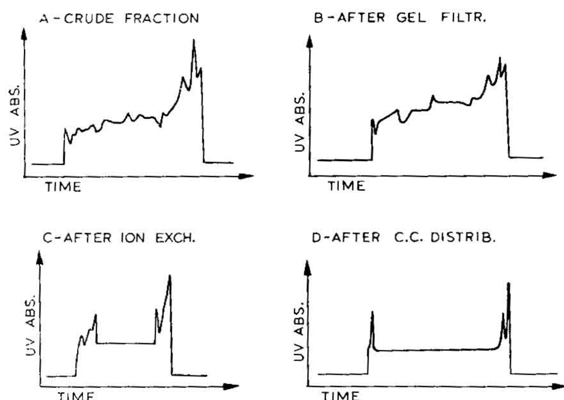

Fig. 9. Isotachophoretic separation of vasoactive intestinal peptide during different stages of the purification procedure. Leading ion, 0.005 M KAc titrated to pH 5.1 with HAc, 0.25% methylcellulose; terminating ion, 0.005 M α-alanine; capillary length, 23 cm; separation time, 9 min.

5.6. Proteins

The application of capillary isotachophoresis to proteins has been investigated intensively only during the last 2 years. Under suitable operating conditions, the different protein species of a sample arrange themselves between the leading and terminating electrolytes according to their net electrophoretic mobilities. Not much useful information can be gained from such an experiment because the UV beam, however narrow, cannot resolve the different protein zones, which follow each other without interruption and all of which absorb the UV light. In order to arrive at useful separation patterns, a spacer mobility gradient must be created between the leading and terminating electrolytes. The different protein species constituting the sample are then interspaced with other ions that have mobilities in the same region as those of the proteins under separation. A prerequisite is that these spacer ions must show no UV absorbance and that there must be a sufficient number of them with different mobilities[10].

The commercial preparations of synthetic peptides used for isoelectric focusing are suitable as spacer preparations. Some of the amino acids can also be used as

discrete spacers to split up a complex protein separation pattern into more easily interpretable sub-groups.

If the operating parameters are sufficiently optimized and a true dynamic equilibrium has been reached, then the integration of the peak surfaces produced by pure protein species is directly proportional to the absolute amount of protein in the peaks[11].

Fig. 10A shows a typical separation pattern obtained with only 0.6 μl of human serum. The large broad peak is albumin and all of the peaks with mobilities slower than that of the amino acid valine are mobility sub-fractions of immunoglobulin G. The pattern of immunoglobulin sub-fractions depends on the composition of the mobility spacer gradient used, but it is extremely reproducible.

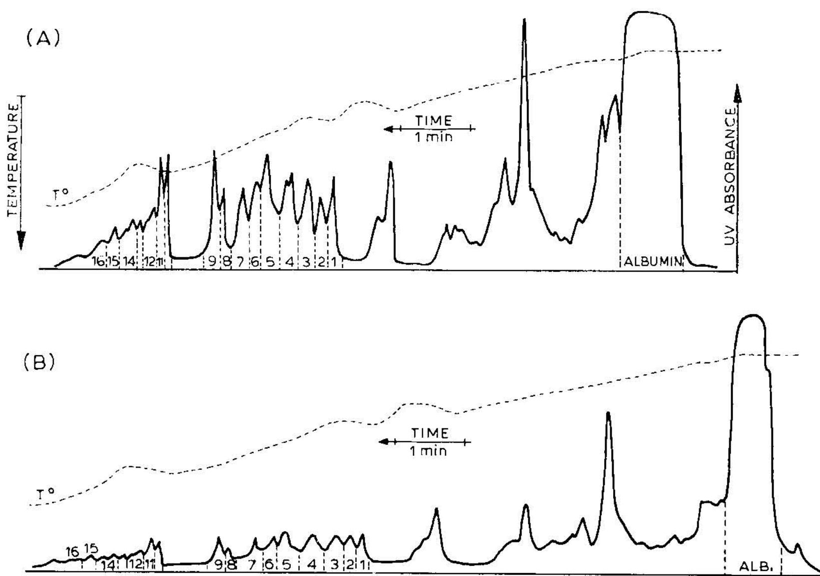

Fig. 10. Isotachophoresis of proteins. Upper pattern, 0.6 μl of human serum; lower pattern, 2 μl of 15-fold concentrated cerebrospinal fluid from the same subject. G = glycine spacer; V = valine; A = β-alanine. Leading electrolyte, 0.005 *M* morpholinoethanolsulphuric acid adjusted to pH 9 with aminopropanediol, 0.4% methylcellulose; terminating electrolyte, 0.005 *M* animocaproic acid adjusted to pH 10.8 with barium hydroxide; temperature, 12°; capillary length, 23 cm; separation time, 25 min.

Fig. 10B presents the pattern obtained by injection of 2 μl of about 15-fold concentrated cerebrospinal fluid from the same subject. As the peak surfaces are a direct measure of the amount of protein, and by taking into account the sample volumes and concentration factor, the permeability coefficients of the blood/cerebrospinal fluid can be calculated for several protein species.

Fig. 11A is the separation pattern obtained from the soluble eye lens proteins from a young mouse. Fig. 11B shows the same results obtained under the same conditions but with the proteins from the lens of a very old mouse. Influence of age on the protein composition can thus be demonstrated in a very short time, using very small

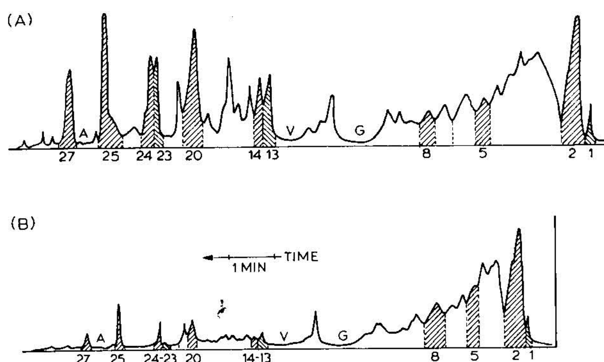

Fig. 11. Isotachophoresis of soluble eye lens proteins from mouse. Injected: 2 μl of 1% solution. Conditions as in Fig. 10. (A), very young mouse; (B), very old mouse.

amounts of protein and without making use of denaturation or coloration of the separated protein fractions.

6. CONCLUSIONS

A fraction collector device applicable in capillary isotachophoresis has recently become available[12]. The sample zones are transferred to a moving cellulose acetate strip without appreciable loss of resolution. This renders possible the application of special detection and identification techniques. Owing to the extremely small amounts of material concerned, the most promising application of this technique lies with radioactively labelled compounds.

Preparative isotachophoresis in columns of polyacrylamide gels is a new, standardized, high-resolution technique, more specifically suited to protein chemistry. Preparative isotachophporesis can compete with many other protein separation techniques. Much valuable time, effort and materials can certainly be saved if the separation conditions for such preparative work are first studied by capillary isotachophoresis.

7. SUMMARY

Isotachophoresis is an electrophoretic technique in which the different sample ions are separated, under the influence of a strong electric field, according to their effective electrophoretic mobilities; all ions are, at equilibrium, moving with the same constant speed between two different electrode ions.

The separated components are detected directly in the capillary, either by thermometry, potentiometry, resistance measurement or by UV light absorption. Practically any charged molecule (organic acids, nucleic acids, antibiotics, amino acids, peptides and proteins) can be separated and in most instances be quantified, in a very short time, by this new analytical technique.

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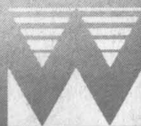

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