

CHROM. 8468

GRADIENT ELUTION IN LIQUID CHROMATOGRAPHY

VII. COMPARISON OF DIFFERENT MOBILE PHASES IN ADSORPTION CHROMATOGRAPHY

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(Received May 27th, 1975)

SUMMARY

Theoretical assumptions concerning the influence of the composition of binary mobile phases on capacity ratios in adsorption chromatography have been verified for binary mobile phases composed of ethanol-*n*-pentane; *n*-propanol-*n*-heptane; dioxane-*n*-heptane and chloroform-*n*-heptane. Calculated and experimental values of retention characteristics in gradient elution chromatography have also been compared for these systems. The agreement between experiment and theory was satisfactory, just as it was for a mobile phase composed of cyclohexane and ethyl acetate (as described in Part III of this series). Practical aspects of the solvent systems tested as the mobile phase in gradient elution chromatography are discussed, and the relationship of plate number to composition of mobile phase is considered.

INTRODUCTION

A theory that makes possible estimation of retention characteristics (retention volume, band width, plate number and resolution) in gradient elution liquid chromatography was described in Parts I and II of this series^{1,2}. Its practical utility in adsorption chromatography was tested by using model experiments with four *N,N*-dimethyl-*p*-aminobenzeneazobenzoyl amides, which were chromatographed on columns packed with silica (Porasil A) in a binary mobile phase of ethyl acetate and cyclohexane^{3,4}; the experimental elution characteristics were in good agreement with the theory.

In the present work, the theory has been verified for adsorption chromatography using several other mobile phases, mainly those compatible with a UV detector.

THEORETICAL

In the theoretical approach verified in this paper, the validity of a simple relationship between the concentration (mole fraction) of the more efficient eluting

component in the binary mobile phase (c) and the capacity ratio of a sample compound (k') is assumed; this relationship is expressed by:

$$k' \approx k'_0 \cdot c^{-n} \quad (1)$$

where k'_0 and n are constants¹.

In the derivation of equations for retention volume, retention ratio, peak width, plate number and resolution in gradient elution chromatography, it was further assumed that no change occurred in the composition of the mobile phase during transport from the outlet of the gradient-generating device to the column and that the gradient-generating device was able to produce any required mathematical form of the relationship between the concentration (c) of the more efficient eluting component in the binary mobile phase at the outlet of the gradient-generating device and the volume (V) of mobile phase delivered. This relationship was chosen as follows²:

$$c = (A^{\frac{1}{\kappa}} + B \cdot V)^{\kappa} \quad (2)$$

Here, A , B and κ are adjustable constants that characterize the shape of the gradient function and are defined as:

$$A = c_0 \quad (3)$$

and:

$$B = \frac{c_k^{\frac{1}{\kappa}} - c_0^{\frac{1}{\kappa}}}{V_f} \quad (4)$$

where c_0 is the initial concentration (c) at the beginning of gradient elution and V_f is the volume of eluate where $c = c_k$; an arbitrary value can be chosen for c_k (with advantage, $c_k = 1$ or $c_k = 0$).

By using eqs. 1 and 2, the following relationships for retention volume ($V_{R(g)}$, $V'_{R(g)}$), peak width ($w_{(g)}$), plate number ($N_{(g)}$) and resolution of two compounds 1 and 2 ($R_{s(g)}$) in gradient elution chromatography were derived²:

$$V_{R(g)} = V_m + V'_{R(g)} \quad (5)$$

$$V'_{R(g)} \approx \frac{1}{B} \cdot \left[(2 \cdot n + 1) \cdot B \cdot k'_0 \cdot V_m + A^{\frac{\kappa \cdot n + 1}{\kappa}} \right]^{\frac{1}{\kappa \cdot n + 1}} - \frac{A^{\frac{1}{\kappa}}}{B} \quad (6)$$

$$w_{(g)} \approx \frac{4V_m}{\sqrt{N}} \cdot \left\{ 1 + k'_0 \cdot \left[A^{\frac{1}{\kappa}} + B \cdot (V'_{R(g)} - V_2) \right]^{-\kappa \cdot n} \right\} \quad (7)$$

$$N_{(g)} = 16 \cdot \left(\frac{V'_{R(g)} + V_m}{w_{(g)}} \right)^2 \quad (8)$$

$$R_{s(g)} = 2 \cdot \frac{V'_{R(g)2} - V'_{R(g)1}}{w_{(g)2} + w_{(g)1}} \quad (9)$$

In these equations, V_m represents the total volume of the mobile phase in the column; N is the plate number in this column in isocratic elution chromatography under otherwise identical conditions; V_z is the volume of connecting tubing between the outlet of the gradient-generating device and the top of the column; and $V'_{R(g)1}$, $V'_{R(g)2}$, $w_{(g)1}$ and $w_{(g)2}$ are the retention volumes and peak widths of sample compounds 1 and 2, respectively. To a first approximation, N was assumed not to depend on the composition of the binary mobile phase, circumstances that had been approximately obtained in the system cyclohexane-ethyl acetate³. In some practical separations, however, the plate number can be influenced by the composition of the mobile phase; in other words, N can depend on the capacity ratios (retention volumes) of the sample compounds.

If diffusion of solute in the stationary liquid phase (or in the "stagnant" mobile phase in the pores of the support material) or adsorption-desorption kinetics controls mass transfer during the chromatographic operation, the corresponding contribution to the total plate height will depend on the capacity ratio of the solute⁵. For stationary liquid-phase diffusion, a simplified equation for the contribution to plate height, H , can be written:

$$H = \frac{k'}{(1 + k')^2} \cdot \frac{d^2 \cdot v}{D_s} \quad (10)$$

where D_s is the stationary-phase diffusion coefficient, v is the mobile-phase velocity and d is the approximate thickness of the liquid layer on the support material.

By assuming that diffusion in the stationary liquid phase predominates over the other contributions to the total solute-peak broadening, eqn. 10 can be rearranged as follows:

$$N = \frac{L}{H} = \frac{(1 + k')^2}{k'} \cdot \frac{L \cdot D_s}{d^2 \cdot v} \approx \frac{L \cdot D_s}{d^2 \cdot v} \cdot (2 + k') \approx C + D \cdot V'_R \quad (10a)$$

where L is the length of the column. It is assumed that the values of k' are not too low, so that the term $1/k'$ is lower than the sum $(2 + k')$ and can be neglected.

The separation of protonated *N,N*-dimethyl-*p*-aminobenzeneazobenzoic acid esters and amides by cation-exchange chromatography⁶ is an example of diffusion-controlled separation.

The contributions of the "stagnant" mobile-phase diffusion and of adsorption-desorption kinetics can complicate the relationship between N and V'_R , but, as the exact mathematical form of these effects is not well known, we took eqn. 10a as a basis for further considerations.

If this relationship is respected in the equations for peak width in isocratic

and stepwise elution chromatography (see eqn. 17 in ref. 2 and eqn. 5 in ref. 4), these equations acquire somewhat altered forms:

$$w \approx \frac{4 V_m \cdot (k'_0 \cdot c^{-n} + 1)}{\sqrt{C + D \cdot V_m \cdot k'_0 \cdot c^{-n}}} \quad (\text{for isocratic elution}) \quad (11)$$

and

$$w \approx \frac{4 V_m \cdot (k'_0 \cdot c_n^{-n} + 1)}{\sqrt{C + D \cdot V_m \cdot k'_0 \cdot c_n^{-n}}} \quad (\text{for stepwise elution}) \quad (12)$$

By inserting into eqn. 7 the plate number as it would be in isocratic elution chromatography with the same composition of mobile phase as the actual composition in the moment of elution of the peak maximum in gradient elution chromatography, the equation for peak width in gradient elution chromatography can be written in the following form:

$$w_{(g)} \approx \frac{4 V_m \cdot \{1 + k'_0 \cdot [A^{\frac{1}{\alpha}} + B \cdot (V'_{R(g)} - V_2)]^{-\alpha n}\}}{\sqrt{C + D \cdot V_m \cdot \{1 + k'_0 \cdot [A^{\frac{1}{\alpha}} + B \cdot (V'_{R(g)} - V_2)]^{-\alpha n}\}}} \quad (13)$$

The peak width in combined two-step elution chromatography, isocratic in the first step (volume of mobile phase used in this step = V_1 ; capacity ratio = k'_1) followed by the second (gradient) step, can be written as:

$$w \approx \frac{4 V_m \cdot \left\{1 + k'_0 \cdot \left[A^{\frac{1}{\alpha}} + B \cdot \left(V'_{R(g)} - V_1 \cdot \frac{k'_1}{1 + k'_1}\right)\right]^{-\alpha n}\right\}}{\sqrt{C + D \cdot V_m \cdot \left\{1 + k'_0 \cdot \left[A^{\frac{1}{\alpha}} + B \cdot \left(V'_{R(g)} - V_1 \cdot \frac{k'_1}{1 + k'_1}\right)\right]^{-\alpha n}\right\}}} \quad (14)$$

In these instances, the resolution can be calculated by combining eqns. 11–14 with the well-known eqn. 9.

