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# GRADIENT ELUTION IN LIQUID CHROMATOGRAPHY

## IV. VERIFICATION OF THE THEORETICAL RELATIONSHIPS FOR ELUTION CHARACTERISTICS (RETENTION VOLUME, BAND WIDTH, RESOLUTION, PLATE NUMBER) IN ADSORPTION CHROMATOGRAPHY ON SILICA USING STEPWISE AND COMBINED (STEPWISE–GRADIENT) ELUTION AND SOME CONSIDERATIONS CONCERNING SOLVENT-PROGRAMMED AND ISOCRATIC ELUTION CHROMATOGRAPHY

PAVEL JANDERA and JAROSLAV CHURÁČEK

*Department of Analytical Chemistry, University of Chemical Technology, Pardubice (Czechoslovakia)*

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SUMMARY

The verification of the theoretical equations for the elution characteristics in stepwise and combined two-step isocratic–gradient elution chromatography is described for four N,N-dimethyl-*p*-aminobenzeneazobenzoyl amides chromatographed on Porasil A using a binary mobile phase consisting of ethyl acetate and cyclohexane. Good agreement between the experimental and calculated values was found. The resolutions in isocratic, simple gradient, stepwise and combined two-step isocratic–gradient elution are compared and the results achieved in the gradient elution chromatography of some N,N-dimethyl-*p*-aminobenzeneazobenzoyl amides and esters on silica are presented.

## INTRODUCTION

In the earlier parts of this series[1–3] we presented the theory that enables the retention characteristics in isocratic and gradient elution liquid chromatography to be estimated. This theory was verified for the adsorption chromatography of N,N-dimethyl-*p*-aminobenzeneazobenzoyl amides on silica in binary mobile phases consisting of cyclohexane and ethyl acetate.

Similar calculations are also possible for stepwise elution chromatography and for combined chromatography consisting in two consecutive steps, isocratic and gradient elution[2]. The verification of this theory is considered in this paper, using the same chromatographic system as in Part III[3]. The separations achieved using these techniques are compared with those in isocratic and gradient elution chromatography from both the theoretical and practical points of view.

## THEORETICAL

The theoretical calculations of the retention characteristics in isocratic, stepwise, gradient elution and combined two-step elution in adsorption and ion-exchange chromatography are based on a simple equation[1] which proved to describe well the relationship between the concentration, $c$ (molarity or mole fraction) of the more efficient eluting component in the binary mobile phase and the capacity ratio, $k'$, of the sample compound:

$$k' \approx k'_0 \cdot c^{-n} \tag{1}$$

where $k'_0$ and $n$ are constants and $k'_0$ represents the capacity ratio, $k'$, of the sample compound in the mobile phase when $c = 1$. Using this equation, the relationships for the retention characteristics in stepwise and combined two-step (isocratic and gradient) elution chromatography were derived in Part II[2] and are as follows.

### *Stepwise elution chromatography*

Retention volumes, $V_R$, $V'_R$:

$$V_R = V'_R + V_m \tag{2}$$

and

$$V'_R \approx \sum_{i=1}^{n-1} V_i \cdot \left(k'_i \cdot \frac{1 + k'_n}{1 + k'_i} - k'_n\right) + V_m \cdot k'_n \tag{3}$$

Peak width, $w$:

$$w = \frac{4\,V_m}{\sqrt{N}} \cdot (k'_0 \cdot c_n^{-n} + 1) \tag{4}$$

Resolution, $R_s$, of two compounds, 1 and 2:

$$R_s = 2 \cdot \frac{V'_{R2} - V'_{R1}}{w_2 + w_1} \tag{5}$$

The actual plate number achieved in stepwise elution chromatography can be calculated according to the well known equation

$$N = 16 \left(\frac{V_R}{w}\right)^2 \tag{6}$$

It is assumed that the peak is eluted in one step, the $n$th step. $k'_n$ and $c_n$ relate to the values of $k'$ and $c$ in the $n$th step and $k'_i$ is used for $k'$ in step $i$. $V_t$ is the total

volume of the mobile phase used in step $i$; $V_m$ is the volume of the mobile phase in the column and $N$ is the plate number of the column in isocratic elution chromatography.

*Chromatography using elution with mobile phase of a constant composition in the first step followed by gradient elution in the second step*

Retention volumes, $V'_{R(g)}$, $V_{R(g)}$:

$$V'_{R(g)} \approx \frac{V_1 \cdot k'_1}{1 + k'_1} + \frac{1}{B} \times$$

$$\times \left[(\varkappa \cdot n + 1) \cdot B \cdot k'_0 \cdot \left(V_m - \frac{V_1}{1 + k'_1}\right) + A^{\frac{\varkappa \cdot n + 1}{\varkappa}}\right]^{\frac{1}{\varkappa \cdot n + 1}} - \frac{A^{\frac{1}{\varkappa}}}{B} \tag{7}$$

and (8)

$$V_{R(g)} = V'_{R(g)} + V_m$$

Peak width, $w_{(g)}$:

$$w_{(g)} \approx \frac{4\,V_m}{\sqrt{N}} \cdot \left\{1 + k'_0 \cdot \left[A^{\frac{1}{\varkappa}} + B \cdot \left(V'_{R(g)} - V_1 \cdot \frac{k'_1}{1 + k'_1}\right)^{-\varkappa \cdot n}\right]\right\} \tag{9}$$

Plate number, $N_{(g)}$:

$$N_{(g)} = 16 \cdot \left(\frac{V'_{R(g)} + V_m}{w_{(g)}}\right)^2 \approx$$

$$\approx N \cdot \left(\frac{V'_{R(g)}}{V_m} + 1\right)^2 \cdot \frac{}{\left\{1 + k'_0 \cdot \left[A^{\frac{1}{\varkappa}} + B \cdot \left(V'_{R(g)} - V_1 \cdot \frac{k'_1}{1 + k'_1}\right)\right]^{-\varkappa \cdot n}\right\}^2} \tag{10}$$

Resolution, $R_{s(g)}$, of two compounds, 1 and 2:

$$R_{s(g)} = 2 \cdot \frac{V'_{R(g)2} - V'_{R(g)1}}{w_{(g)2} + w_{(g)1}} \tag{11}$$

where $k'_1$ is the capacity ratio, $k'$ (constant), of the sample compound in the first step using a volume $V_1$ of the mobile phase, and $A$, $B$ and $\varkappa$ relate to the parameters of the gradient function in the second step (eqn. 2).

