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STEPWISE GRADIENT DEVELOPMENT IN THIN-LAYER CHROMATOGRAPHY

III*. A COMPUTER PROGRAM FOR THE SIMULATION OF STEPWISE GRADIENT ELUTION

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SUMMARY

A general equation for the final R_F value of a solute chromatographed under conditions of stepwise gradient elution with one void volume of mobile phase has been derived. The elution process and the distance travelled by the spot as a function of eluent volume are illustrated graphically for retention–eluent composition relationships typical of a displacement adsorption mechanism or for reversed-phase chromatography. A computer program (in BASIC) is given for the simulation of stepwise gradient thin-layer chromatography. The program can be used for the optimization of stepwise gradient programs by computer simulation of the elution process.

INTRODUCTION

In Parts I[1] and II[2] it was demonstrated that a sandwich chamber with a glass distributer[3,4] simplifies the use of continuous [5,6] and stepwise[2,6] gradient elution in thin-layer chromatography (TLC), as the eluent is delivered to the layer by a capillary siphon from a small container, or is introduced directly, in small portions, under the distributer[1,2,5,6]. Qualitative rules for the modification of the gradient profile were formulated in Part I[1]; however, it would be advantageous to have a mathematical model of the process involved.

Numerous mathematical considerations concerning gradient elution have been mostly restricted to continuous column chromatography[7–10]. Several workers[11–17] considered the movement of zones under conditions of stepwise elution and derived corresponding equations that took into account the delayed overtaking of the solute band by the consecutive zones of increased concentration of the modifier. In this paper analogous considerations are applied to the migration of consecutive zones of the mobile phase and the corresponding migration of the solute band in TLC where

* For Part II, see ref. 2.

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the elution stops when the front of the mobile phase has reached the far end of the plate. The mathematical relationship between the R_F values of solutes, the applied gradient program and the retention–eluent composition relationships involved are presented as a computer program that permits the study of the chromatographic process and the resulting separation of the components of the sample on the computer screen.

THEORETICAL

Gradient elution is used when the sample to be chromatographed contains components with a wide range of retention parameters. Consider a twenty-component mixture with capacity factors k' of the components forming a geometrical progression, the divisor being equal to 2 [$k'(j) = 0.5k'$ $(j - 1)$] and exponentially dependent on the modifier concentration (molar or volume fraction c), in accordance with the Snyder–Soczewiński model of adsorption[18–22]:

$$a(j) = 25.6/2^j \text{ (solute No. } j = 1,2, \ldots, 20) \quad (1)$$

$$\log k'(j) = \log a(j) - m \log c;\ k'(j) = a(j)c^{-m};\ R_{F(j)} = 1/[1 + a(j)c^{-m}] \quad (2)$$

where $a(j) = k'(j)_{\text{mod}}$ for $c = 1.0$ (pure modifier).

The log k' *vs.* log c plots of the twenty solutes are given in Fig. 1, which has

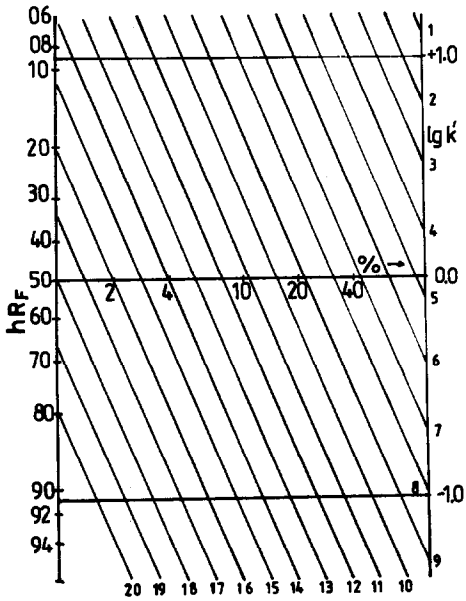


Fig. 1. Family of linear log k' *vs.* log c_{mod} plots for hypothetical solutes 1–20 with capacity factors forming a geometrical progression according to eqns. 1 and 2); slope = −2. For isocratic elution only ten solutes give R_F values in the range 0.04–0.96 (left-hand ordinate).