EXPERIMENTAL

The instrumentation, operating conditions and compounds chromatographed in this work were essentially the same as those used in Part III of this series³. The glass column (400 × 3.0 mm) was packed with Porasil A(60), 37–75 μm (Waters Ass., Milford, Mass., U.S.A.); for this column, V_m was 2.00 ml, and the volume of connecting tubing between the mixing chamber of the gradient pump and the injection port of the column (V_2) was 0.30 ml.

The mobile phases tested were: I, *n*-pentane-ethanol; II, *n*-heptane-*n*-pro-

panol: III, *n*-heptane-dioxane; IV, *n*-heptane-chloroform; and V, cyclohexane-ethyl acetate.

All solvents were of reagent grade and were distilled before use; *n*-propanol and dioxane were dehydrated by distillation with metallic sodium and kept in a solvent reservoir fitted with a calcium chloride guard-tube. The components of the binary mobile phase were mixed directly from two solvent reservoirs in the required constant ratio by means of a gradient pump (isocratic elution) or in a volume ratio changing with time according to the selected mathematical function (eqn. 2) drawn on a sheet of paper fastened on to the rotating drum of the photoelectric curve-follower of the gradient-generating pump³. As the flow-rate of the mobile phases tested was independent of their composition, the volume contractions connected with mixing of the two solvent components were not significant and could be neglected.

RESULTS AND DISCUSSION

Properties of different binary solvent systems as mobile phases for gradient elution in adsorption chromatography

Binary mobile phases useful in gradient elution chromatography should be so chosen as to meet certain requirements and restrictions, which are given as follows:

(1) The sample compounds should be soluble in the binary mobile phase.

(2) The components of the binary mobile phase should be completely miscible over the whole concentration range used for elution, and should not react with one another or with the sample.

(3) The solvents should not interfere with detectors used. As, in spite of some published data⁷, a differential refractometer cannot be used with advantage in connection with gradient elution, only mass-transport (wire) and photometric detectors would seem to be useful for this technique. Most common solvents are sufficiently more volatile than the chromatographed compounds and can be used without difficulty with mass-transport detectors. Similarly, as almost all common solvents are transparent in the visible region, the use of photometric detectors operating in this range gives rise to no difficulties; such detectors, however, can only be used for the detection of coloured substances. The most frequently used photometric detectors, therefore, operate in the UV region (at 254 or 280 nm, or at any continuously adjustable wavelength). Thus, if serious base-line shifts are to be avoided, the components of a binary mobile phase should exhibit negligible absorption at the wavelength used.

(4) The main advantage of gradient elution chromatography consists in the improved separation of mixtures containing compounds differing widely in retention. To take full advantage of the technique, it is important to control the capacity ratios of the sample compounds over a wide range in the course of elution. This means that the eluting power of the mobile phase must be adjustable within sufficiently broad limits. In adsorption chromatography, this can most conveniently be achieved by using as components of the binary mobile phase two compounds that differ sufficiently in polarity. Moreover, the larger the difference in polarities between these two components, the better should be the validity of eqn. 1 (see ref. 1). The generation of more complex gradients using several solvents with gradually increasing polarities is somewhat difficult in practice when using commercially available equipment.

(5) In adsorption chromatography, a constant activity of adsorbent is assumed in eqn. 1. The change in adsorbent activity due to preferential adsorption of the more polar solvent of the binary mobile phase may to some extent interfere with the validity of eqn. 1, mainly in the low concentration range; it is therefore necessary to check the validity of eqn. 1 for each combination of solvents.

Further, it is important that the amount of water adsorbed on the surface of the adsorbent is not significantly changed during gradient elution; otherwise, there will be differences between the experimental retention volumes and the values calculated by using eqn. 6. A constant content of water (which does not change significantly with time) can be attained provided that the adsorbent is thoroughly pre-equilibrated with the two components of the mobile phase. These components should not differ much from each other in water content. With this aim, the more polar component of the mobile phase, which is always more miscible with water should be freed from excess of water (the less polar component can be saturated with water).

(6) Another assumption involved in the derivation of eqn. 1, *i.e.*, that the composition of the mobile phase remains constant during transport from the gradient-generating device to the outlet of the column when gradient elution is applied, is not strictly valid in practice. The more polar component of a binary mobile phase is sorbed preferentially on the adsorbent; consequently, the composition of the mobile phase changes with time as the mobile phase advances through the column. This preferential adsorption (the "solvent-demixing effect") becomes more significant as the difference in polarity between the components of the binary mobile phase increases. It has been observed that sudden changes in polarity of the mobile phase during elution can give rise to problems, namely, incomplete separation of some sample components, peak splitting, and even the occurrence of "ghost" peaks⁸. To eliminate these difficulties, some workers prefer to use a set of several (Snyder and Saunders^{9,10} used six; Scott and Kucera¹¹ used twelve) individual solvents with gradually increasing polarities for the generation of concentration gradients.

To investigate the influence of the "solvent-demixing effect" in a binary mobile phase composed of *n*-heptane and *n*-propanol (solvents with a large difference in polarities), we compared the change of the concentration of *n*-propanol with time during gradient elution at the outlet of a column of Porasil A (900 × 2 mm) and in the same equipment without the column, using a UV detector. The detector response was virtually identical in both instances when a linear gradient was run from 0 to 100% of *n*-propanol in 0.5 or 1 h. The curve measured with the column installed showed a delay 0.2–0.5 ml higher than the column void volume (see Table I). The situation was different when a linear gradient was run from 0 to 10% of *n*-propanol in 0.5 or 1 h. In this instance, the detector response at the outlet of the column was divided into two parts (see Fig. 1). In the first part, the concentration of *n*-propanol increased much more slowly than the concentration profile in the system without the column. This part was followed by a sudden large increase in detector response. Then, the response of the detector soon decreased to the level predicted in the experiment without the column, and the second part of the concentration profile was again virtually identical in both experiments.

To ascertain the nature of "peaks" on the detector-response curves in experiments with the column, the composition of the mobile phase leaving the UV detector was determined by an independent analytical method (gas chromatography). The re-

TABLE I

THE INFLUENCE OF "SOLVENT-DEMIXING EFFECT" IN GRADIENT ELUTION ADSORPTION CHROMATOGRAPHY ON PORASIL A

A linear gradient (0.5 h) was run from 0 to 100% and from 0 to 10%, respectively, of *n*-propanol in *n*-heptane using τ column (900 \times 2 mm) packed with Porasil A(60), 37-75 μ m, and in the system where the column was replaced by a tube (0.2 mm I.D.; 0.3 ml in volume). A gradient pump (PPM 68005; Workshops of the Czechoslovak Academy of Sciences, Prague) delivered mobile phase to a Waters M6000 high-pressure pump. The response of a UV detector (254 nm, Waters Associates) at the outlet of the column or of the tube was registered, and the delay between start-up of the gradient and the beginning of the concentration change registered by the detector was measured. Predicted delay values were calculated from the void volumes of the individual instrument components (determined in independent experiments), *viz.*, PPM pump, 2.50 ml; mixing chamber and connecting tubing, 0.30 ml; column and injection port, 3.05 ml; tubing inserted instead of column, 0.30 ml.