If the volume of the connecting tubing between the outlet of the gradient-generating device and the top of the column ($V_z$) cannot be neglected, it must be added to the volume of the mobile phase in the first step, $V_1$, in the calculations concerning both stepwise and combined two-step isocratic gradient elution.

## EXPERIMENTAL

The instrumentation and operating conditions used in this work were the same as those used in Part III[3].

*Column*

The columns used were (a) glass, 400 × 3.0 mm, packed with Porasil A(60), 37–75 μm (spherical) (Waters Ass., Framingham, Mass., U.S.A.), $V_m = 2.00$ ml; (b) glass, 400 × 2.75 mm, packed with Kieselgel (Merck, Darmstadt, G.F.R.) (irregular particle shape), screened to obtain the fraction under 50 μm, which was separated from fine powder particles by repeated decantation with water; $V_m = 1.08$ ml. The volume of the connecting tubing between the mixing chamber of the gradient pump and the injection port of the column, $V_z = 0.67$ ml.

*Chromatographed compounds*

The coloured derivatives of N,N-dimethyl-*p*-aminobenzeneazobenzoic acid[4] were used individually or as a synthetic mixture, dissolved in ethyl acetate (*ca.* 1 mg/ml of each compound). The volume of the sample solution was 8 μl.

## RESULTS AND DISCUSSION

*Verification of the theoretical equations*

A synthetic mixture of four N,N-dimethyl-*p*-aminobenzeneazobenzoyl amides [the dimethyl-, diethyl-, di-(*n*-propyl)- and di-(*n*-butyl)amide] was used as the sample to be separated. In the experiment with stepwise elution, the concentration of ethyl acetate in the mobile phase was gradually increased in three steps from $c_1 = 0.20$ in the first step ($V_1 = 8.90$ ml), to $c_2 = 0.67$ in the second step ($V_2 = 2.79$ ml) and $c_3 = 1.00$ in the third step. The concentration $c$ is expressed as the volume ratio of ethyl acetate in cyclohexane in the verification experiments described, as it has been found that eqn. 1 has equal validity for both the volume ratio and mole fraction of ethyl acetate in the mobile phase[3]. The expression of the concentration in the form of the volume ratio is more convenient, as the gradient pump can mix the solvents in volume ratios only.

The separation achieved and the dependence of $c$ on time (or volume of the eluate) are shown in Fig. 1.

Fig. 2 shows the separation and the dependence of $c$ on the volume of the eluate for the two-step combined isocratic–gradient elution. The concentration of ethyl acetate was constant ($c = 0.20$) in the first step ($V_1 = 7.41$ ml) and a linear gradient of the concentration of ethyl acetate was used in the second step ($\varkappa = 1$; $A = c_0 = 0.2$; $B = 0.063936$).

The experimental values of retention volume, peak width, resolution and plate number in both experiments are compared with the values calculated according to eqns. 4–11 in Table I. The agreement between the experimental and calculated values is satisfactory. The differences between the calculated and experimental values of $w$ of the diethylamide and di-(*n*-propyl)amide in the stepwise elution experiment and, consequently, the differences between the corresponding values of $N$ and $R_s$, can be attributed to the errors in the evaluation of the widths of the experimental peaks,

