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Computer-aided optimization of gradient multiple development thin-layer chromatography
III. Multi-stage development over a constant distance

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Abstract

A theoretical model of multiple development over a constant distance is presented. The model is verified with experimental results. The final R_F values are calculated by computer program for known retention vs. eluent composition relationships for the solutes. The predicted and experimental R_F values were compared and showed satisfactory agreement.

Keywords: Computer-assisted chromatography; Optimization; Gradient multiple development; Amino acids

1. Introduction

Multiple chromatography entails any procedure involving repeated development of a chromatographic adsorbent layer with one or more solvents [1].

The technique of unidimensional multiple chromatography (UMC) first was proposed by Jeanes et al. [2] and later investigations were continued by Thoma [3,4]. Recently, multiple chromatography has become of great interest [5,6]. UMC has some advantages over normal development, as pointed out by Poole and Poole [7]: (1) greater efficiency owing to the zone refocusing mechanism, (2) greater separation capacity owing to (1) and the more optimum use of solvent selectivity and (3) improved sample detectability by scanning densitometry owing to smaller separated zones. However, there are some weak points associated with multiple development. First, there is no adequate model to use as the basic for method development; the multi-step process requires longer separation times. Attempts have been made [8,9] to formulate a simple physical model to describe the migration of the solutes zones, their dispersion and other phenomena that may distort the development process and which can be included in the model. In this paper, the simple model is applied to describe the migration of the solute zones in multiple development over a constant distance. The computer-assisted method development can be used to accelerate the calculation in the case multi-component mixtures and application of mixed solvents.

2. Theory

We assume that the plate is developed a

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number of times in the same detection and that the plate is dried after each development, the mobile phase then being completely removed from the adsorbent layer (Fig. 1). Further assumptions are as follows: the adsorption layer has identical properties along the whole length (thickness, phase ratio, activity, packing density); the elution strength of mobile phase is constant during the programme or it is varied according to a programme; the development distance is also constant in each development; after each development the plate is dried and brought to such a state that the eluent delivered in the next step

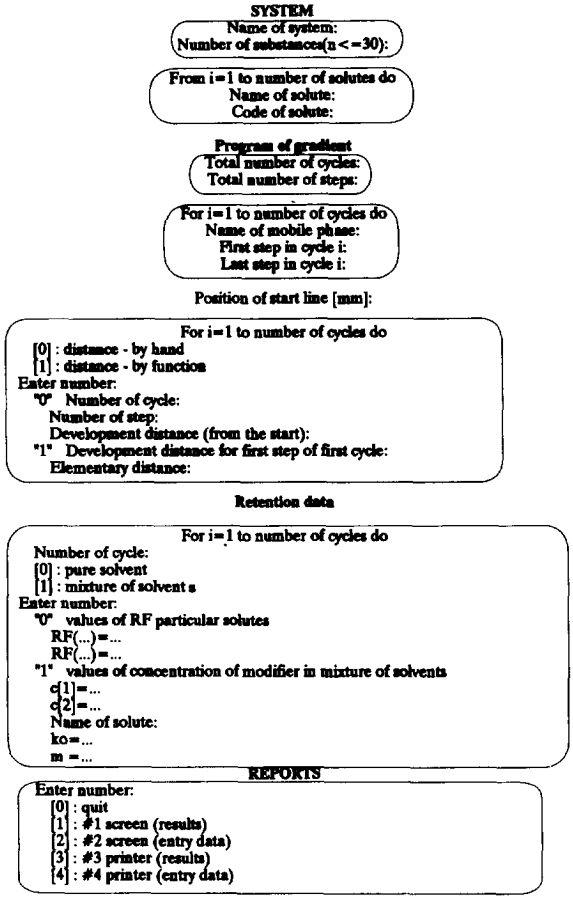

Fig. 1. Schematic diagram of migration of solutes in two-stage development over a constant distance. (a) Migration of solute D during the first development; (b) during the second development the spot remains in the same place until it is reached by the front of the second eluent; (c) then the zone migrates in the second mobile phase.

does not change its properties; the relationships between retention of solutes and the properties of the eluents (concentration, eluent strength) are known; the solutes are not decomposed and their loss is insignificant; the solvent demixing process in the case of mixed eluents is negligible to a first approximation or can be taken into account in the equation; and the whole elution process can be divided into cycles within which steps are discerned. As a cycle we understand a number of steps in which the same eluent type is used, differing only in the concentration of the modifier; the eluent is the solvent (containing one or more components) and the modifier is the more polar (stronger) component of the eluent. In the practical realization of the multiple development process, some deviations from these simplifying assumptions may be observed.