Gradient	Flow-rate of mobile phase (ml/min)	Delay (ml)			Deviation from predicted value (ml)
		Column	Tubing	Difference	
0 to 100% <i>n</i> -propanol	0.96	5.95	3.17	2.78	+0.03
	0.46	6.11	3.21	2.90	+0.15
	0.16	6.10	3.14	2.96	+0.21
0 to 10% <i>n</i> -propanol	0.96	5.96	3.08	2.88	+0.13
	0.46	6.40	3.09	3.31	+0.56
	0.16	6.45	3.02	3.42	+0.67
	Predicted value	5.85	3.10	2.75	—

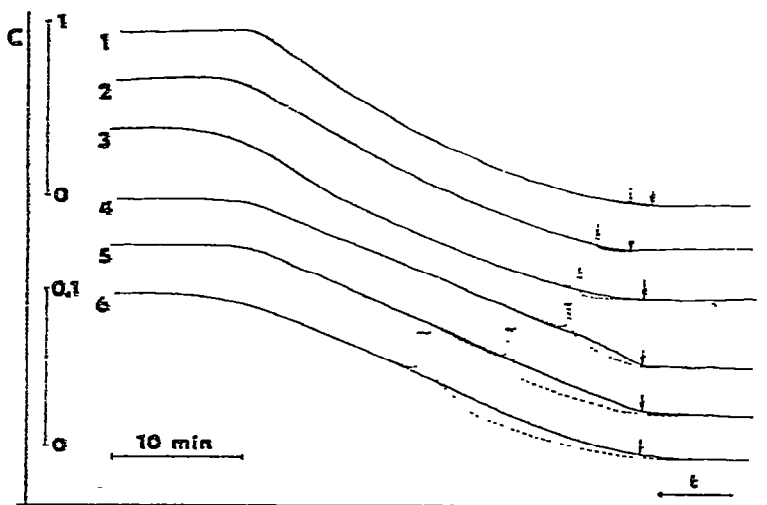


Fig. 1. Concentration profiles measured in gradient elution experiments with and without a column (900 \times 2 mm) of Porasil A (37-75 μ m); the experimental method is described in the legend to Table I. Full lines represent the concentration change of *n*-propanol in experiments with tubing in place of the column and broken lines show this change in experiments with the column installed. The points considered as the beginning of concentration change are marked. These points were taken as the basis of the data in Table I. Detector: UV (254 nm; Waters Associates); range 0.16 absorbance units (full-scale) (aufs) (curves 1-3) and 0.02 aufs (curves 4-6). Concentration change from 0 to 100% of *n*-propanol in *n*-heptane (0.5 h): curves 1-3; from 0 to 10% of *n*-propanol in *n*-heptane (0.5 h): curves 4-6. Flow-rate of mobile phase: 0.96 ml per min for curves 1 and 4; 0.46 ml per min for curves 2 and 5; 0.16 ml per min for curves 3 and 6. c = Volume % of *n*-propanol \times 0.01; t = time (min).

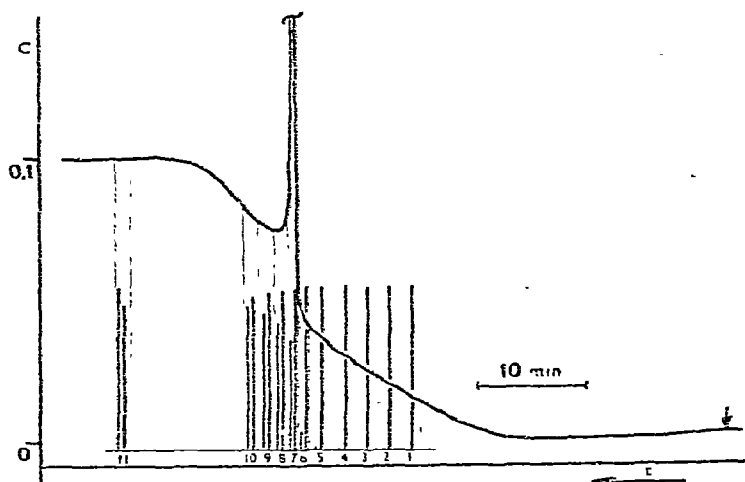


Fig. 2. Comparison of a concentration profile in gradient elution chromatography measured by means of a UV detector with the results of gas chromatographic analysis of the individual fractions. Column: Porasil A (37–75 μm), 900 \times 2 mm. Mobile phase: linear gradient from 0 to 10% (v/v) of *n*-propanol in *n*-heptane accomplished in 0.5 h. Flow-rate: 0.40 ml per min. Detector: UV (254 nm; Waters Associates); range 0.02 aufs. The full curve represents the record of the UV detector. Below the curve, 11 fractions (0.4–0.8 ml each) taken for gas chromatographic analysis are marked. For each fraction, the concentration of *n*-heptane (first vertical line) and that of *n*-propanol (second vertical line) are traced inside the corresponding interval (the sensitivity of the instrument for *n*-propanol was $64 \times$ greater than the sensitivity for *n*-heptane). c = Volume % of *n*-propanol \times 0.01; t = time (min).

sults of gas chromatographic analysis of narrow fractions of detector effluent are shown in Fig. 2, in which the heights of the peaks corresponding to the two components of the mobile phase are shown for each fraction. It can be seen that a sudden increase in *n*-propanol concentration occurs in fractions 6 and 7, and a further moderate increase is observed in the following fractions. Gas chromatographic data show no sharp "peak" of *n*-propanol concentration; therefore, the "peaks" on the detector-response curves can be attributed to turbulence in the detector cell due to mixing of the *n*-propanol front with *n*-heptane rather than to a sharp increase in the concentration of *n*-propanol above 10% (v/v). The occurrence of a "false peak" is less significant, but still distinct, at lower sensitivities of the detector (see Fig. 1), and very small peaks due to turbulence in the detector cell were observed in earlier experiments with spectrophotometric detection in the visible region at a low sensitivity level^{3,4}.

The experiments described above show that the "solvent-demixing effect" can cause significant deviation from the predicted concentration gradient function in the low concentration region (0–3%, by vol., of *n*-propanol in *n*-heptane). This effect can seriously influence the retention volumes of the compounds eluted early, but no significant changes in elution behaviour of the more strongly retained sample components are to be expected. As will be seen from the following discussion, in mobile phases containing strong solvents in low concentration, eqn. 1 is not always strictly obeyed. The deviations from this equation, together with the "solvent-demixing effect", make the use of binary mixtures containing less polar solvents (such as chloroform, dioxane and dichloromethane) desirable if the sample components are weakly retained by the column.

Nevertheless, the good agreement between experimental retention volumes and the values calculated from eqn. 6 in the current (see the following discussion) and previous³ experiments with different mobile phases indicates that this effect does not significantly influence the results. This experience led us to omit attempts to introduce mathematical corrections for preferential adsorption into the theoretical equation for retention volume in gradient elution chromatography.

With respect to the above rules, we compared several different solvent systems as binary mobile phases. Cyclohexane and ethyl acetate, which had been tested in previous work, would interfere with the UV detector operating at 254 nm, as the upper range of absorption of ethyl acetate is about 260 nm, and cyclohexane often contains significant amounts of UV-absorbing impurities (e.g., benzene). From a variety of other solvents, those commercially available with minimum light absorption at 254 nm were selected as suitable components of binary mobile phases for adsorption chromatography.

Several solvents of different polarities were tested as the more efficient eluting components of the binary mobile phases. Methanol could not be used in mixtures with non-polar hydrocarbons because of its limited solubility in these solvents. The sample compounds studied showed only limited solubility in a mobile phase composed of 1,2-dichloroethane and *n*-heptane and were very strongly retained on the column. For this reason, this mobile phase (which can be useful in other practical separations) could not be studied here.

When *n*-pentane was a component of the mobile phase (in mixtures with ethanol), bubble formation occurred in the plunger block of the gradient pump; this would cause serious flow irregularities if experiments were run at ambient temperature. This difficulty could be eliminated by cooling the plunger block with a mixture of ice and solid carbon dioxide, but, as such cooling was inconvenient, systems containing *n*-pentane were not used in experiments with gradient elution. *n*-Heptane was selected as the best non-polar component available for the binary mobile phases. The remaining solvent systems studied were: *n*-heptane-*n*-propanol; *n*-heptane-dioxane and *n*-heptane-chloroform. The polarities of the more efficient eluting components decrease⁴ in the above order, and so did the total polarity change that could be obtained during gradient elution.