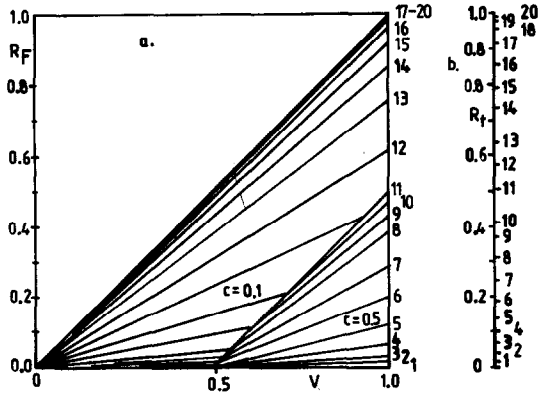


Fig. 2. (a) Two-step development of the hypothetical mixture (Fig. 1). Lines of unit slope: migration of the fronts of the first (c = 0.1) and second (c = 0.5) zones of the stepwise gradient; the remaining lines represent the migration of the individual compounds of the mixture, accelerated in the second zone. Solutes 9–11 are accumulated near the front of the more concentrated mobile phase. (b) R_F values of the hypothetical series of solutes after five-step development (c = 0.05, 0.1, 0.2, 0.5, 1.0).

a parallel R_F axis subordinated to the right-hand-side log k' axis; m = 2. It can be seen that no isocratic eluent can separate all the components: pure modifier (c = 1.0) separates well solutes 1–7 and the less polar solutes are accumulated near the solvent front; for c = 0.1 (10%), solutes 7–14 are well separated, the remaining ones being accumulated either near the start line or the front line; for c = 0.02 (2%), solutes 1–10 are accumulated on the start line. Thus, only half of the components can be satisfactorily separated by isocratic elution.

The separation can be greatly improved already by two-step gradient elution[23,24] (Fig. 2). However, the distribution of spots along the chromatogram is uneven, some spots being accumulated near the front of the more concentrated eluent

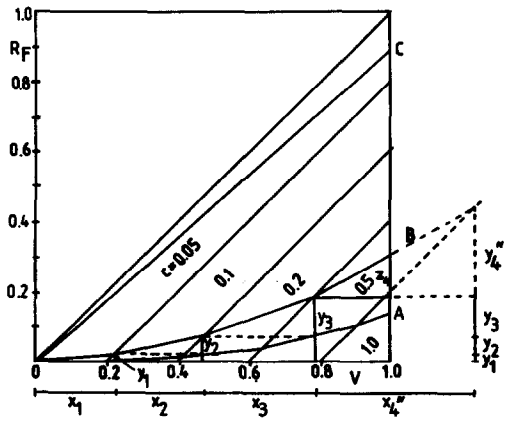


Fig. 3. Five-step development, equal volumes of the eluent fraction (0.2 of the void volume). Lines of unit slope: fronts of consecutive concentration zones. Exponential gradient program: 0.05, 0.1, 0.2, 0.5, 1.0; retention *vs.* modifier concentration relationships corresponding to eqn. 2 for m = 1.

owing to the steep gradient of elution strength. In the case illustrated in Fig. 2, the mixture (Fig. 1) was eluted to half the length of the plate with 10% modifier and the elution continued with 50% modifier ($c = 0.5$).

A still better distribution of spots can be obtained with multi-step gradients and especially with continuous gradient programs. In earlier papers[1,2,5,6], 8–10-step or continuous gradients were used; to find a compromise between the smoothness of the gradient profile and simplicity of the elution procedure, a five-step gradient seems to be the most convenient. As will be demonstrated below, such a profile does not cause accumulation of spots near the boundaries of the consecutive concentration zones. Such a process will be considered theoretically and then generalized.