2.1. The migration process

We assume that for the purposes of computer simulation we have to know the relationships between the retention of the sample components and the properties (composition) of the eluents used. For pure solvents it is simplest to give the R_F values of the solutes. On the other hand, when binary eluents are used, it is convenient to give the parameters of the retention vs. modifier concentration plots, from which the R_F values can be calculated from Eqs. 1 and 2 [10].

$$\log k_{(i,j)} = \log k_{0(j)} - m_{(j)} \log C_{(i)} \quad (1)$$

Eq. 1 is known as Snyder–Soczewiński (S–S) equation.

$$k_{(i,j)} = k_{0(j)} + k_{1(j)} C_{(i)} + k_{2(j)} C_{(i)}^2 \quad (2)$$

In simulation procedures, the R_F values are introduced into the computer program or are calculated by suitable procedures.

When planning a multiple development programme, we introduce the number of cycles and steps. Then the development distances for consecutive steps are given, and also the eluent compositions used in the consecutive cycles and steps. The computer program calculates the R_F values of solutes in the consecutive steps.

Let us consider the process of multiple development for a simple n-step cycle. The migration of the solutes is given by the following equations.

For the first and successive steps, the distance travelled by the mobile phase front measured from the sample origin (first step) to the highest position on the plate is $z_{(i)}$ and it is constant. The distance migrated by the mobile phase in the first step and taken into consideration for calculation of migration distances for all solutes is the same and is equal to $\nu_{e(i,j)}$. This follows from the fact that all solutes are applied on the starting line.

The migration distances (from the start line) of solutes are

$$y_{(1,j)} = \nu_{e(1,j)} R_{F(1,j)} \tag{3}$$

where

$$\nu_{e(1,j)} = z_{(1)} = z_{(2)} = \cdots z_{(k)} \cdots = z_{(n)} \tag{3a}$$

After development, the chromatogram is dried so that the spots retain their positions attained after first development. Therefore, we can write that the sum of the paths of a given solute is equal to the distance travelled in the first step. For $i = 1$, we have

$$s_{(1,j)} = \sum_{i=1}^{1} y_{(i,j)} = y_{(1,j)} \tag{4}$$

$$s_{(1,j)} = z_{(1)} R_{F(1,j)} \tag{5}$$

The next step is carried out according to the programme adopted.

As after the first development the solutes have various positions on the plate, then the distance travelled by the mobile phase front in the second step and considered for calculation of the migration distance is different for each solute, depending on the distance travelled in the first sept. Therefore,

$$\nu_{e(2,j)} = z_{(2)} - s_{(1,j)} \tag{6}$$

The migration path in the second step is

$$y_{(2,j)} = \nu_{e(2,j)} R_{F(2,j)} \tag{7}$$

and the total path after two steps is

$$s_{(2,j)} = \sum_{i=1}^{2} y_{(i,j)} \tag{8}$$

Introducing Eqs. 6, 7 and 5 into Eq. 8, we obtain an equation for the sum of migration paths after two developments:

$$s_{(2,j)} = s_{(1,j)} + [z_{(2)} - s_{(1,j)}] R_{F_{(2,j)}} \tag{9}$$

Analogous equations are obtained for n-step development:

$$\nu_{e(n,j)} = z_{(n)} - s_{(n-1,j)} \tag{10}$$

$$y_{(n,j)} = \nu_{e(n,j)} R_{F(n,j)} \tag{11}$$

$$y_{n,j)} = [z_{(n)} - s_{(n-1,j)}] R_{F_{(n,j)}} \tag{12}$$

and

$$S_{(n,j)} = \sum_{i=1}^{n} y_{(n,j)} = s_{(n-1,j)} + [z_{(n)} - s_{(n-1,j)}] R_{F_{(n,j)}} \tag{13}$$

Analysing Eqs. 12 and 13, we see that these are typical recurrent equations in which the $(k-1)$th value for the sum of the distances travelled by a solute is necessary to calculate the kth value.