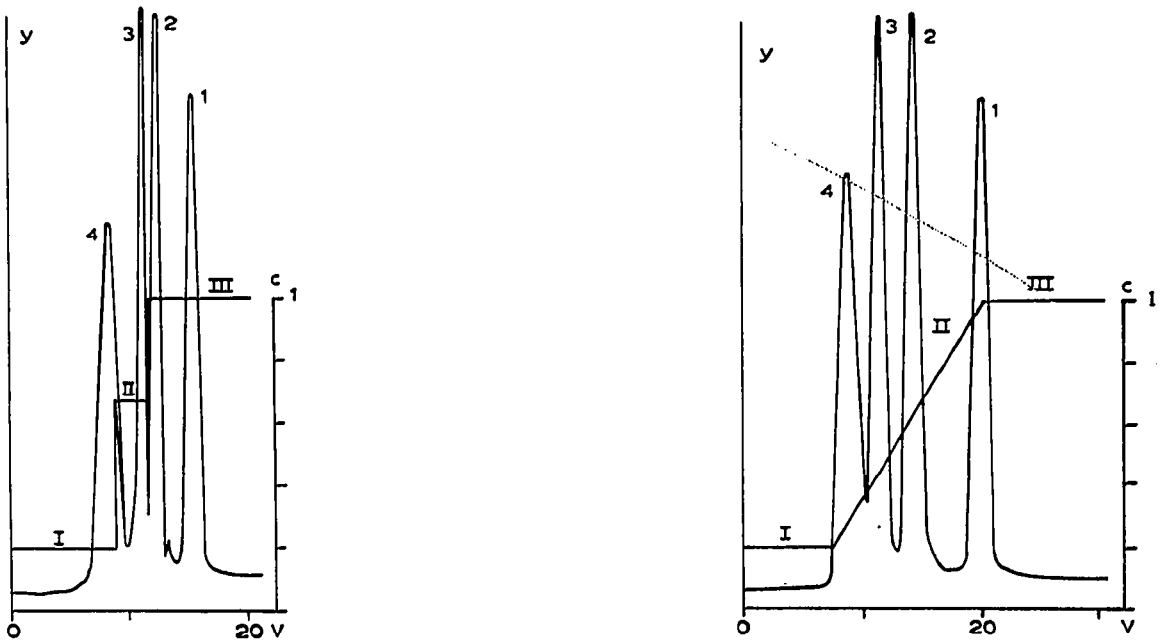



Fig. 1. Separation of a mixture of the dimethylamide (1), diethylamide (2), di-(*n*-propyl)amide (3) and di-(*n*-butyl)amide (4) of N,N-dimethyl-*p*-aminobenzeneazobenzoic acid in stepwise elution chromatography on a column (400 × 3.0 mm) packed with Porasil A ($V_m$ = 2.00 ml) with ethyl acetate–cyclohexane as the mobile phase. Flow-rate: 38.5 ml/h. Detection: photometric, 440 nm. Step I: volume, 8.90 ml; volume ratio of ethyl acetate, 0.20. Step II: volume, 2.79 ml; volume ratio of ethyl acetate, 0.67. Step III: volume ratio of ethyl acetate, 1.00. $V$ = volume of the eluate (ml); $y$ = detector response. The figure also shows the dependence of the concentration ($c$) (volume ratio) of ethyl acetate at the inlet of the column on $V$.

Fig. 2. Separation of a mixture of the dimethylamide (1), diethylamide (2), di-(*n*-propyl)amide (3) and di-(*n*-butyl)amide (4) of N,N-dimethyl-*p*-aminobenzeneazobenzoic acid in combined two-step elution chromatography (first step, isocratic; second step, gradient) on a column (400 × 3.0 mm) packed with Porasil A ($V_m$ = 2.00 ml) with ethyl acetate–cyclohexane as the mobile phase. Flow-rate: 38.5 ml/h. Detection: photometric, 440 nm. Step I: volume, 7.41 ml; volume ratio of ethyl acetate, 0.20. Step II: gradient parameters, $\varkappa = 1$, $A = c_0 = 0.2$, $B = 0.063936$. Step III: volume ratio of ethyl acetate, 0.20 (this step was applied after the elution of the last compound had been accomplished). $V$ = volume of the eluate (ml); $y$ = detector response. The figure also shows the dependence of the concentration ($c$) (volume ratio) of ethyl acetate at the inlet of the column on $V$.

which are very narrow and partially overlap. The relative differences in the experimental and calculated values of $V'_{R(g)}$, $R_{s(g)}$ and $w_{(g)}$ in the combined elution experiment are less than 10% for all of the compounds, with the exception of the $w_{(g)}$ value for the di-(*n*-propyl)amide. The agreement between the calculated and experimental plate numbers is also satisfactory.

The experiments described show that eqns. 4–11 can be used successfully in order to predict the elution characteristics in chromatography involving different consecutive elution steps with a constant or changing composition of the mobile phase. It has been ascertained that eqn. 1 provides a good basis even for rather complex calculations in the system studied. These calculations could be of practical value, as stepwise and combined elution make a significant contribution to the pro-

TABLE I

EXPERIMENTAL AND CALCULATED VALUES OF THE ELUTION CHARACTERISTICS OF N,N-DIMETHYL-*p*-AMINOBENZENEAZOBENZOYL AMIDES IN ADSORPTION CHROMATOGRAPHY ON PORASIL A USING STEPWISE AND COMBINED TWO-STEP ISOCRATIC–GRADIENT SOLVENT-PROGRAMMED ELUTION

A, stepwise elution; step I: $V_1 = 8.90$ ml, $c_1 = 0.20$; step II: $V_2 = 2.79$ ml, $c_2 = 0.67$; step III: $c_3 = 1.00$. $V_i$ is used for the total volume of the mobile phase in step $i$ and $c_i$ denotes a constant concentration (volume ratio) of ethyl acetate in the mobile phase (cyclohexane + ethyl acetate). B, combined two-step isocratic–gradient elution; step I: $V_1 = 7.41$ ml, $c_1 = 0.20$ (isocratic); step II: gradient elution with the gradient parameters $\varkappa = 1$, $A = c_0 = 0.2$, $B = 0.063936$ (isocratic step III ($c_3 = 1.0$) in Fig. 2 was not used for the elution). Column dimensions, 400 × 3 mm; $V_m = 2.00$ ml. Adsorbent: Porasil A(60), 37–75 μm. Flow-rate of mobile phase (ethyl acetate–cyclohexane): 38.5 ml/h. Temperature: 20–22°. Detection: photometric, 440 nm. Chromatographed derivatives: 1 = dimethylamide; 2 = diethylamide; 3 = di-(*n*-propyl)amide; 4 = di-(*n*-butyl)amide; *ca.* 8 μg each. The experimental values represent the arithmetic means from three experiments.