Fig. 3 represents the migration of the fronts and zones of a mobile phase composed of five fractions of eluent of exponentially increasing concentration of the modifier (five-step gradient). Equal volumes ($v = 0.2$) of the five fractions are applied so that the consecutive fronts of the concentration zones are lower by 0.2 unit relative to the preceding ones. The ordinate axis represents the migration along the TLC plate and the abscissa the volume of eluent absorbed by the layer; the void volume of the layer is assumed to be equal to 1.

Consider the migration of the spot of solute B from the start line. The spot migrates in the first zone of eluent longer than would be expected from $v(1) = 0.2$, as the front of the next concentration zone must first overtake it[12,14,15]; the spot migrates a distances $y(1)$ in the first zone and the corresponding volume of mobile phase, $x(1)$, is equal to $0.2 + y(1)$. The coordinates of the first intersection point $[x(1), y(1)]$ can be calculated by analytical geometry from the equation of the migration of the spot [$y = R_{F(1)} \cdot x(1)$, where $R_{F(1)}$ is the R_F value of solute in the first concentration zone] and that of the front of the second concentration zone [$y(1) = x(1) - 0.2$]. The solution gives

$$x(1) = \frac{0.2}{1 - R_{F(1)}} \text{ and } y(1) = \frac{0.2\ R_{F(1)}}{1 - R_{F(1)}} = \frac{0.2}{k'(1)} \tag{3}$$

where $y(1)$ is the fractional ΔR_F value of the spot travelled in the first concentration zone of the mobile phase. It is evident from Fig. 3 that

$$x(1) = 0.2 + y(1) \tag{3a}$$

The discussion is repeated for the second concentration zone, in which the R_F value of the solute is $R_{F(2)}$. Transferring the origin of coordinates to the former intersection point $[x(1), y(1)]$ we obtain $y(2) = 0.2/k'(2)$; the volume of mobile phase corresponding to this migration distance is equal to $x(2) = 0.2 + y(2) = 0.2/(1 - R_{F(2)})$ (see Fig. 3). The total distance (R_F) travelled by the solute spot in zones 1 and 2 is equal to $y(1) + y(2)$.

By analogous reasoning we obtain for the third concentration zone $y(3) = 0.2/k'(3)$ and $x(3) = 0.2 + y(3) = 0.2/(1 - R_{F(3)})$. After migration in three gradient zones, the R_F value is $y(1) + y(2) + y(3)$, which corresponds to absorption by the layer of eluent fractions with a total volume $x(1) + x(2) + x(3) = 0.6 + y(1) + y(2) + y(3)$.

For single development of the layer $x = 1.0$. It can be seen from Fig. 3 that

the intersection of the solute migration line [slope = $R_{F(4)}$] in zone No. 4 with the migration line of the front of zone No. 5 occurs beyond the area of the diagram so that the calculated $y(4)$ value would be fictitious, and could be obtained only by continued elution with the last eluent fraction until $x = x(1) + x(2) + x(3) + x(4) > 1$. This fact requires the introduction of suitable corrections to the equation describing the migration of solute spot under stepwise gradient conditions.

It follows from the discussion of the case under considerations (Fig. 3, five consecutive eluent fractions equal to 0.2 of the total volume of the solvent in the layer) that the total volume of mobile phase corresponding to i migration steps is

$$x = \sum_i x(i) = \sum_i [0.2 + y(i)] = 0.2 \sum_i \frac{1}{1 - R_{F(i)}} \tag{4}$$

and the hypothetical R_F of a solute travelling through i zones is

$$y = R_F = \sum_i y(i) = 0.2 \sum_i 1/k'(i) \tag{5}$$

To find the real R_F value of solute it must be assumed that the last, say the hth, development step is incomplete:

$$\sum_{i=1}^{h-1} x(i) < 1, \text{ but } \sum_{i=1}^{h} x(i) > 1 \text{ and } y = R_F = 0.2 \sum_{i=1}^{h-1} 1/k'(i) + z(h) \tag{6}$$