Verification of eqn. 1

The results of verification of eqn. 1 in the four binary mobile phases tested are shown in Table II and in Figs. 3 and 4. The figures show graphs of the logarithms of retention volumes (V'_R) of the sample compounds against the logarithms of the mole fraction and the volume concentration of the more polar component in the binary mobile phase. Significant deviations from linearity were observed in the mobile phase composed of ethanol and *n*-pentane in both high-polarity ($x \geq 0.33$) and low-polarity ($x < 0.04$) regions, and partly in the mixture of *n*-propanol and *n*-heptane in the high-polarity region ($x \geq 0.51$). The deviations in the binary mobile phase composed of ethanol and *n*-pentane could be influenced by the water content (3-4%) of the ethanol used. It seems probable that part of the water in the ethanol became irreversibly sorbed on the adsorbent during the experiments. Consequently, the activity of the Porasil could be decreased in later experiments as compared with earlier ones. This behaviour was partly confirmed in experiments repeated with a mobile

TABLE II
EXPERIMENTAL VALUES OF THE CONSTANTS n AND k'_0 OF EQN. 1 AND OF THE CORRELATION COEFFICIENTS RK FOR THE RELATIONSHIP BETWEEN $\log k'$ AND $\log c$

Compounds: N,N-dimethyl-*p*-aminobenzencarboxyl amides; I, dimethylamide; 2, diethylamide; 3, di-*n*-propylamide; 4, di-*n*-butylamide. Mobile phase: I, *n*-pentane-ethanol; II, *n*-heptane-*n*-propanol; III, *n*-heptane-dioxane; IV, *n*-heptane-chloroform; V, cyclohexane-ethyl acetate. Column: Porasil A(60), 37-75 μ m, 400 \times 3 mm; $V_m = 2.00$ ml. Flow-rate: 38.5 ml/h. Pressure: ≈ 10 atm. The values in the Table were calculated from plots of $\log k'$ against the log of the mole fraction (a) and the log of the volume concentration (b) of the more efficient eluting component of the binary mobile phase.

Mobile Compound phase	1				2				3				4			
	n	k'_0	RK	n	k'_0	RK	n	k'_0	RK	n	k'_0	RK	n	k'_0	RK	
Ia	0.8506	1.3184	-0.9911	0.6732	0.8828	-0.9943	0.6302	0.5535	-0.9964	0.6280	0.3909	-0.9983				
	0.8088	0.8706	-0.9954	0.6167	0.6250	-0.9961	0.6050	0.4010	-0.9977	0.6018	0.2844	-0.9978				
IIa	1.1869	0.8626	-0.9989	1.0252	0.4949	-0.9981	1.0597	0.1806	-0.9994	1.0359	0.1266	-1.0000				
	1.0972	0.5413	-0.9993	0.9792	0.2404	-0.9985	1.0296	0.1025	-0.9992	1.0112	0.0711	-1.0000				
IIIa	2.2272	0.7284	-0.9974	2.1651	0.2974	-0.9986	2.2019	0.1565	-0.9980	2.0067	0.1584	-0.9998				
	1.9379	0.4837	-0.9989	1.9132	0.1920	-0.9997	1.9462	0.1002	-0.9993	1.8033	0.0988	-0.9996				
IVa	2.4061	0.7477	-0.9989	2.4042	0.4063	-0.9797	2.3175	0.2449	-0.9990	2.2568	0.1895	-0.9986				
	1.9044	0.5278	-0.9992	1.9065	0.2915	-0.9997	1.8552	0.1829	-0.9995	1.7708	0.1492	-0.9974				
Va	1.7929	1.5230	-0.9996	1.8203	0.5252	-0.9981	1.8805	0.2453	-0.9976	1.6712	0.1915	-0.9994				
	1.7100	1.4889	-0.9996	1.7535	0.5088	-0.9978	1.8172	0.2357	-0.9971	1.6207	0.1835	-0.9992				

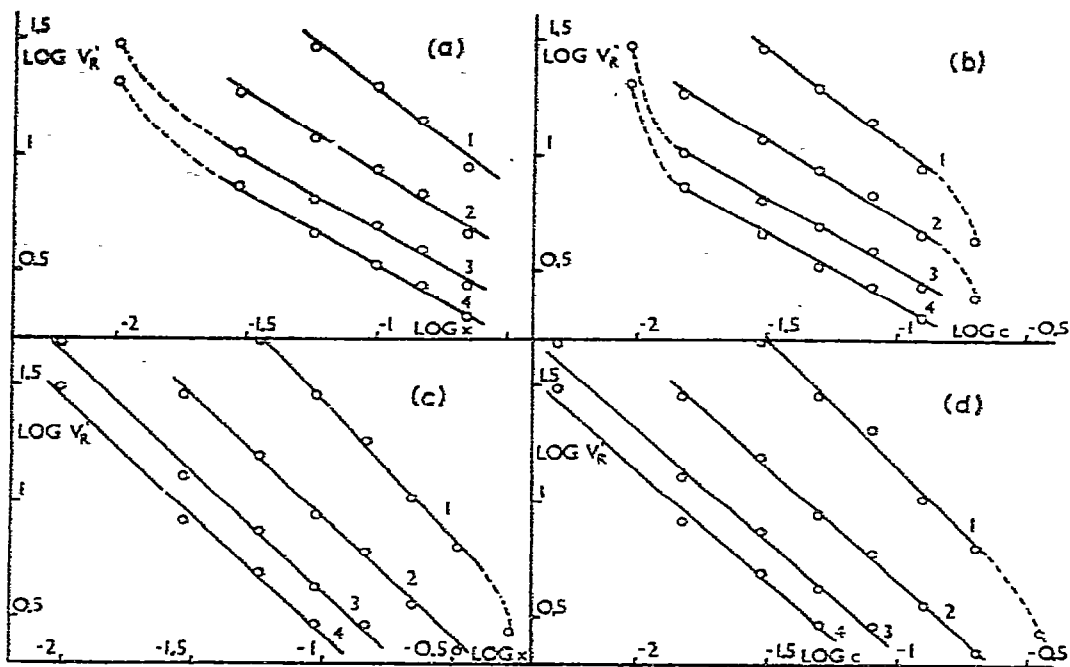


Fig. 3. Relationship between retention volumes, V'_R (ml), of *N,N*-dimethyl-*p*-aminobenzeneazobenzoyl amides and concentration of the more efficient eluting component in the binary mobile phase (ethanol-*n*-pentane in a and b; *n*-propanol-*n*-heptane in c and d) during chromatography on Porasil A. The concentration is expressed as the mole fraction (x) in a and c and as the volume concentration (c) in b and d. Compounds: 1 = dimethylamide; 2 = diethylamide; 3 = di-*n*-propylamide; 4 = di-*n*-butylamide. Operating conditions as in Table II.

phase of equal composition after two or three weeks of experiments with mobile phases of different composition. The retention-volume values were slightly (but significantly) higher in the repeated experiments, which indicates a slight decrease in the activity of the adsorbent with time (in contrast to the assumptions made in the derivation of eqn. 1).

No change in activity was observed in experiments with *n*-propanol and *n*-heptane; *n*-propanol, like the other more polar components of the binary solvent systems (except ethanol), was dehydrated. The graphs of $\log V'_R$ against $\log x$ or $\log c$ are linear over the whole concentration range studied for mobile phases composed of *n*-heptane-chloroform or *n*-heptane-dioxane, and almost linear for mobile phase containing *n*-heptane-*n*-propanol.

Linearity of the graphs in Figs. 3 and 4 is equally good if the concentration is expressed in volume units instead of mole fractions, as it is evidenced by the correlation coefficients in Table II. A similar linearity was observed in earlier experiments with mixtures of ethyl acetate and cyclohexane, where the differences between the two concentration expressions were not highly significant, in contrast to the mobile phases studied now.

The conclusion to be drawn from the above experiments is that eqn. 1 is valid for all the mobile phases studied within the practically useful concentration limits,

