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ROLE OF EXTRA-COLUMN VOLUME IN THE EFFICIENCY OF HIGH-SPEED LIQUID CHROMATOGRAPHY

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SUMMARY

The role of extra-column contributions to the height equivalent to a theoretical plate, H, in high-speed analysis is pointed out. A simple equation, usually applied to band broadening in the column, was used to demonstrate that extra-column contributions influence not only H but also, to a varying extent, coefficients of the equation. Coefficients C in the equation $H = f(u)$ were determined experimentally with the use of sorbents with particle diameters of 6 and 3.2 μm. Columns 55 and 30 mm long were connected to spectrophotometric and electrochemical detectors, respectively, having detection cell volumes of 20, 10 and 8 μl and 7 nl.

C as a function of the capacity ratio, k, follows the theoretical dependence only when a cell having a volume small enough with respect to the volumetric variance of the peak is used. For the work at higher flow-rates of the mobile phase (important for high-speed analysis), it is necessary to optimize the size of the detection cell. It is shown that under the experimental conditions given, an increase in the detection cell volume leads to an increase in H at the minimum of the dependence on the mobile phase flow-rate, but also, in contrary to expectation, to a decrease in C. As a consequence, at higher flow-rates of the mobile phase, lower values of H are found with the use of a detector with larger detection cell, which makes it possible to increase the speed of the analysis. The detection cell volume also affects the character of the dependence of H on u significantly.

INTRODUCTION

Increasing the speed of the analysis while maintaining or even increasing the efficiency of chromatographic separations is one of the important trends in the development of liquid chromatography. The desired result is mostly achieved by decreasing the particle size of the sorbent used in the column. It is several years since the optimal particle diameter was characterized[1] and estimated from theoretical analyses[2] to be in the approximate range d_p = 1–3 μm. The preparation of columns with sorbents having d_p = 1.8 μm (ref. 3) and 2 μm (ref. 4) and the instrumentation for columns with sorbent having d_p = 3 μm (refs. 5–13) have received considerable at-

tention in the last 5 years. With regard to small volumetric variances of the solute peak at the outlet of efficient columns packed with sorbents with small particle diameters, the demands on the instrument are similar to those imposed on the use of small-bore columns (*e.g.*, refs. 14–18).

In this work, we investigated the effects of extra-column volume, particularly that of the detector cell, on the height equivalent to a theoretical plate depending on the linear velocity of the mobile phase in the column.

EXPERIMENTAL

The chromatograph used was composed of the following elements. A VCM 300 pump (Development Workshop, Czechoslovak Academy of Sciences, Prague, Czechoslovakia) was used for the mobile phase. Two manometers connected via a stainless-steel capillary were used to damp the pulses. A Valco valve (Valco Instruments, Houston, TX, U.S.A.) with a 0.2-μl internal loop was used to sample solutes on the column. The coupling between the valve and the column was a stainless-steel capillary, 20 × 0.15 mm I.D. Glass columns of two types were used. Columns of 55 × 4 mm I.D. were made of a glass tubing of 7.5 mm O.D., provided in the lower section with connections to the detector consisting of a stainless-steel capillary (20 × 0.25 mm I.D.). These columns can be operated at pressures of 15–20 MPa and are designed so that the column can be fitted inside a suspension reservoir and filled under pressures up to 40 MPa[19]. Columns of 30 × 4 mm I.D. (Fig. 1) were designed as one unit together with an electrochemical detector[17,20] and, in order to increase the applicable pressure, they were sealed into a metallic casing. The influence of permeability of the outlet frit on the total column permeability increases[21] with re-
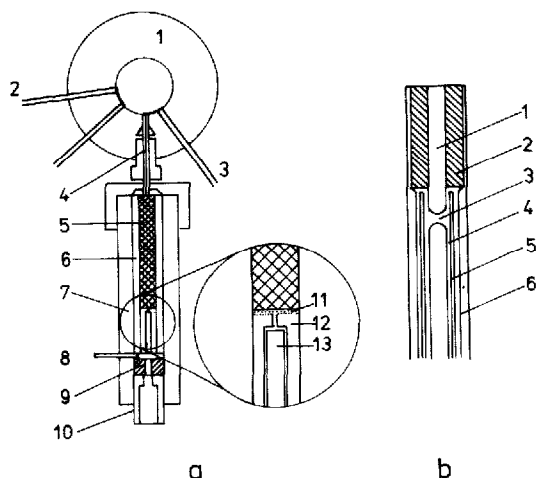

Fig. 1. (a) Arrangement of the chromatographic column with a Valco sampler and an electrochemical detector. 1 = Valco valve (0.2 μl); 2 = sample inlet; 3 = mobile phase inlet; 4 = connecting capillary, length 22 mm, I.D. 0.15 mm; 5 = column packing, length 30 mm; 6 = glass tube, I.D. 4 mm; 7 = metallic casing of the column; 8 = mobile phase outlet; 9 = seal; 10 = holder of electrode connector; 11 = stainless-steel nets; 12 = electrode holder; 13 = electrode. (b) Detail of the electrochemical detector electrode. 1 = Pt wire, O.D. 0.5 mm; 2 = glass tube; 3 = soldered joint; 4 = contact wire; 5 = PTFE insulation; 6 = electrode body of stainless-steel tube, O.D. 1.6 mm.

duction in the column length and with increasing flow-rate. The most advantageous solution consisted in the use of two ring-shaped stainless-steel nets, 2-μm mesh, covered with a filter-paper ring to prevent leakage of the sorbent. After packing, the sorbent at the column inlet was covered with a ring of filter-paper. The outlet of 30 mm long columns was created by a 1 mm long capillary, I.D. 0.25 mm. This capillary ended in a cylindrical cavity, I.D. 1.8 mm, into which the electrode of the electro-chemical detector, O.D. 1.6 mm (Fig. 1), was inserted.