and the real migration path $z(h)$ of the solute spot in the last (hth) incomplete stage is calculated from the proportion (see Fig. 3 for solute B, $h = 4$):

$$\frac{z(h)}{y(h)} = \frac{1 - \sum_{i=1}^{h-1} x(i)}{x(h)}; \tag{7}$$

$$z(h) = \frac{1 - \sum_{i=1}^{h-1} x(i)}{1 + k'(h)} = R_F(h) \left[1 - \sum_{i=1}^{h-1} x(i) \right]$$

It should be mentioned that only the solutes of very high initial $k'(i)$ values [very low $R_{F(1)}$ values] migrate through all five zones of mobile phase concentrations, so that their final R_F is below 0.2 (Fig. 3, solute A). The number of zones through which the solute spot migrates also depends on the eluent volume fractions used, *i.e.*, the step lengths and heights of the concentration program (gradient steepness) and the slope of the log k' *vs.* log c plot (eqn. 2). On the other hand, a solute of high $R_{F(1)}$ value for the first concentration zone [in the case illustrated, when $R_{F(1)} > 0.8$] migrates all the time in the first zone and its R_F value is then not $1/k'$ (fictitious R_F) but $1/(1 + k')$, in accordance with the well known equation (see Fig. 3, solute C).

The equations can be generalized for a solute (j) for any number of steps (i)

and fractional volumes of the eluent, $v(i)$ [$\sum_i v(i) = 1$]. The total volume of mobile phase corresponding to $y(j)$ is

$$x(j) = \sum_{i=1}^{h} x(j,i) = \sum_{i=1}^{h} [v(i) + y(j,i)] = \sum_{i=1}^{h} \frac{v(i)}{1 - R_{F(j,i)}} \tag{8}$$

The actual final R_F value of solute j (considering that the last, hth, development step is incomplete) is

$$R_F = \sum_{i=1}^{h-1} y(j,h) + z(j,h) = \sum_{i=1}^{h-1} \frac{v(i)}{k'(j,i)} + z(j,h) \tag{9}$$

(for $\sum_{i=1}^{h-1} x(j,i) < 1$)

$$z(j,h) = y''(j,h) \frac{1 - \sum_{i=1}^{h-1} x(j,i)}{x''(j,h)} = \frac{1 - \sum_{i=1}^{h-1} x(j,i)}{1 + k'(j,h)}$$

$$= R_{F(j,h)} \left[1 - \sum_{i=1}^{h-1} x(j,i) \right] \tag{10}$$

(the double primes mean that the value is fictitious, *i.e.*, beyond the migration diagram).

The corresponding computer program for eqns. 9 and 10 in BASIC is given in Table I and can be used to analyse the paths of the series of solutes under stepwise gradient conditions for various parameters (slope of log k' *vs.* log c plots, eqn. 1), lengths [$v(i)$] and heights [$c(i)$] of the gradient program (for any number of steps n). In the authors' laboratory a ZX Spectrum + personal computer was used. The paths of the individual solutes from the series are shown on the monitor screen and the numerical values of the final R_F coefficients are printed. The program can be modified for other sets of solutes (other versions of eqn. 1) and retention–modifier concentration equations. For instance, for reversed-phase systems of the type octadecylsilica–water + methanol another type of retention–eluent composition relationship is frequently observed[8–10,21–22]:

$$\log k' = \log a - mc;\ k' = a \cdot 10^{-mc};\ R_F = \frac{1}{1 + a \cdot 10^{-mc}} \tag{11}$$

where c is the concentration of modifier (methanol) in volume fractions, a is the k'_w value for pure water as eluent (for $c = 0$) and the slope m is equal to $\log k'_w - \log k'_{mod} = \log k'_w/k'_{mod}$. For the hypothetical model mixture a similar geometrical progression of $a(j)$ values can be chosen, *e.g.*,

$$a(j) = \frac{2^{18}}{2^j} = \frac{262\,144}{2^j}\ (j = 1, 2, \ldots, 20) \tag{12}$$