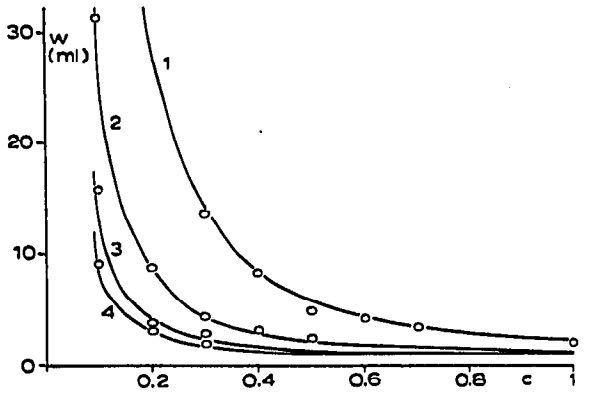
| *Experiment* | *Step* | *Derivative* | $V'_R$ *(ml)* | | $w$ *(ml)* | | $N$ | | $R_s$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* | *Calc.* | *Exptl.* |
| A | III | 1 | 13.76 | 13.88 | 2.09 | 1.99 | 910 | 1019 | 2.16 | 1.97 |
| | III | 2 | 10.35 | 10.39 | 1.06 | 1.56 | 2172 | 1009 | 1.24 | 0.79 |
| | II | 3 | 9.04 | 9.27 | 1.05 | 1.29 | 1769 | 1221 | 1.20 | 1.30 |
| | I | 4 | 6.64 | 6.42 | 2.95 | 3.10 | 137 | 118 | | |
| B | II | 1 | 18.05 | 17.73 | 2.47 | 2.25 | 1054 | 1230 | 2.59 | 2.53 |
| | II | 2 | 12.24 | 12.53 | 2.01 | 1.86 | 803 | 976 | 1.48 | 1.49 |
| | II | 3 | 9.11 | 9.71 | 2.22 | 1.83 | 401 | 655 | 0.96 | 1.03 |
| | I | 4 | 6.64 | 7.21 | 2.95 | 3.02 | 137 | 149 | | |

gramming of capacity ratios of sample compounds during elution in order to increase the resolution while maintaining the analysis time as short as possible (requiring a minimal volume of the mobile phase for the elution).

*Comparison of the resolution in chromatography using different modes of elution*

*Isocratic elution.* As far as eqn. 1 can be used to describe the influence of the concentration of the more efficient eluting component in the binary mobile phase on the capacity ratios of sample compounds, an increase in concentration will cause decreases in retention volume, peak width and resolution ($n > 0$). If sample components have different values of $n$, a change in the composition of the mobile phase will influence the retention ratio (separation factor), $\alpha$. The change in $\alpha$ brings about corresponding change in resolution. The situation is different with compounds that have similar values of $n$ and in this case the retention ratio is not dependent on the composition of the mobile phase, which is able to influence the resolution only by means of the capacity ratio, $k'$. In such an instance, $R_s$ can acquire values between an upper ($c = 0$) and a lower ($c = 1$) limit for given two components of the mobile phase, column and flow-rate. The maximum $R_s$ would be achieved for $c = 0$, at a cost of maximum separation time (if any elution could be accomplished under this condition).

Fig. 3 shows the dependence of the peak widths of the four N,N-dimethyl-*p*-aminobenzeneazobenzoyl amides studied on the volume ratio ($c$) of ethyl acetate in the mobile phase (cyclohexane + ethyl acetate) on the column packed with Porasil A. The dependence of the resolution on the volume ratio of ethyl acetate is shown



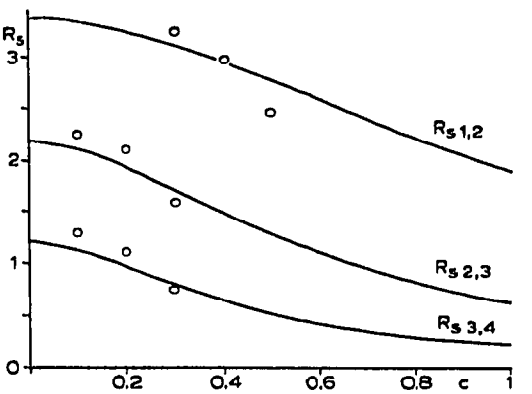




Fig. 3. Relationship between the peak width ($w$) of N,N-dimethyl-*p*-aminobenzeneazobenzoyl amides and the concentration ($c$, volume ratio) of the more efficient eluting agent (ethyl acetate) in the binary mobile phase cyclohexane–ethyl acetate in chromatography on Porasil A (37–75 $\mu$m). Derivative: 1 = dimethylamide; 2 = diethylamide; 3 = di-(*n*-propyl)amide; 4 = di-(*n*-butyl)amide. Column dimensions: 400 × 3.0 mm; $V_m$ = 2.00 ml. Flow-rate: 38.5 ml/h. Detection: photometric, 440 nm. The points represent the experimental values and the curves were calculated from the average values of $n$ and $k'_0$ (Table III in ref. 3).

Fig. 4. Relationship between the resolution ($R_s$) and the concentration ($c$, volume ratio) of the more efficient eluting agent (ethyl acetate) in the binary mobile phase cyclohexane–ethyl acetate in chromatography on Porasil A (37–75 $\mu$m). The subscripts to $R_s$ on the curves indicate the derivatives listed in Fig. 3. The operating conditions are as in Fig. 3. The points represent the experimental values and the curves were calculated from the average values of $n$ and $k'_0$ (Table III in ref. 3).

in Fig. 4. The experimental points are located close to the calculated curves. The limits of the $R_s$ values are presented in Table II. The maximum acceptable concentration of ethyl acetate (or of any more efficient eluting component of the binary mobile phase) for the resolution required (within the above limits) can be calculated using the equation

$$c = \left(\frac{k'_{02}}{2}\right) \cdot \left(\frac{\sqrt{N}}{2R_s} \cdot \frac{\alpha_0 - 1}{\alpha_0} - \frac{\alpha_0 + 1}{\alpha_0}\right)^{\frac{1}{n}} \qquad (12)$$

where $\alpha_0 = k'_{02}/k'_{01}$ ($k'_0$ = 2.056 for the dimethylamide, 0.554 for the diethylamide, 0.253 for the di-(*n*-propyl)amide and 0.166 for the di-(*n*-butyl)amide, and $n \approx 1.86$ for all of the compounds[3]).