The sum of the distances travelled by a solute after n-step development is equal to the final R_F value:

$$R_{FG(j)} = s_{(n,j)} \tag{14}$$

This equation forms the basis to elaborate a computer program that simulates the multiple development process over a constant distance.

The program, written in Pascal, is represented in Fig. 2. It allows for the calculation of the final R_F values and the migration distance. The knowledge of the R_F vs. i (number of steps) relationships for a chosen gradient programme permits the investigation by computer simulation of the effect of the number of steps and variation of eluent composition on the final distribution of spots along the chromatogram. The program elaborated permits the simulation of multiple development both for a constant development distance and for increasing development distances for consecutive steps. When an identical eluent is applied in each development step, the

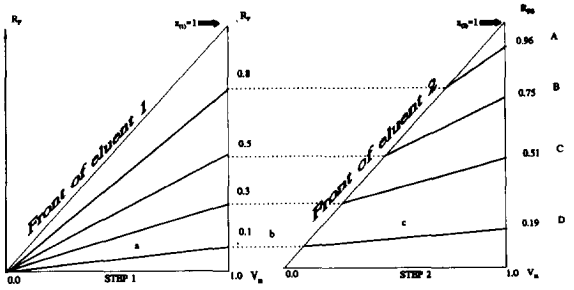

Fig. 2. Flow diagram of the computer program for calculation of the final values of R_{FG} in multi-stage development over a constant distance.

description of the migration process is considerably simplified and can be expressed by an analytical equation [3,6]. When eluents of various properties and compositions are applied, the migration of spots can be calculated from recurrent equations and a simple program for the calculation of final R_F values from the introduced data can be formulated.

The program consists of the following parts: in the first, the characteristics of the system and solutes are introduced, and in the second, the parameters of the gradient programme. When development to a constant distance is applied, the parameters defining cycles, steps and the type and composition of the eluent are introduced. The third part contains the retention characteristics of the solute as a function of the composition of the mobile phase. It may introduce the R_F values corresponding to the consecutive concentrations or eluents or apply the equation which describes the retention over the full range.

3. Experimental

A horizontal sandwich chamber of the DS type (Chromdes, Lublin, Poland) was used [11]. Precoated plates (silica gel Si 60_{F254}; Merck, Darmstadt, Germany) were activated at 100°C for 1 h and cooled in a desiccator. As eluents, solutions of ethanol in ethyl acetate with constant addition of 2% of acetic acid were used. The solutes (DNP derivatives of amino acids; BDH, Poole, UK; Table 1) were spotted on dry plates as ca. 0.01% solutions in the eluent. After each development (to a distance of 90 mm) the plates were dried for 0.5 h in a stream of air. The solutes were visible in daylight and the migration distances were measured manually ($\pm 0.02 R_F$ units).

Table 1
DNP-Amino acid reference collection

No.	Compound	Code
1	2,4-Dinitro-phenyl-L-α-alanine	DNP1
2	2,4-Dinitro-phenyl-L-asparagine	DNP4
3	2,4-Dinitro-phenyl-DL-glutamic acid	DNP7
4	N,N'-Di-(2:4-dinitro-phenyl)-L-lysine	DNP15
5	2,4-Dinitro-phenyl-L-serine	DNP20
6	2,4-Dinitro-phenyl-L-valine	DNP21

4. Results and discussion

Prediction of the separation of a mixture of solutes under conditions of multiple development is possible when the relationship between retention of the solutes and the properties of the mobile phase is known. In the experiments ternary eluents containing acetic acid, ethyl acetate and ethanol were used. The concentration of acetic acid was constant at 2% (v/v). Ethanol was the modifier with a content varying from 0 to 75%. R_F values for isocratic runs are given in Table 2. The retention vs. modifier concentration relationship can be represented as an equation: the Snyder–Soczewiński equation (Eq. 1) was

Table 2
R_F values (×100) obtained in isocratic development with the system ethyl acetate–ethanol–2% acetic acid and silica gel