TABLE I

COMPUTER PROGRAM FOR NUMERICAL AND GRAPHICAL REPRESENTATION OF STEPWISE GRADIENT ELUTION

The program is written in BASIC for operation on a Sinclair ZX Spectrum + microcomputer making use of all facilities of this version of BASIC. For this reason, adaptation to other microcomputers has not been taken into consideration. The program requires a screen monitor and a printer as peripherals. All data would be read from the keyboard and every reading is proceeded by a suitable explanatory text. An arbitrary number of concentration steps and an arbitrary number of solutes may be used for simulation. The volumes of portions of the eluent and their concentrations may be arbitrary. Only one subroutine for drawing the diagram is used. Coincidence of notations of variables in the program and the text was preserved if it did not lead to misunderstanding. The results of the program are the diagram of gradient concentrations, the diagram of the paths of solutes and the final R_F values for each substance. It should be noted that the program below is for normal-phase systems. To alter it for reversed-phase systems substitute steps 240 and 260 in the program as follows:

240 LPRINT "THE R-P SYSTEM"

260 DEF FN $k(j,i)$ = 262 144/((2↑j) ∗ (10↑($c(i)$ ∗ m)))

```
 10 LPRINT
 20 LPRINT "STEPWISE GRADIENT"
 30 LPRINT
 40 INPUT "THE NUMBER OF STEPS n=";n
 50 LPRINT "THE NUMBER OF STEPS n=";n
 60 LPRINT
 70 INPUT "THE NUMBER OF SOLUTES b=";b
 80 LPRINT "THE NUMBER OF SOLUTES b=";b
 90 LPRINT
100 DIM c(n): DIM v(n): DIM k(b,n): DIM R(b,n)
110 DIM x(b,n): DIM y(b,n): DIM s(b,n): DIM z(b,n)
120 LPRINT
130 LPRINT "THE CONCENTRATION OF MODIFIER ON i-th STEP"
140 LPRINT
150 FOR i=1 TO n
160 INPUT "c=";c(i),"v=";v(i)
170 PRINT "c(";i;")=";c(i),"v(";i;")=";v(i)
180 NEXT i
190 COPY
200 CLS
210 LPRINT
220 GO SUB 1000
230 LPRINT
240 LPRINT "THE S-P SYSTEM"
250 LPRINT
260 DEF FN k(J,i)=25.6/((2↑J)*(c(i)↑m))
270 DEF FN R(J,i)=INT (1000/(1+FN k(J,i)))/1000
280 INPUT "THE SLOPE m=";m
290 LPRINT "THE SLOPE m=";m
300 LPRINT
310 LPRINT "THE DISTANCE TRAVELLED BY SPOTS AFTER n DEVELOPMENT STEPS
320 LPRINT
330 FOR J=1 TO b
340 FOR i=1 TO n
350 LET x(J,i)=v(i)/(1-FN R(J,i))
360 LET y(J,i)=v(i)/FN k(J,i)
370 NEXT i
380 LET s=0
390 FOR i=1 TO n
400 LET s=s+x(J,i)
410 LET s(J,i)=s
420 NEXT i
430 FOR i=1 TO n
440 IF i>=2 AND s(J,i)>=1 THEN  GO TO 520
450 IF s(J,i)>=1 THEN  GO TO 480
460 NEXT i
470 GO TO 640
480 LET R=FN R(J,i)
490 PLOT 10,10: DRAW 160,R*160
500 LPRINT "Rf(";J;")=";R
510 GO TO 640
520 LET z(J,i)=(1-s(J,i-1))*FN R(J,i)
530 LET R=0
540 FOR P=1 TO i-1
550 LET R=R+y(J,P)
560 NEXT P
570 LET R(J,i)=INT (1000*(R+z(J,i)))/1000
580 LPRINT "Rf(";J;")=";R(J,i)
590 PLOT 10,10
600 FOR h=1 TO i-1
610 DRAW x(J,h)*160,y(J,h)*160
620 NEXT h
630 DRAW (1-s(J,i-1))*160,z(J,i)*160
640 NEXT J
650 LET v=0
660 FOR i=1 TO n
670 LET v=v+v(i)
680 PRINT AT 21,v*20;" ";v
```