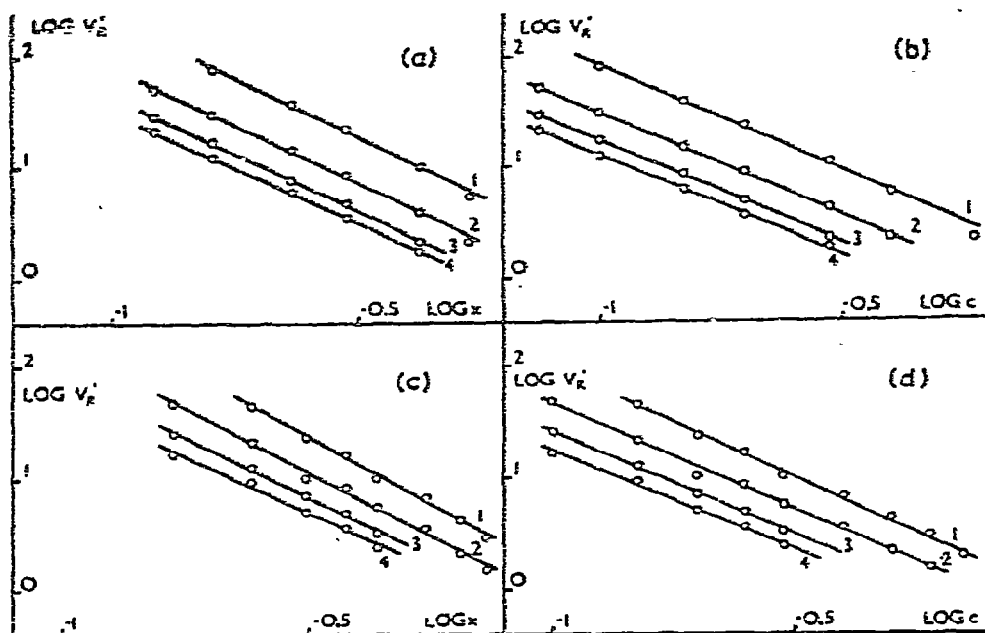


Fig. 4. Relationship between retention volumes (V'_R) of *N,N*-dimethyl-*p*-aminobenzeneazobenzoyl amides and the concentration of the more efficient eluting component in the binary mobile phase (dioxane-*n*-heptane in a and b; chloroform-*n*-heptane in c and d) during chromatography on Porasil A. The concentration is expressed as the mole fraction (x) in a and c and as the volume concentration (c) in b and d. Compounds 1 to 4 as in Fig. 3. Operating conditions as in Table II.

and, moreover, that volume concentrations can be used in calculations according to this equation, even when they differ significantly from the mole fractions.

The experimental values of the coefficients n and k'_0 in eqn. 1 in different mobile phases are shown in Table II. In the derivation of eqn. 1, as the simplification of Snyder's model of adsorption, the parameter n represents the ratio of the area of adsorbent surface occupied by an adsorbed molecule of sample compound to that occupied by an adsorbed molecule of the more polar of the solvents in the mobile phase. Snyder's concept of adsorption assumes mono-layer adsorption on a continuous and homogeneous surface of the adsorbent^{10,12}.

During the preparation of Parts I-III of this series¹⁻³ for publication, Soczewinski and Golkiewicz¹³ published an equation, virtually identical with eqn. 1, to describe adsorption on discrete adsorption sites of equal energy. The latter model was recognized as better suited to describe adsorption on silica in binary systems containing strong solvents than the model of Snyder¹⁰. In the model of Soczewinski and Golkiewicz, n represents the number of adsorbable functional groups in a molecule of sample compound.

Comparison of the coefficients n in Table II seems to support the suitability of the model of Soczewinski and Golkiewicz to the adsorption systems studied. The values of n in mobile phases containing ethanol and *n*-propanol are close to unity, while in other mobile phases (in which dioxane, chloroform and ethyl acetate are the more polar components) n values are rather close to 2. *N,N*-Dimethyl-*p*-aminoben-

zeneazobenzoyl amides contain two polar adsorbable functional groups (the amido and the tertiary amino groups). In solutions containing alcohols, solvation is likely to occur with a hydrogen bond, which can block one of these groups (probably the amido group), so that only one (the amino group) is available for adsorption. This effect cannot occur in mobile phases containing chloroform, dioxane or ethyl acetate, so that in these phases two functional groups are available for adsorption.

The parameter k'_0 is influenced not only by the polarity of the more efficient eluting solvent, but also by the activity of the adsorbent. The differences in activity of Porasil between each set of experiments with the different mobile phases studied were evidently large enough to change the order of k'_0 in the different mobile phases, so that this order did not agree with the order of polarities of the more efficient eluting components. This may be partly because the values of k'_0 are relatively close to one another in the binary mobile phases tested.

Gradient elution

The validity of eqns. 6-9 in gradient elution chromatography with binary mobile phases composed of *n*-heptane and *n*-propanol, *n*-heptane and dioxane and *n*-heptane and chloroform was tested experimentally in a way analogous to that used with the system cyclohexane-ethyl acetate³. The volume concentration of the more polar solvent in *n*-heptane was changed continuously according to the gradient function (eqn. 2) during elution. The parameters of the gradient functions tested are given in Tables III-IX. In addition, experiments were performed with two mobile phases in which the mole fraction instead of the volume concentration of the stronger solvent was changed according to eqn. 2. For this purpose, the gradient function was so constructed that the corresponding volume concentrations were calculated for the 40

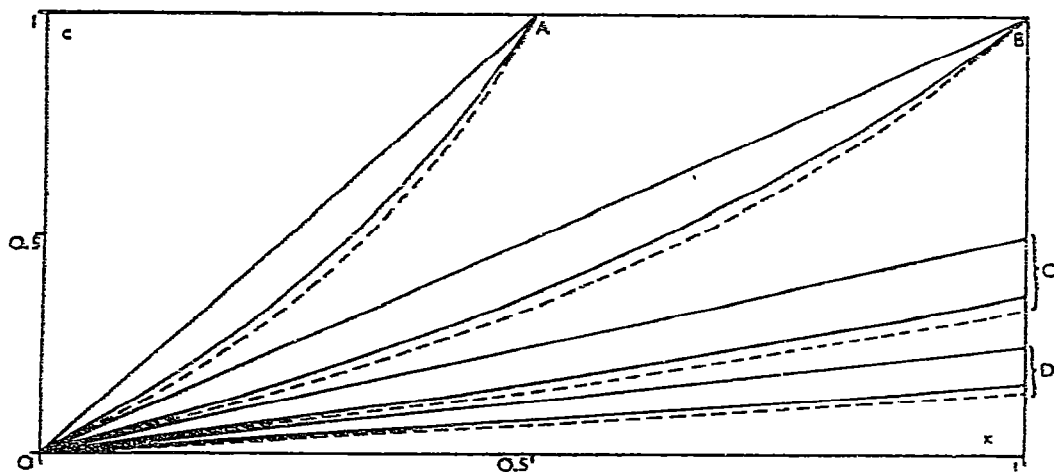


Fig. 5. Change in the volume concentration (c) of the more efficient eluting component with time (gradient function) for the linear change in the corresponding mole fraction in the binary mobile phases dioxane-*n*-heptane (full curves) and *n*-propanol-*n*-heptane (broken curves). The full lines represent the linear change in the volume concentration with time. $x = t/t_0$, where t is the time elapsed from the beginning of the gradient programme and t_0 is the time necessary for complete change of the concentration; $c = 0$ to 1. Parameters of the gradient: $A = 0$; $\alpha = 1$; $B = 0.051948$ (A), 0.025974 (D), 0.012987 (C) and 0.0064935 (D).

TABLE III

EXPERIMENTAL AND CALCULATED VALUES FOR ELUTION CHARACTERISTICS OF N,N-DIMETHYL-*p*-AMINOBENZENEAZOBENZENE ZOYL AMIDES IN ADSORPTION CHROMATOGRAPHY ON PORASIL A USING MOBILE PHASE *n*-PROPANOL-*n*-HEPTANE Gradient elution, $\alpha = 0.5$; $\lambda = c_0 = 0$; $V_y = 38.5$ ml. Column: 409 \times 3 mm; $V_m = 2.00$ ml; Porasil A(60), 37–75 μ m. Flow-rate of mobile phase: 38.5 ml/h. Detection: photometric, 440 nm. Chromatographed compounds: 1 = di-*n*-butylamide; 2 = di-*n*-propylamide; 3 = diethylamide; 4 = dimethylamide; ca. 8 μ g each. The experimental values represent the arithmetic means from three experiments.

c_A	B	Compound	V'_{100} (ml)		v_{100} (ml)		$N_{(a)}$		R_{100}		
			Calc.	Exptl.	Calc.*	Exptl.	Calc.*	Exptl.	Calc.*	Exptl.	
0.25	0.0016234	1	3.11	3.11	1.69	1.41	145	209	0.52	0.65	—
		2	4.10	3.79	2.14	1.63	129	223	1.03	1.30	—
		3	6.60	5.95	2.73	2.22	159	240	2.01	2.36	2.51
		4	13.59	11.75	4.22	3.71	219	283	—	—	—
0.5	0.0061935	1	1.95	—	1.38	1.15	132	188	0.39	0.50	—
		2	2.56	2.59	1.75	1.29	109	201	0.84	1.10	—
		3	4.18	3.93	2.11	1.67	138	220	1.57	1.96	—
		4	8.31	7.40	3.16	2.54	171	263	—	—	—
1.0	0.025974	1	1.22	—	1.18	0.99	119	171	0.28	0.36	—
		2	1.60	1.72	1.49	1.07	94	181	0.66	0.88	—
		3	2.65	2.53	1.70	1.32	120	200	1.18	1.55	—
		4	5.09	4.52	2.42	1.83	137	240	—	—	—

* Values calculated using eqns. 13, 8 and 9.