Solute	Modifier (ethanol) concentration (%)					
	0	5	10	25	50	75
DNP1	28	35	41	44	58	79
DNP4	2	5	7	15	22	40
DNP7	10	16	19	33	40	61
DNP15	19	27	37	54	61	82
DNP20	9	13	19	30	40	65
DNP21	12	18	24	40	49	79

Table 3
Parameters k_0 and m of the Snyder–Soczewiński equation $\log k_{(i,j)} = \log k_{0(j)} - m(j)\log c_{(i)}$, calculated from data presented in Table 2

Solute	k_0	m	r
DNP1	0.334	0.76	0.8985
DNP4	1.340	1.03	0.9786
DNP7	0.601	0.89	0.9636
DNP15	0.218	0.96	0.9384
DNP20	0.532	0.99	0.9461
DNP21	0.266	1.19	0.9245

chosen. The constants (intercepts, slopes) of the equation were calculated by the least-squares method and are given in Table 3. To describe the data obtained under isocratic conditions, a second-degree polynomial was also applied; its constants are given in Table 4. The description of retention data in the form of equations permits the calculation of R_F values for any modifier concentration within the concentration range to which the equation can be applied.

Alternatively, the choice of a suitable composition of the mobile phase can be made from the plot of R_M ($\log k$) values against the composition of the binary mobile phase. In the case of multiple development, an essential problem is the choice of optimum eluent compositions and the calculation of the minimal number of developments necessary to achieve an appropriate spacing of spots along the chromatogram and adequate separation of consecutive pairs of solutes. The choice of the composition of the eluent and the number of developments required can be made by trial and error or by computer simulation of the multiple, development process. The first method is tedious and time consuming; in computer simulation, the main problem is the correctness of the program and the agreement of experimentally obtained and theoretically calculated final R_F values.

Table 4
Parameters of the polynomial calculated from data presented in Table 2

Solute	k_0	k_1	k_2	r
DNP1	2.24	−4.94	3.20	0.9579
DNP4	35.42	−153.95	150.00	0.8632
DNP7	7.45	−25.63	22.86	0.9373
DNP15	3.54	−12.86	11.67	0.9333
DNP20	8.54	−30.06	26.74	0.9443
DNP21	6.11	−21.88	19.45	0.9419

The basis of computer simulation of the process of multiple development (MD) is Eq. 14; it requires the introduction of the R_F value of a given solute for the chromatographic system applied. Simulation is applied to various numbers of developments and the final R_F values are calculated after each development. The simulation process is concluded when one of the solutes reaches $R_F = 0.80$. Eq. 14 was verified experimentally using R_F values found (a) after the first development (method 1), (b) calculated from the S–S equation (method 2) on the basis of isocratic data and (c) calculated from the polynomial (method 3) on the basis of isocratic data. The computer program was applied to the simulation of MD to compare the experimental and calculated R_F values which are given in Table 5.

Let us consider method 1, in which the R_F values obtained after the first development are substituted into Eq. 14. The simulation was performed for four developments; comparison of the measured and calculated values shows satisfactory agreement (Table 5): the differences are <0.1 R_F units.

Method applies the R_F values calculated form the S–S equation. The differences between the experimental and calculated R_F values are larger than those for method 1 and are in the range 0.05–0.17 R_F units. It can be seen that the differences between the final R_F values determined experimentally and calculated from Eq. 14 are proportional to differences in R_F values substituted in Eq. 14 calculated form Eq. 1 and R_F values obtained from isocratic analysis. The differences between the initial R_F values calculated from the S–S equation and determined in isocratic analysis can be interpreted as follows. Depending on the modifier concentration, two situations can arise. The first case corresponds to the situation in which the modifier concentrations lies in the concentration range for which

Table 5
Comparison of simulated and experimental data obtained in isocratic and multiple development for mobile phase of composition 5% ethanol–ethyl acetate–2% acetic acid