*Simple gradient elution.* The parameters $A$ ($A = c_0$, the initial concentration of the more efficient component of the mobile phase) and $B$ (the slope of the gradient) affect the elution characteristics in simple gradient elution chromatography in a similar manner to the concentration of the more efficient component in the mobile phase ($c$) in isocratic elution chromatography. An increase in $A$ or $B$ will cause a decrease in retention volumes, peak widths and resolutions of the chromatographed compounds. The peak width shows a tendency to reach a constant value at high $B$ values, as shown in Fig. 5 for the four N,N-dimethyl-*p*-aminobenzeneazobenzoyl amides in the system studied. $R_{s(B)}$ values reach a maximum at $B$ values near to zero (Fig. 6). These maximum values are the same as the $R_s$ values achieved when using

TABLE II

THEORETICAL LIMITS OF RESOLUTION OF THE DIMETHYL- (1) DIETHYL- (2) DI-(*n*-PROPYL)- (3) AND DI-(*n*-BUTYL)- (4) AMIDES OF N,N-DIMETHYL-*p*-AMINOBENZENEAZOBENZOIC ACID THAT CAN BE ACHIEVED IN A BINARY MOBILE PHASE CONSISTING OF ETHYL ACETATE AND CYCLOHEXANE

Column: 400 × 3 mm, $V_m$ = 2.00 ml; packed with Porasil A, 37–75 μm.

| *Compounds* | *Theoretical limits of* $R_s$ | |
|---|---|---|
| | *Lower* | *Upper* |
| 1,2 | 1.90 | 3.36 |
| 2,3 | 0.62 | 2.18 |
| 3,4 | 0.22 | 1.21 |

elution with the mobile phase containing the more efficient eluting component at a concentration $c = c_0 = A$.

The increase in the value of the parameter $\varkappa$ is connected with increasing retention volumes, while both peak widths and resolution are not much influenced. It is probable that there are certain values of $\varkappa$ that make it possible to achieve maximum resolution. The experimental values of the resolution of the four N,N-dimethyl-*p*-aminobenzeneazobenzoyl amides studied are higher at $\varkappa = 1.0$ and $\varkappa = 2.0$ than at $\varkappa = 0.5$ or $\varkappa = 4.0$ (ref. 3).

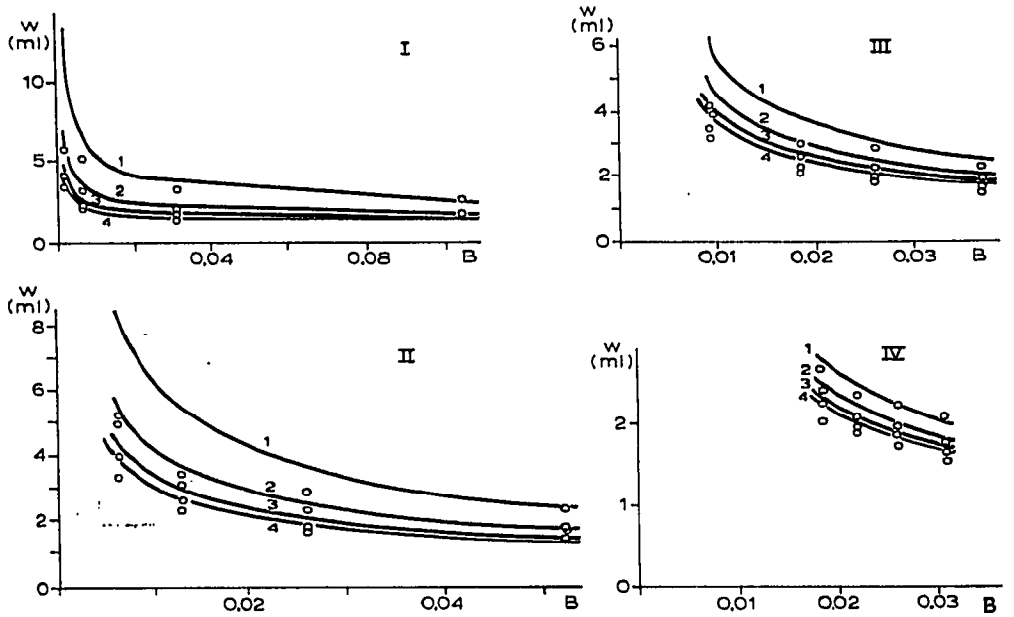



Fig. 5. Relationship between the peak width ($w$) of N,N-dimethyl-*p*-aminobenzeneazobenzoyl amides and the parameter $B$ in gradient elution chromatography on Porasil A (37–75 μm) in the binary mobile phase cyclohexane–ethyl acetate. Parameters of the gradient elution: $A = c_0 = 0$; $V_y$ = 38.5 ml; I, $\varkappa = 0.5$; II, $\varkappa = 1.0$; III, $\varkappa = 2.0$; IV, $\varkappa = 4.0$. The numbers on the curves indicate the derivatives listed in Fig. 3 and the operating conditions are also identical. The points represent the experimental values and the curves are calculated from the average values of $n$ and $k'_0$ (Table III in ref. 3).