(Continued on p. 70)

TABLE I (*continued*)

```
 690 PLOT 10+v*160,10: DRAW (1-v)*160,(1-v)*160
 700 CIRCLE 10+i+160/n,10,1
 710 CIRCLE 171,10+i*160/n,1
 720 NEXT i
 730 PRINT AT 10,24;"Rf=0.5"
 740 PRINT AT 0,24;"↑";"Rf"
 750 PRINT AT 21,24;"-->";"V,X,S"
 760 NEXT i
 770 PLOT 10,10: DRAW 160,0: DRAW 0,160: DRAW -160,0: DRAW 0,-160
 780 COPY
 790 CLS
 800 INPUT "REPEAT PROFILE OF GRADIENT a$=1 OR REPEAT ANOTHER SLOPE a$=0
";";";"a$=";a$
 810 IF a$="1" THEN  GO TO 10
 820 IF a$="0" THEN  GO TO 240
 830 STOP
 840 RUN 10
1000 LPRINT "THE PROFILE OF STEPWISE GRADIENT"
1010 LPRINT
1020 FOR i=1 TO n
1030 IF i>=2 THEN  GO TO 1070
1040 PLOT 0,c(i)*160: DRAW 160/n,0
1050 PLOT 0,0: DRAW 0,c(i)*160
1060 GO TO 1090
1070 PLOT (i-1)*160/n,c(i)*160: DRAW 160/n,0
1080 PLOT (i-1)*160/n,c(i-1)*160: DRAW 0,(c(i)-c(i-1))*160
1090 NEXT i
1100 PLOT 0,0: DRAW 0,160: DRAW 160,0: DRAW 0,-160: DRAW -160,0
1110 FOR i=1 TO n
1120 PRINT AT 21,(i-1)*20/n;i
1130 PRINT AT (21-20*c(i)),20;"_(";i;")=";c(i)
1140 NEXT i
1150 COPY
1160 CLS
1170 LPRINT
1180 LPRINT
1190 RETURN
```

STEPWISE GRADIENT

THE NUMBER OF STEPS n= 5

THE CONCETRATION OF MODIFIER ON i-th STEP AND THE VOLUME OF ELUENT ON i-th STEP

c (1) =.05 v (1) =0.2
c (2) =0.1 v (2) =0.2
c (3) =0.2 v (3) =0.2
c (4) =0.5 v (4) =0.2
c (5) =1 v (5) =0.2

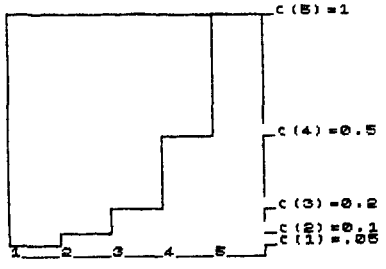
THE S-P SYSTEM

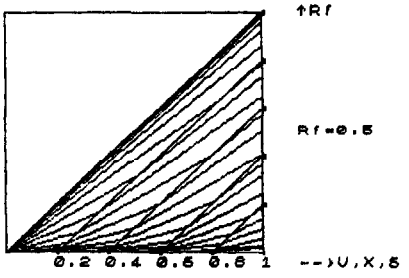
THE SLOPE m=2

THE DISTANCE TRAVELLED BY SPOTS AFTER n DEVELOPMENT STEPS

Rf(1)=.018
Rf(2)=.035
Rf(3)=.062
Rf(4)=0.1
Rf(5)=0.144
Rf(6)=0.186
Rf(7)=0.245
Rf(8)=0.316
Rf(9)=0.368
Rf(10)=0.407
Rf(11)=0.504
Rf(12)=0.573
Rf(13)=0.647
Rf(14)=0.735
Rf(15)=0.791
Rf(16)=0.864
Rf(17)=0.927
Rf(18)=0.962
Rf(19)=0.98
Rf(20)=0.99