** Values calculated using eqns. 7, 8 and 9.

TABLE IV

EXPERIMENTAL AND CALCULATED VALUES FOR ELUTION CHARACTERISTICS OF N,N-DIMETHYL-*p*-AMINOBENZENEAZO-BENZOYL AMIDES IN ADSORPTION CHROMATOGRAPHY ON PORASIL A USING MOBILE PHASE *p*-PROPANOL-*n*-HEPTANE

Gradient elution, $\alpha \approx 1.0$; $A = \epsilon_p = 0$; $V_f = 38.5$ ml. Chromatographed compounds and other operating conditions as in Table III.

c_s	B	Compound	V'_{100} (ml)		v_{100} (ml)		N_{100}		R_{100}		Calc.**	Exptl.	Calc.**	Exptl.	
			Calc.	Exptl.	Calc.*	Exptl.	Calc.*	Exptl.	Calc.*	Exptl.					
0.25	0.0064935	1	6.75	6.22	2.27	1.87	239	352	0.65	0.80	0.80	—	—	—	—
		2	8.36	7.59	2.68	2.12	238	380	1.14	1.40	1.40	—	—	—	—
		3	11.79	10.97	3.31	2.75	271	401	2.26	2.58	2.58	391	638	2.81	2.81
		4	20.62	19.42	4.53	4.08	399	492	—	—	—	—	—	—	—
0.5	0.012987	1	4.77	4.16	1.85	1.53	215	313	0.55	0.69	0.69	—	—	—	—
		2	5.88	5.09	2.24	1.71	199	340	1.01	1.28	1.28	—	—	—	—
		3	8.36	7.44	2.67	2.16	240	368	1.89	2.29	2.29	307	434	2.18	2.18
		4	14.35	12.90	3.65	3.06	321	457	—	—	—	—	—	—	—
1.0	0.025974	1	3.36	—	1.55	1.29	190	276	0.45	0.57	0.57	—	—	—	—
		2	4.14	3.85	1.91	1.41	166	300	0.88	1.14	1.14	—	—	—	—
		3	5.94	5.26	2.20	1.74	208	332	1.56	1.98	1.98	217	402	1.91	1.91
		4	9.98	9.31	2.97	2.35	261	417	—	—	—	—	—	—	—
2.0	0.051948	1	2.37	—	1.35	1.12	167	242	0.36	0.46	0.46	—	—	—	—
		2	2.91	3.07	1.66	1.21	139	263	0.74	0.98	0.98	—	—	—	—
		3	4.21	3.96	1.86	1.45	179	295	1.27	1.66	1.66	232	355	1.58	1.58
		4	6.95	6.61	2.45	1.85	213	373	—	—	—	—	—	—	—

* Values calculated using eqns. 13, 8 and 9.

** Values calculated using eqns. 7, 8 and 9.

TABLE V

EXPERIMENTAL AND CALCULATED VALUES FOR ELUTION CHARACTERISTICS OF *N,N*-DIMETHYL-*p*-AMINOBENZENEAZOREN-
ZOYL AMIDES IN ADSORPTION CHROMATOGRAPHY ON FORASIL A USING MOBILE PHASE *n*-PROPANOL-*n*-HEPTANE

Gradient elution, $\kappa = 2.0$; $A = t_0 = 0$; $V_y = 38.5$ ml. Chromatographed compounds and other operating conditions as in Table III

c_A	B	Compound	$V'_{K(0)} (ml)$		$w_{(0)} (ml)$		$N_{(0)}$		$R_{(0)}$	
			Calc.	Exptl.	Calc.*	Exptl.	Calc.*	Exptl.	Calc.*	Exptl.
0.5	0.018366	1	10.97	11.03	2.40	1.97	465	693	0.66	0.82
		2	12.66	12.65	2.71	2.14	468	747	1.11	1.37
		3	15.89	15.89	3.12	2.56	527	778	2.06	2.46
		4	22.97	22.41	3.78	3.19	700	981		2.52
1.0	0.025974	1	8.70	8.72	2.08	1.71	422	623	0.59	0.74
		2	10.02	9.85	2.40	1.85	402	675	1.02	1.29
		3	12.63	12.39	2.71	2.19	466	715	1.83	2.26
		4	18.11	17.47	3.29	2.67	597	908		2.27
2.0	0.036732	1	6.90	6.55	1.83	1.51	378	555	0.52	0.66
		2	7.94	7.65	2.14	1.62	344	604	0.93	1.20
		3	10.04	9.84	2.38	1.89	410	649	1.61	2.04
		4	14.27	13.76	2.89	2.26	509	831		2.14

* Values calculated using eqns. 13, 8 and 9.

** Values calculated using eqns. 7, 8 and 9.

TABLE VI

EXPERIMENTAL AND CALCULATED VALUES FOR ELUTION CHARACTERISTICS OF N,N-DIMETHYL-*n*-AMINO BENZENEF AZOBENZYL AMIDES IN ADSORPTION CHROMATOGRAPHY ON PORASIL A USING MOBILE PHASE *n*-PROPANOL-*n*-HEPTANE

Gradient elution, $\alpha = 1.0$; $A = c_0 = 0$; $V_y = 38.5$ ml, as in Table IV, but mole fraction (and not volume concentration) of *n*-propanol in *n*-heptane changed in linear manner with time. Chromatographed compounds and other operating conditions as in Table III.

c_A	B	Compound	$V_{R(0)} (ml)$		$v_{(0)} (ml)$		$N_{(0)}$		$R_{(0)}$		Exptl.	Calc.**	Exptl.	Calc.**	Exptl.	Calc.**
			Calc.	Exptl.	Calc.*	Exptl.	Calc.*	Exptl.	Calc.*	Exptl.						
0.25	0.0064935	1	9.37	7.79	2.81	2.29	2.23	263	394	308	0.73	0.89	0.38	0.89	0.38	
		2	11.56	9.64	3.20	2.64	2.62	288	423	316	1.72	2.03	1.51	2.03	1.51	
		3	18.05	14.28	4.33	3.76	3.51	343	454	344	2.11	2.29	2.98	2.29	2.98	
		4	28.24	25.77	5.34	5.13	4.19	513	555	439						
0.5	0.012987	1	6.58	6.72	2.21	1.82	—	241	354	—	0.23	0.78	—	0.78	—	
		2	8.10	7.82	2.62	2.06	—	238	383	—	1.53	1.87	—	1.87	—	
		3	12.71	10.95	3.42	2.86	2.89	295	422	323	1.74	2.02	2.49	2.02	2.49	
		4	19.39	18.42	4.26	3.75	3.13	403	520	426						
1.0	0.025974	1	4.63	4.82	1.81	1.50	—	215	314	—	0.52	0.66	—	0.66	—	
		2	5.67	5.57	2.18	1.66	—	197	341	—	1.33	1.69	—	1.69	—	
		3	8.95	7.75	2.75	2.23	2.19	254	386	320	1.42	1.73	2.18	1.73	2.18	
		4	13.31	12.60	3.41	2.80	2.23	322	478	688						
2.0	0.051948	1	3.25	—	1.52	1.27	—	190	275	—	0.42	0.54	—	0.54	—	
		2	3.97	4.09	1.86	1.38	—	164	300	—	1.13	1.48	—	1.48	—	
		3	6.30	5.55	2.25	1.78	1.83	218	347	272	1.13	1.44	1.77	1.44	1.77	
		4	9.14	8.68	2.77	2.15	1.71	259	430	625						

* Values calculated using eqns. 13, 8 and 9.

** Values calculated using eqns. 7, 8 and 9.

TABLE VII

EXPERIMENTAL AND CALCULATED VALUES FOR ELUTION CHARACTERISTICS OF N,N-DIMETHYL-*p*-AMINOBENZENEAZOBENZYL AMIDES IN ADSORPTION CHROMATOGRAPHY ON PORASIL A USING MOBILE PHASE DIOXANE-*n*-HEPTANE Gradient elution, $\alpha = 1.0$; $A = C_0 = 0$; $V_1 = 38.5$ ml. Chromatographed compounds and other operating conditions as in Table III.