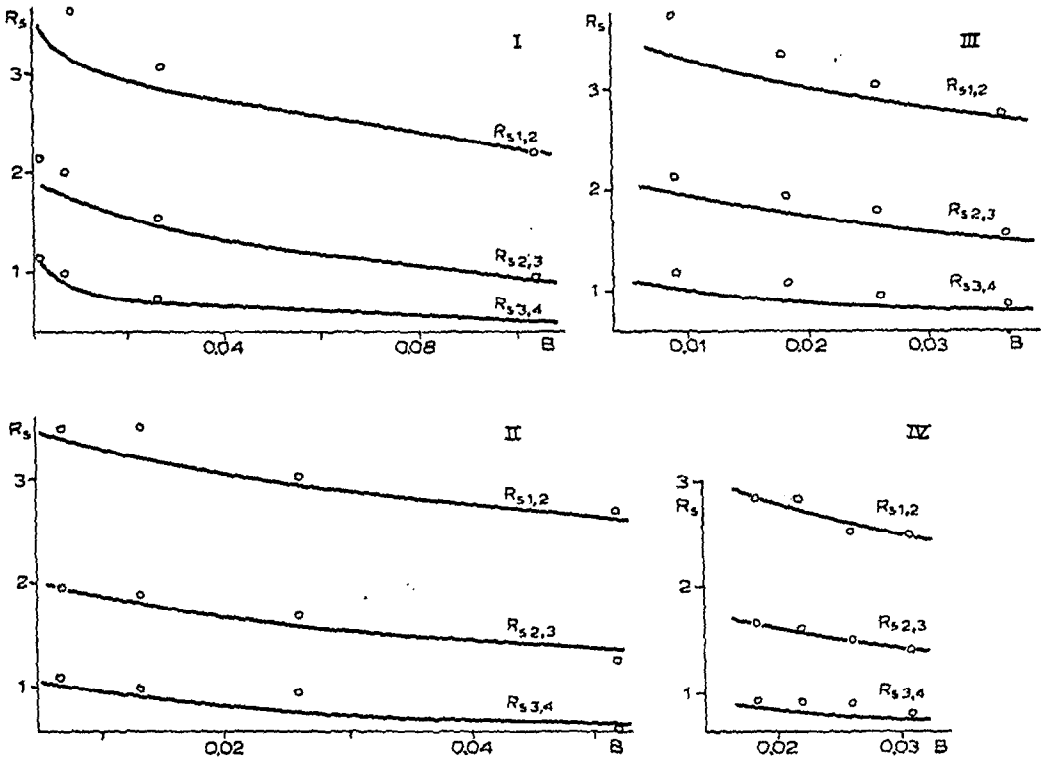



Fig. 6. Relationship between the resolution ($R_s$) of N,N-dimethyl-*p*-aminobenzeneazobenzoyl amides and the parameter $B$ in gradient elution chromatography on Porasil A (37–75 $\mu$m) in the binary mobile phase cyclohexane–ethyl acetate. The parameters of the gradient elution are as in Fig. 5. The subscripts to $R_s$ on the curves indicate the derivatives listed in Fig. 3 and the operating conditions are also identical. The points represent the experimental values and the curves are calculated from the average values of $n$ and $k'_0$ (Table III in ref. 3).

It can be shown that the resolution of compounds with similar values of $n$ will be lower in gradient elution with binary mobile phases than the maximum theoretical value in isocratic elution chromatography (at $c_0 = A$ for $B > 0$). Thus, simple gradient elution does not represent any significant improvement over isocratic elution in the separation of two-component mixtures. However, gradient elution has clear advantages if multicomponent mixtures are to be separated. The appropriate choice of the gradient function enables a more regular positioning of the peaks of the sample components in the chromatogram to be achieved, and consequently a good resolution is obtained in a shorter time in comparison with isocratic elution.

The optimization of the gradient function would be possible through calculation of the parameters $A$, $B$ and $\varkappa$ for two values of resolution required for two different "critical" pairs of sample components, or the calculation could be based on the required resolution for one pair of sample components and the maximum permissible analysis time. Such calculations lead to a system of equations that is impossible to solve in the explicit form and the use of a computer is required. The experimental results encouraged us to assume that calculations according to the theoretical equa-

tions for the elution characteristics[3] are precise enough to allow for such calculations to give results that have practical value.

*Stepwise elution.* Stepwise elution, consisting in consecutive isocratic steps with different compositions of the mobile phase, makes it possible to achieve separations in less time than with the other modes of elution if the sequence of volumes and compositions of the mobile phase in each step is correctly chosen. The calculation of these parameters for a required resolution or analysis time, based on eqn. 1, is not as complicated as with gradient elution and the relationships can be expressed in the explicit form. The separation of the four N,N-dimethyl-*p*-aminobenzeneazobenzoyl amides shown in Fig. 1 is close to the optimum conditions and requires only *ca.* 17 ml of the mobile phase, which is a significantly lower volume than in isocratic or gradient elution with a comparable resolution (see ref. 3 for comparison).

A serious disadvantage of stepwise elution is that a sudden discontinuous change in concentration of the more efficient component in the mobile phase can cause significant irregularities in the shapes of peaks of the compounds eluted. This is connected with difficulties in evaluating chromatograms for the purpose of quantitative analysis. Sometimes even "false" peaks can be found. Such peaks do not represent real individual compounds and are, in fact, the remaining compounds from incomplete elution in the previous step, which are swept from the column by the front of the mobile phase containing a higher amount of the more efficient eluting component. The small peak eluted after compound No. 2 in Fig. 1 is an example of a "false" peak and can be attributed to the remainder of compound 2 after elution in step II, which is eluted by pure ethyl acetate at the beginning of step III. (Fig. 1 shows the concentration of ethyl acetate in the mobile phase at the inlet of the column, and the retardation of a volume equal to $V_m$ (2.00 ml) in comparison with the chromatographic trace must be considered.) "False" peaks can lead to an erroneous interpretation of the chromatogram.