Solute	R_F isocratic			Final R_F after 4 developments				$\Delta R_F = R_{F(exp.)} - R_{F(calc.)}$		
	Experiment	Calculated		Experiment	Calculated			S–S development	Polynomial	
		S–S	Polynomial		After first development	S–S	Polynomial			
DNP1	0.35	0.28	0.33	0.89	0.88	0.81	0.86	0.01	0.08	0.03
DNP15	0.27	0.24	0.26	0.85	0.79	0.75	0.78	0.06	0.10	0.07
DNP21	0.18	0.14	0.20	0.70	0.63	0.53	0.67	0.07	0.17	0.03
DNP7	0.16	0.13	0.14	0.64	0.58	0.50	0.53	0.06	0.14	0.11
DNP20	0.13	0.11	0.12	0.51	0.50	0.44	0.47	0.01	0.07	0.04
DNP4	0.05	0.04	0.03	0.23	0.23	0.18	0.14	0.00	0.05	0.09

S-S: values R_F calculated from Eq. 1. Polynomial: values R_F calculated from Eq. 2.

the R_F values were determined by the isocratic method; in the second case, the modifier concentration is outside the concentration range for which the isocratic experiments were carried; then larger deviations of R_F values between theoretical and experimental final R_F values after multiple development.

The S–S equation assumes linear relationships between retention and concentration of modifier (strictly, log k and log C_{mod}). In practice, deviations from linearity are frequently observed, which lead to differences between calculated and experimental R_F values.

Method 3 is based on R_F values calculated from the polynomial equation. This method should be more accurate when the log k vs. log C_{mod} relationships are non-linear. The comparison of calculated and experimental R_F values indicates smaller discrepancies ($\Delta R_F = 0.03–0.11$) than in method 2 ($\Delta R_F = 0.05–0.17$), but larger than in method 1 ($\Delta R_F = 0.01–0.07$).

Comparing the agreement of the calculated and experimental R_F values, the three methods discussed for the case illustrated can be ordered in the sequence 1, and 2. Method 1 requires that each time the isocratic experiment is carried out and that a large number of chromatographic isocratic data for the chromatographic system chosen are collected (in order to have a complete characterization of the behaviour of the components of the mixture). For methods 2 and 3 a minimum amount of data is sufficient for the characterization of the system (three isocratic R_F values) to determine the parameters of the S–S and polynomial equations. Of course, a greater number of isocratic R_F values increases the accuracy of the methods and informs about the linearity of the S–S equation.

If the R_F values substituted into Eq. 14 for simulation originate from methods 2 and 3, then the simulation process can be carried out with division into cycles (see the Theoretical section) in which various compositions of the mobile phase are applied and in an extreme case a complete (qualitative) change of the mobile phase can be applied. Thus, simulations permit the investigation of numerous systems and the investigation of the effect of the number of developments on the final separation.

In the process of multiple development, the plate is dried after each development. It is assumed that the conditions of the development process should be identical for each development. To verify this assumption, the migration paths were calculated for each step and compared with the experimental values. The migration paths for each step were calculated from the isocratic R_F values obtained with 10% ethanol as modifier. The results are presented in Table 6. The comparison of migration paths calculated and determined experimentally after each step shows that the calculated paths are slightly larger

Table 6
Comparison of calculated and experimentally measured migration distances of solutes obtained after different numbers of developments

Number of developments	MD(mm)											
	DNP1		DNP15		DNP21		DNP7		DNP20		DNP4	
	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.
1	37	37	34	33	22	22	17	17	16	17	6	6
2	59	59	54	54	38	38	31	31	29	31	12	12
3	71	72	65	67	50	50	42	42	40	42	16	17
4	76	79	76	76	61	60	53	51	49	51	21	22

Experimental data obtained for mobile phase of composition 10% ethanol–ethyl acetate–2% acetic acid. Calculated data obtained from R_F values measured in isocratic experiment.

than the experimental paths. The calculated final values are also larger. These results are contrary to those given in Table 5, where the experimental R_F values were larger than the theoretical data. In this case the assumptions of identical properties of the adsorbent after each development were probably not completely justified. Especially for components of the mobile phase such as acetic acid, the drying process may be not able to remove it completely or to a constant degree owing to its low volatility and strong adsorption on the silica surface. The activity of the adsorbent can thus be different after each development and the differences in R_F values sum up. On the other hand, the increased concentration of the modifier and its incomplete removal may cause the formation of a stationary phase, causing a decrease retention relative to the theoretically calculated values. The full explanation of this phenomenon would require determinations of calculated and experimental values for the full range of modifier concentrations.