α	B	Compound	$V_{R(0)} (ml)$		$w_{(0)} (ml)$		$N_{(0)}$		$R_{SC(0)}$			
			Calc.	Exptl.	Calc.*	Exptl.	Calc.*	Exptl.	Calc.*	Exptl.	Calc.**	Exptl.
0.25	0.0054935	1	20.68	19.71	3.09	2.93	861	780	878	0.84	0.78	0.64
		2	23.30	22.04	3.15	4.34	1029	870	491	1.47	1.36	1.27
		3	28.40	27.04	3.80	3.54	1024	897	1077	2.57	2.38	2.44
		4	39.57	36.99	4.88	4.62	1160	973	1026			
0.5	0.012987	1	13.24	12.64	2.25	2.36	736	677	616	0.66	0.63	0.62
		2	14.74	14.11	2.27	2.42	867	754	709	1.30	1.24	1.20
		3	18.02	17.12	2.78	2.84	831	793	865	2.28	2.17	2.35
		4	25.05	23.74	3.38	3.03	1023	882	1155			
1.0	0.025974	1	8.48	8.10	1.70	1.76	607	566	—	0.50	0.47	—
		2	9.32	9.15	1.71	1.81	704	625	—	1.11	1.08	—
		3	12.43	11.00	2.09	2.07	663	673	799	1.97	1.91	2.00
		4	15.86	15.00	2.42	2.57	875	770	1002			
2.0	0.051948	1	5.43 [*]	5.18	1.35	1.39	483	456	—	0.5	0.34	—
		2	5.90	5.98	1.35	1.42	551	498	—	0.91	0.90	—
		3	7.25	7.13	1.63	1.58	516	548	926	1.63	1.60	1.44
		4	10.04	9.01	1.80	1.90	719	644	976			

* Values calculated using eqns. 13, 8 and 9.

** Values calculated using eqns. 7, 8 and 9.

TABLE VIII

EXPERIMENTAL AND CALCULATED VALUES FOR ELUTION CHARACTERISTICS OF N,N-DIMETHYL-*p*-AMINOBENZENEAZOBENZOYL AMIDES IN ADSORPTION CHROMATOGRAPHY ON PORASIL A USING MOBILE PHASE DIOXANE-*n*-HEPTANE

Gradient elution, $\alpha = 1.0$; $A \infty \epsilon_0 = 0$; $V_p = 38.5$ ml as in Table VII, but mole fraction (and not volume concentration) of dioxane in *n*-heptane changed in linear manner with time. Chromatographed compounds and other operating conditions as in Table III.

ϵ_A	B	Compound	$V'_{R(0)}$ (ml)		$w_{(0)}$ (ml)		$N_{(0)}$		$R_{(0)}$			
			Calc.	Exptl.	Calc.*	Exptl.	Calc.*	Exptl.	Calc.*	Exptl.		
0.25	0.0064935	1	28.38	27.21	3.75	3.95	1052	944	1269	0.95	0.89	0.89
		2	31.96	30.25	3.77	4.14	1296	1076	1328	1.54	1.41	1.72
		3	38.30	36.77	4.44	4.87	1319	1097	1459	2.75	2.50	—
		4	52.24	—	5.70	6.26	1451	1200	—	—	—	—
0.5	0.012987	1	17.87	17.45	2.64	2.76	907	829	1144	0.75	0.70	0.79
		2	19.84	19.34	2.64	2.85	1095	940	1156	1.37	1.30	1.55
		3	23.84	23.35	3.18	3.30	1059	978	1453	2.43	2.29	2.82
		4	32.38	31.46	3.84	4.16	1278	1093	1864	—	—	—
1.0	0.025974	1	11.25	11.54	1.93	2.01	751	696	857	0.56	0.53	0.65
		2	12.32	12.74	1.92	2.05	889	783	1005	1.19	1.15	1.29
		3	14.84	15.22	2.33	2.33	838	833	1210	2.09	2.02	2.34
		4	20.07	20.06	2.67	2.86	1090	955	1669	—	—	—
2.0	0.051948	1	7.08	7.50	1.49	1.54	595	559	—	0.38	0.37	—
		2	7.65	8.20	1.47	1.55	690	621	—	0.98	0.97	—
		3	9.23	9.48	1.77	1.73	645	676	1328	1.73	1.70	1.89
		4	12.44	12.01	1.94	2.05	890	794	1558	—	—	—

* Values calculated using eqns. 13, 8 and 9.

** Values calculated using eqns. 7, 8 and 9.

TABLE IX
 EXPERIMENTAL AND CALCULATED VALUES FOR ELUTION CHARACTERISTICS OF N,N-DIMETHYL-*p*-AMINOBENZENEAZOBEN-
 ZOYL AMIDES IN ADSORPTION CHROMATOGRAPHY ON FORASIL A USING MOBILE PHASE CHLOROFORM-*n*-HEPTANE
 Gradient elution, $\alpha = 1.0$; $\lambda = c_0 = 0$; $V_s = 38.5$ ml. Chromatographed compounds and other operating conditions as in Table III.

c_0	B	Compound	$V'_{n(0)}$ (ml)		$w_{(0)}$ (ml)		$N_{(0)}$		$R_{1(0)}$				
			Calc.	Exptl.	Calc.*	Exptl.	Calc.*	Exptl.	Calc.**	Exptl.			
0.25	0.0064935	1	23.35	25.36	3.74	3.61	698	2.43	790	2035	0.89	0.91	0.98
		2	26.79	28.11	3.78	3.93	879	3.18	856	1434	1.49	1.38	1.53
		3	32.64	33.28	3.85	4.57	1224	3.56	921	1572	2.07	1.74	—
		4	41.37	—	4.35	5.50	—	—	995	—	—	—	—
0.5	0.012987	1	14.99	17.62	2.87	2.57	561	—	696	—	0.72	0.78	—
		2	17.08	18.51	2.94	2.77	674	—	757	—	1.25	1.22	—
		3	20.71	21.69	2.86	3.16	1011	2.79	824	1153	1.80	1.57	1.97
		4	26.14	28.38	3.18	3.74	1252	4.01	904	918	—	—	—

* Values calculated using eqns. 13, 8 and 9.

** Values calculated using eqns. 7, 8 and 9.

chosen mole fractions used in eqn. 2 and plotted against time in the graph used for generation of the gradient. This is illustrated by Fig. 5, in which the straight lines represent the change of mole fraction with time ($\alpha = 1.0$) and the curves show the corresponding changes in volume concentration for the two solvent systems.

The results of all the gradient-elution experiments are given in Tables III-IX. Here, the experimental values of retention volume, peak width, plate number and resolution are compared with the values calculated from eqns. 5-9 using the constants n and k'_0 determined in experiments with isocratic elution. About 85% of the experimental retention volumes differ from the calculated values by less than 10% (rel.), which is in agreement with results for the mobile phase cyclohexane-ethyl acetate⁵. The agreement between the experimental and calculated values of $w_{(g)}$, $N_{(g)}$ and $R_{s(g)}$ is also similar to that in experiments with cyclohexane-ethyl acetate.

Figs. 6 and 7 show further evidence for the validity of eqn. 6. As has been shown³, the graph of $\log V'_{R(g)}$ against $\log B$ should be linear for gradient elution experiments in which eqn. 2 applies and $A = 0$. Such is the case in Figs. 6 and 7, which show these graphs for mobile phases composed of *n*-propanol and *n*-heptane, and of dioxane and *n*-heptane, respectively, for different values of α .