*Combined two-step isocratic–gradient elution.* The use of combined two-step elution, isocratic in the first step and gradient in the second step, eliminates the difficulties connected with sudden changes in the composition of the mobile phase. Fig. 2 shows an example of such an elution, as, in fact, compound No. 1 was completely eluted in step II and the third, isocratic step ($c = 1$) was not utilized for the separation. The resolution is comparable with the resolution obtained in the experiment with stepwise elution (Fig. 1), but the total volume of the mobile phase required was greater (*ca.* 22 ml). In this case, no "false" peaks were observed. The resolution of the di-(*n*-propyl)- and diethylamide and of the diethyl- and dimethylamide was better than in the experiment with stepwise elution. The resolution of the di-(*n*-butyl)- and di-(*n*-propyl)amide is lower in combined two-step elution, owing to the shorter first (isocratic) step ($c = 0.2$) in comparison with the first step with the same composition of the mobile phase in the stepwise elution experiment (7.41 and 8.90 ml, respectively).

The parameters of the combined two-step elution for a required resolution can be calculated as follows. First, the concentration of the more efficient component in the mobile phase is calculated for the first step in the same manner as in isocratic or stepwise elution, considering the required resolution of the two compounds eluted first. This concentration also represents the value of the parameter $A = c_0$ in the second, gradient step. The remaining parameters, $B$ and $\varkappa$, must be calculated by an

approximative method for the required resolution of a further two "critical" compounds, or for the maximum permissible time of the analysis.

*Separation of N,N-dimethyl-p-aminobenzeneazobenzoyl amides and esters by gradient elution chromatography on silica*

Gradient elution separation of N,N-dimethyl-*p*-aminobenzeneazobenzoyl derivatives of lower aliphatic secondary amides on Porasil A with different gradient parameters was described in Part II of this series[2]. It was possible to achieve successful separations of various other derivatives of N,N-dimethyl-*p*-aminobenzeneazobenzoic acid using gradient elution. The values of the retention volumes, $V'_{R(g)}$, for a number of compounds studied by gradient elution chromatography on a column (400 × 2.75 mm; $V_m$ = 1.08 ml) packed with Kieselgel (<50 $\mu$m) are surveyed in Table III for two linear concentration gradients of ethyl acetate in cyclohexane requiring 1 and 2 h, respectively, for the total concentration change from pure cyclohexane to pure ethyl acetate ($\varkappa = 1$; $A = 0$; $B = 0.012987$ and 0.025974, respectively). A number of 3–5-component mixtures of these derivatives could be resolved. In gradient elution with a total time of 2 h, a retention ratio, $\alpha$, of about 1.3 is sufficient to yield

TABLE III

RETENTION VOLUMES, $V'_{R(g)}$, OF N,N-DIMETHYL-*p*-AMINOBENZENEAZOBENZOYL ESTERS AND AMIDES IN GRADIENT ELUTION CHROMATOGRAPHY ON KIESELGEL (< 50 $\mu$m) IN ETHYL ACETATE–CYCLOHEXANE BINARY MOBILE PHASE

$\varkappa = 1$; $A = 0$; $B = 0.025974$ and 0.012987. Column dimensions: 400 × 2.75 mm; $V_m$ = 1.08 ml. Flow-rate: 0.643 ml/min. Operating pressure: *ca.* 10 atm. Sample volume and concentration: 10 $\mu$l, 0.5 g/l. Detection: photometric, 440 nm.

| *Derivative* | $V'_{R(g)}$ *(ml)* | |
|---|---|---|
| | *B = 0.025974* | *B = 0.012987* |
| *m*-Cresyl ester | 8.10 | 11.53 |
| *o*-Cresyl ester | 8.48 | 12.30 |
| Benzyl ester | 8.48 | — |
| $\alpha$-Naphthyl ester | 9.25 | — |
| *p*-Nitrophenyl ester | 9.63 | 15.16 |
| *p*-Methoxyphenyl ester | 10.59 | 15.54 |
| *m*-Nitrophenyl ester | 10.59 | 15.74 |
| *o*-Nitrophenyl ester | 12.31 | 18.47 |
| 2,4-Dinitrophenyl ester | 28.95 | 40.77 |
| 2,6-Dinitrophenyl ester | 29.71 | 43.06 |
| *p*-Chloroanilide | 12.12 | 18.47 |
| $\alpha$-Naphthylamide | 12.12 | 18.60 |
| Anilide | 12.50 | 18.81 |
| *p*-Toluidide | 13.84 | — |
| N-Ethylanilide | 13.86 | — |
| *p*-Methoxyanilide | 14.60 | 23.44 |
| Benzylanilide | 14.99 | 23.98 |
| N-Methylanilide | 15.74 | 24.14 |
| *m*-Nitroanilide | 15.75 | 24.17 |
| *p*-Nitroanilide | 27.78 | 37.58 |
| *o*-Nitroanilide | 28.09 | 38.23 |
| *p*-Amidoacetanilide | 28.30 | 38.47 |