Another issue which should be considered is the number of developments. The process is repeated and the errors of single developments can sum up. In the system investigated the components of mobile phase are polar solvents (ethanol, ethyl acetate and acetic acid) with limited differences in polarities and the solvent demixing process is negligible to first approximation; the expected R_F values of the demixing fronts are high, which should not interfere with the migration of solutes of low and moderate R_F values, especially for first developments.

The three methods used for calculation give the same sequence of solutes and similar differences in R_F values between successive solutes. Considering the reasonable agreement between the R_F values calculated and measured experimentally in the investigated system, the following method of optimization could be proposed. By means of the polynomial form or S–S equation the R_F values for different concentrations of the modifier can be calculated. Then these values can be used for simulation of the multi-stage development under various conditions. It is easy to investigate the influence of the number of steps, development distance and combination of the different compositions of mobile phase used in the particular cycles on the final distribution of spots along the chromatogram.

Multiple development over a constant distance is an effective technique for the separation of mixtures which is due to a mechanism involving spot reconcentration and increased distance of development. The utilization of the potential advantages of the technique is much easier when an adequate model for the process of multiple development is at hand, which permits the prediction of final R_F values and the distribution of spots along the chromatograms without preliminary experiments, which in the case of non-op-

timum conditions are time consuming. The utilization of the model of the process of multiple development combined with computer-aided simulation experiments permits the full utilization of the existing optimization procedures. The fair agreement between the theoretically predicted and experimentally found data applies to systems in which the demixing effects of the mobile phase are insignificant; however, the maintenance of reproducible and controlled conditions of consecutive developments in a condition the fulfillment of which determines the agreement between calculated and experimental results.

The model presented in this study requires further verification by the investigation of various solute groups and adsorbent–eluent systems, especially those for which solvent demixing and variation of adsorbent activity may play a significant role.

Symbols

$c_{(i)}$	concentration of modifier for the ith step
$k_{0(j)}$	capacity factor of solute j for unit concentration of modifier (pure modifier) for normal-phase systems
$k_{(i),(j)}$	capacity factor of solute j for the ith step
$m_{(j)}$	slope of the log–log plot for solute j
$k_{0(j)}, k_{1(j)}, k_{2(j)}$	coefficients in polynomial
$R_{F(i,j)}$	R_F value for solute j corresponding to the ith concentration of modifier
$R_{FG(i)}$	final R_F value of solute j is gradient development
v_m	void volume of layer corresponding to the development distance $z_{(n)}$
$v_{e(i,j)}$	distance migrated by the front of the mobile phase and used for calculation of the migration distance each solute in particular step
$y_{(i,j)}$	migration distance of solute j in the ith step
$s_{(i,j)}$	total migration distance of solute j after i steps
$z_{(i)}$	development distance of the ith step
$z_{(n)}$	distance migrated by the front of mobile phase in nth step

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References

[1] C.F. Poole and S.K. Poole, Chromatography Today, Elsevier, Amsterdam, 1991, pp. 687–697.
[2] A. Jeanes, C.S. Wise and R.J. Dimler, Anal. Chem., 23 (1951) 415.
[3] J.A. Thoma, Anal. Chem., 35 (1963) 214.
[4] J.A. Thoma, J. Chromatogr., 12 (1963) 441.
[5] C.F. Poole and M.T. Belay, J. Planar Chromatogr., 4 (1991) 345.
[6] B. Szabady, M. Ruszinko and Sz. Nyiredy, in 8th International Symposium on Instrumental Planar Chromatography, Interlaken 1995, Proceedings, Poster G.14.
[7] C.F. Poole and S.K. Poole, Anal. Chem., 66 (1994) 27A.
[8] W. Markowski and E. Soczewiński, J. Chromatogr., 623 (1992) 139.
[9] W. Markowski, J. Chromatogr., 635 (1993) 283.
[10] E. Soczewiński, J. Chromatogr., 388 (1987) 91.
[11] T.H. Dzido, J. Planar Chromatogr., 3 (1990) 199.