THE PROFILE OF STEPWISE GRADIENT





The analysis of stepwise gradient processes in reversed-phase systems requires a suitable modification of the program (included in Table I).

The assumed mathematical model is somewhat simplified; for instance, it does not take into account solvent demixing effects[4,25,26], which are especially significant for the low-concentration region of the gradient profile[4,18] (the effect can be minimized by pre-wetting the layer before spotting the sample[4]). Part of the mobile phase is stagnant in the pores of the adsorbent and the exchange of the stagnant liquid in contact with the new concentration zone may delay the migration of its front and cause some smoothing of the sharp (initial) concentration steps.

CONCLUSION

The computer simulation of stepwise gradient elution can be used for various purposes. The study of migration paths and R_F values of solutes for various gradient programs and retention–modifier concentration relationships is valuable for teaching purposes, as it illustrates the operation of gradients in comparison with isocratic elution and gives general experience in choosing the optimal gradient shape (lengths and heights of the steps). The chromatograms obtained by trial gradient runs can be compared with computer data for model mixtures and modified programs tried by computer simulation to give an improved distribution of the spots.

The examples chosen in this study concern simplified situations (parallel, equidistant log k' *vs.* log c plots). In real systems, more complex situations can be encountered, *e.g.*, the spots may form two groups separated by a wide gap on the chromatogram (ref. 1, Fig. 6c). The situation can be simulated by assuming that the middle solutes (*e.g.*, Nos. 7–13) in the hypothetical series are non-existent and the task is to choose a gradient profile that would secure maximal compression of spots 7–13 and equidistant distribution of the remaining solutes 1–6 and 14–20. Another cause of complications may be differentiation of the slopes (m) of the log k' *vs.* log c plots of the individual solutes, which may lead to crossing of some of the paths and changes in the sequence of the spots. Some more complex situations will be analysed in subsequent papers in this series.

The computer program can also be adapted to stepwise gradient elution in column chromatography (for other programs for optimization of continuous gradients in column chromatography, see ref. 10, p. 485, and ref. 27; several programs for the optimization of TLC are given in ref. 28). The differences are that numerous void volumes of the eluent are used in elution and the process is terminated when the last solute ($j = 1$) leaves the column [*i.e.*, its $\sum_i y(i) = 1$; see refs. 9–17].

LIST OF SYMBOLS

Different symbols are used in the BASIC program for technical reasons (*e.g.*, *Rf* instead of R_F, *s* instead of $\sum$).

j	No. of solute (1–20).
i	No. of elution step (eluent fraction).
$k(j,i)$	capacity factor of solute j in the ith step.
$R(j,i)$	corresponding fraction of solute in the mobile phase.
$Rf(j,i)$	R_F value.

$c(i)$ concentration of modifier (molar or volume fraction) in the ith step.
$v(i)$ volume of eluent introduced in the ith step.
$y(j,i)$ distance (ΔR_F) travelled by solute j in the ith step.
$x(j,i)$ corresponding volume of mobile phase.
$s(j,i)$ total distance (R_F) travelled by solute j after i steps.
$z(j,i)$ fractional distance travelled by solute j in the last (incomplete) step.
$-m$ slope of log k *vs.* log c plot.

$$x(j,i) \quad \frac{v(i)}{1 - Rf(j,i)}$$

$$y(j,i) \quad \frac{v(i)}{k(j,i)}$$

$$z(j,i) \quad R(j,i) \cdot [1 - s(j,i-1)]$$

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