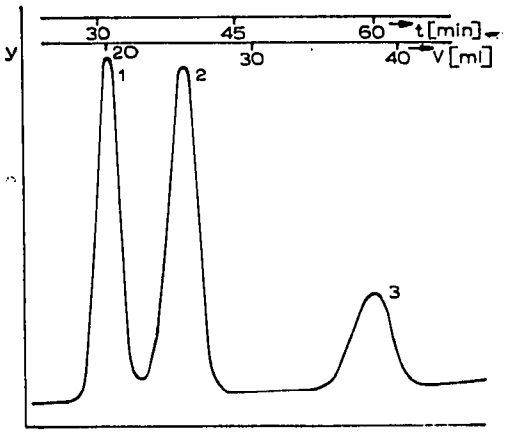



Fig. 7. Chromatographic separation of a mixture of the α-naphthylamide (1), N-methylanilide (2) and *p*-nitroanilide (3) of N,N-dimethyl-*p*-aminobenzeneazobenzoic acid using gradient elution on Kieselgel (< 50 μm) in the binary mobile phase ethyl acetate–cyclohexane. Column dimensions: 400 × 2.75 mm; $V_m$ = 1.08 ml. Flow-rate: 0.643 ml/min. Operating pressure: *ca.* 10 atm. Sample volume: 10 μl. Concentration of the compounds in sample: *ca.* 0.5 g/l. Detection: photometric, 440 nm. Parameters of the gradient: $A = 0$; $\varkappa = 1$; $B = 0.012987$. $y$ = Detector response; $V$ = volume of eluate; $t$ = time elapsed.

a satisfactory separation at a flow-rate of the mobile phase of 0.643 ml/min. Figs. 7–9 show examples of separation of three mixtures under these conditions. The separation of the α-naphthylamide, N-methylanilide and *p*-nitroanilide is illustrated in Fig. 7, that of the anilide, *p*-methoxyanilide and *p*-acetaminoanilide in Fig. 8 and that of the *m*-cresyl ester, *p*-methoxyphenyl ester, *o*-nitrophenyl ester and 2,4- and 2,6-dinitrophenyl esters in Fig. 9.

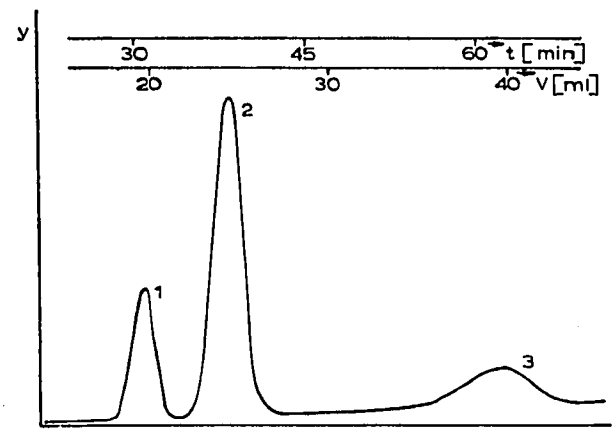



Fig. 8. Chromatographic separation of a mixture of the anilide (1), *p*-methoxyanilide (2) and *p*-amidoacetanilide (3) of N,N-dimethyl-*p*-aminobenzeneazobenzoic acid using gradient elution on Kieselgel (< 50 μm) in the binary mobile phase ethyl acetate–cyclohexane. Operating conditions as in Fig. 7. $y$ = Detector response; $V$ = volume of eluate; $t$ = time elapsed.

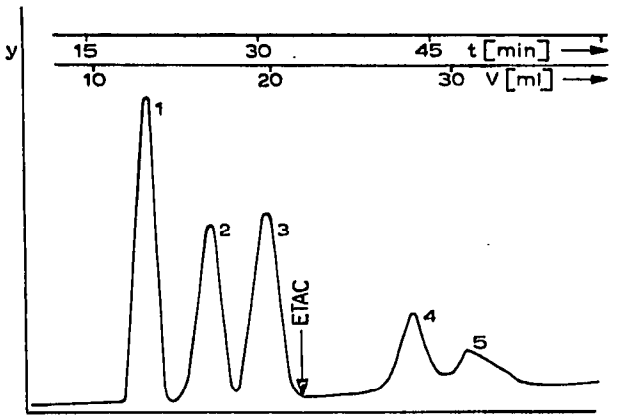



Fig. 9. Chromatographic separation of a mixture of the *m*-cresyl ester (1), *p*-methoxyphenyl ester (2), *o*-nitrophenyl ester (3), 2,4-dinitrophenyl ester (4) and 2,6-dinitrophenyl ester (5) of N,N-dimethyl-*p*-aminobenzeneazobenzoic acid using gradient elution on Kieselgel (< 50 $\mu$m) in the binary mobile phase ethyl acetate–cyclohexane. Operating conditions as in Fig. 7. The elution of the last two compounds was accomplished with pure ethyl acetate (ETAC; from the volume marked). $y$ = Detector response; $V$ = volume of eluate; $t$ = time elapsed.

In order to increase the number of theoretical plates of the column, gradient elution with a less viscous binary mobile phase consisting of *n*-pentane and acetone was tested with some derivatives. A relative increase in $N$ of about 15% was achieved. The retention volumes of the compounds tested were lower than those in cyclohexane–ethyl acetate under identical conditions, as acetone is a stronger eluting agent than ethyl acetate. Therefore, it was possible to decrease the total analysis time required for the separation of the four derivatives of phenols (Fig. 8) by about 10–15% with approximately the same resolution as in cyclohexane–ethyl acetate. It was difficult, however, to avoid bubble formation in the pump when using these low-boiling solvents. The separation of the lower aliphatic esters of N,N-dimethyl-*p*-aminobenzeneazobenzoic acid ($C_1$–$C_9$) on silica was inefficient, because of the small differences in the capacity ratios of the homologous esters studied.

## REFERENCES

1 P. Jandera and J. Churáček, *J. Chromatogr.*, 91 (1974) 207.
2 P. Jandera and J. Churáček, *J. Chromatogr.*, 91 (1974) 223.
3 P. Jandera and J. Churáček, *J. Chromatogr.*, 93 (1974) 17.
4 J. Churáček, *J. Chromatogr.*, 48 (1970) 241.