In Figs. 6 and 7, graphs constructed from data from the experiments using the

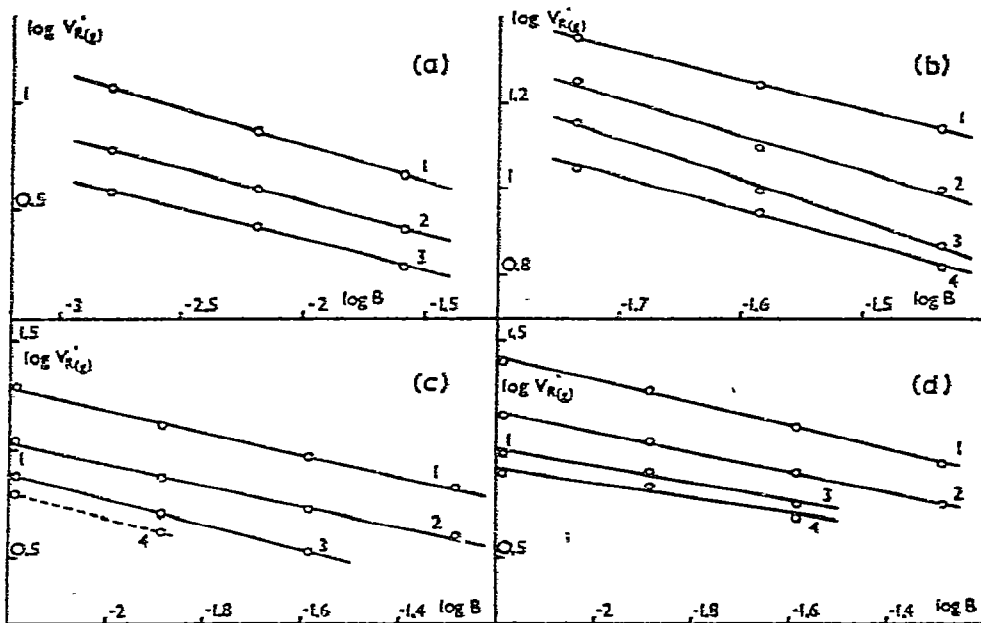


Fig. 6. Relationship between retention volumes, $V'_{R(g)}$ (ml), of *N,N*-dimethyl-*p*-aminobenzeneazobenzoyl amides and parameter B in gradient elution chromatography on Porasil A with the binary mobile phase *n*-propanol-*n*-heptane. Parameters of the gradient elution: $A = c_0 = 0$; $V_f = 38.5$ ml; $\alpha = 0.5$ (a), 2.0 (c) or 1.0 (b and d). In a, b and c the volume concentration of *n*-propanol was changed with time according to the above parameters; d shows the results of experiments in which the mole fraction of *n*-propanol was changed with time according to these parameters, the actual change of volume concentration with time being shown in Fig. 5 (broken curves). Compounds: 1 = dimethylamide; 2 = diethylamide; 3 = di-*n*-propylamide; 4 = di-*n*-butylamide. Operating conditions as in Table III.

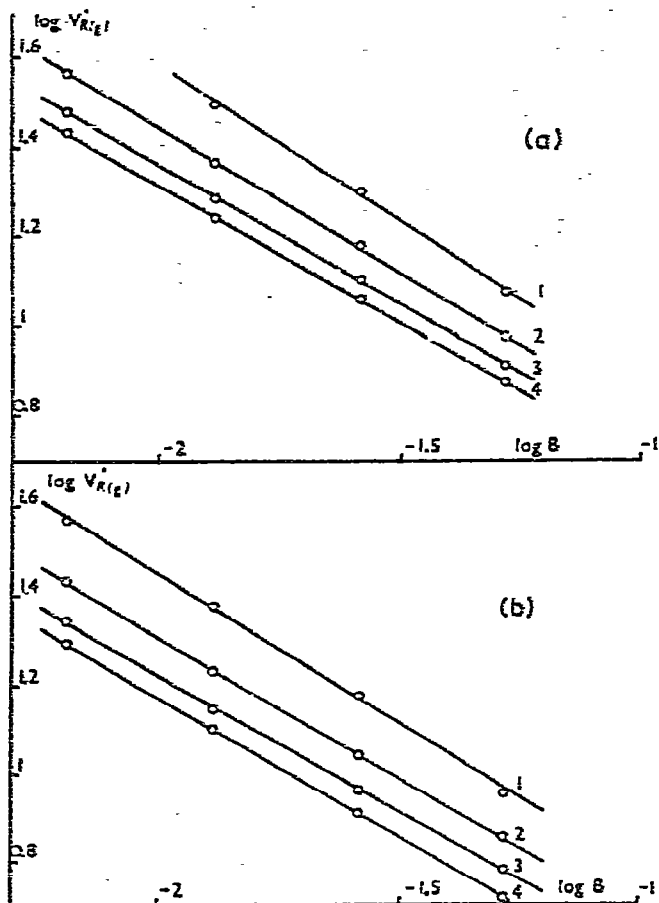


Fig. 7. Relationship between retention volumes, $V'_{R(g)}$ (ml), of *N,N*-dimethyl-*p*-aminobenzeneazobenzoyl amides and parameter B in gradient elution chromatography on Porasil A with the binary mobile phase dioxane-*n*-heptane. Parameters of the gradient elution: $A = c_0 = 0$; $V_s = 38.5$ ml; $\alpha = 1.0$. (a) The mole fraction of dioxane was changed with time according to these parameters, the actual change of volume concentration with time being shown in Fig. 5 (full curves). (b) The volume concentration of dioxane was changed with time according to the above parameters. Compounds: 1 = dimethylamide; 2 = diethylamide; 3 = di-*n*-propylamide; 4 = di-*n*-butylamide. Operating conditions as in Table VII.

gradient curve (eqn. 2) based on the mole fraction of the more polar solvent are compared with those using the volume concentration of this solvent as the basis of the gradient curve ($\alpha = 1$). Comparison of these graphs, as well as comparison of Tables IV and VI (*n*-propanol-*n*-heptane) and Tables VII and VIII (dioxane-*n*-heptane), shows that no significant improvement of results is gained by using mole fractions instead of volume concentrations as the basis for constructing the gradient curves.

The influence of the composition of the mobile phase on the plate number was also tested. The eqns. (11-14) for peak width were derived by assuming a linear change in N with retention volume. Experimental values of N for the four sample compounds studied in four different mobile phases are shown in Table X. The plate numbers are

TABLE X

EXPERIMENTAL VALUES OF PLATE NUMBER (N) OF *N,N*-DIMETHYL-*p*-AMINO-BENZENEAZOBENZOYL AMIDES IN ADSORPTION CHROMATOGRAPHY ON PORASIL A

Column: 400×3 mm; $V_m = 2.00$ ml; Porasil A(60), 37–75 μ m. Flow-rate of mobile phase: 38.5 ml/h. Detection: photometric, 440 nm. Chromatographed compounds: 1 = di-*n*-butylamide; 2 = di-*n*-propylamide; 3 = diethylamide; 4 = dimethylamide; ca. 8 μ g each. Mobile phases: I = ethanol-*n*-pentane; II = *n*-propanol-*n*-heptane; III = dioxane-*n*-heptane; IV = chloroform-*n*-heptane. $c = \%$ (vol.) of the more efficient component in the mobile phase. The experimental values of N represent the arithmetic means from three experiments.

Mobile phase	c	N for compound			
		1	2	3	4
I	0.5	172	354	—	—
	1.5	113	83	192	—
	3.0	79	77	167	318
	5.0	104	88	84	201
	8.0	—	—	124	168
	12.5	—	—	98	130
	20.0	—	—	—	93
II	0.5	76	184	—	—
	1.5	99	99	114	—
	3.0	—	—	114	—
	5.0	—	—	92	131
	8.0	—	—	79	106
	12.5	—	—	—	92
	20.0	—	—	—	89
35.0	—	—	—	58	
III	7.5	161	192	199	—
	10.0	156	176	201	161
	15.0	—	—	162	208
	20.0	—	—	150	195
	30.0	—	—	100	146
	40.0	—	—	—	122
IV	10.0	—	—	227	—
	15.0	128	165	197	240
	20.0	111	130	177	188
	25.0	—	—	160	205
	30.0	—	—	155	189
	40.0	—	—	128	182
	50.0	—	—	—	160
	60.0	—	—	—	148

not constant over the whole concentration range, whereas they were approximately so in experiments with the mobile phase cyclohexane-ethyl acetate³.

The relationship between experimental plate numbers and V'_R was not strictly linear, but it could be approximated to a line because of the significant random error in determining N . The coefficients of the regression lines representing the relationship (eqn. 10a) were calculated using the linear-regression method and were used for further calculations of $w_{(g)}$, $N_{(g)}$ and $R_{s(g)}$ according to eqns. 13, 8 and 9.

The calculated retention characteristics, which are also shown in Tables III–

IX, mostly differed little from data calculated on the basis of one (average) value of N for each combination of sample compound and mobile phase (eqns. 7-9). The agreement between calculated and experimental data was, in some instances, poorer in calculations using eqn. 13. This may be attributable to the way of estimating the constants C and D of eqn. 10a, namely, that all values of N in isocratic elution experiments, including those significantly deviated from the trend followed by other values, were taken as a basis for linear-regression analysis. On the other hand, the approach making use of eqn. 10a is likely to significantly increase the accuracy of calculating retention characteristics if the dependence of N on retention volume (or composition of the mobile phase) is more distinct than in the examples reported here.

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