Two different detectors were used for measurements. An LCD 254 UV detector (Laboratory Instruments, Prague, Czechoslovakia) with a 250 × 0.25 mm I.D., inlet capillary and with an 8-μl cell was connected to a 55 mm long column. An electro-chemical detector in a wall-jet arrangement of a home-made design[17,20] was first connected to the supply from the 55-mm column and later to the 30-mm columns to form one unit (Fig. 1a). The working electrode, 0.2 mm^2 in surface area, was insulated with a glass capillary from the auxiliary electrode formed by a stainless-steel tube (Fig. 1b). The volume of the cell of the electrochemical detector is determined by the distance between the polished cross-section of a Pt wire from the end of the outlet capillary. Provided that the electrode is pushed in almost completely (the gap is 0.05–0.1 mm), the cell volume can be estimated to be *ca.* 7 nl. With the electrode pushed to a distance of 4 mm from the capillary outlet the cell volume increases to 10 μl, and when pushed to a distance of 8 mm the cell volume is about 20 μl.

The mobile phase used was 70–75% of acetonitrile in water and, in order to increase the conductivity, the addition of 0.1 M NaClO$_4$ to 1 l of the mobile phase was necessary. To test the columns, we employed a standard mixture containing 4-aminoazobenzene (0.01%, w/v), 2-aminoazotoluene (0.015%, w/v) and N,N-dimethyl-4-aminoazobenzene (0.02%, w/v) in the mobile phase.

Preparation of sorbent

We used Separon Si C$_{18}$ (d_p = 6 μm) and Separon SIX (d_p = 3.2 μm) spherical silica gels (Laboratory Instruments). The mean numerical particle diameter was determined by measurements of photographs taken with an electron scanning microscope. After drying, Separon SIX was converted to sorbents of RP-8 and RP-18 types by treatment with toluene solutions of octyldimethylchlorosilane and octadecylmethyldichlorosilane, respectively. A toluene solution of trimethylchlorosilane was used for the post-treatment of the sorbents obtained.

Suspension of the sorbent in 10 ml of tetrachloromethane (*ca.* 2.5%, w/v) at a pressure of 15–20 MPa was used to pack the columns. The time necessary for packing was 3 min. Dioxane served as a pressure liquid. After packing, the column was washed with 10 ml of dioxane, 20 ml of water and 20 ml of 0.3 M NaClO$_4$ solution. Freshly packed columns possessed fairly low permeability (column resistance parameter φ = 2000) and a low efficiency (h_{min} = 6–7). A gradual increase in the mobile phase flow-rate led to an increase in both permeability and efficiency. A stationary state was reached after the third cycle of the flow-rate increase within the pressure range ΔP = 0–10 MPa and values of h_{min} = 2.4 and φ = 650–800 were measured, which correspond a value of the separation impedance[22] of E = 3800–4600.

RESULTS AND DISCUSSION

The length of the chromatographic column was selected so that the theoretical plate number would be approximately constant when sorbents with particle diameters of 6 and 3.2 μm, respectively, were chosen. We assume that the reduced height equivalent to a theoretical plate at the optimal mobile phase velocity is $h \approx 2$. Under these circumstances, provided that the extra-column volume has no effect on the height equivalent to a theoretical plate, a 55 mm long column with particles 6 μm in diameter would have 4600–4700 theoretical plates at the optimum of the dependence of H on the mobile phase velocity, and likewise a 30 mm long column packed with particles 3.2 μm in diameter. We had found earlier that the effect of the extra-column volume on the height equivalent to a theoretical plate cannot be considered to be constant within the range of linear velocities used in liquid chromatography[23]. Regarding the possibility of obtaining short analysis times, we studied in particular the effects of the extra-column volume on the height equivalent to a theoretical plate at higher linear velocities.

In order to describe the dependence of the height equivalent to a theoretical plate, H, on the linear velocity, u, we used the simple relationship

$$H = A + B/u + Cu \tag{1}$$

the validity of which within the range of velocities under study we confirmed. This equation is usually used only for the band broadening in the column. Nevertheless, considering that H is a function of the extra-column volume, individual coefficients of the equation must also be a function of this volume. Under these circumstances, the coefficients lose their physical meaning, which can be ascribed to them if only the processes in the column are considered. We determined experimentally both A and C in eqn. 1 by using the plots of the linear regression of H vs. u for higher values of u and calculated B from the relationship

$$B = 2\gamma D_m \tag{2}$$

where D_m (diffusion coefficient) $= 10^{-5}$ cm^2 sec^{-1} and $\gamma = 0.6$. The estimate of B according to eqn. 2 is satisfactory as attention is concentrated on higher mobile phase velocities. The correlation coefficients of C lay in the range 0.9–0.7 and the standard deviation of the determination of A was ca. 10%.

For columns packed with particles of $d_p = 6$ μm we used an electrochemical detector with a cell volume of ca. 7 nl and a spectrophotometric detector with a cell volume of 8 μl. The values of the height equivalent to a theoretical plate measured with the electrochemical detector are listed in Table I.

The coefficient C shows a theoretical dependence on the capacity ratio, k, as is obvious from Table I and Fig. 2. Theoretical values of C were calculated according to the relationship

$$C = E(k) \, d_p^2/D_m$$

$$E(k) = \frac{1 + 6k + 11k^2}{96 (1 + k)^2} \tag{3}$$

TABLE I

COMPARISON OF EXPERIMENTAL AND CALCULATED VALUES OF PARAMETERS IN EQN. 1 FOR THE 55-mm COLUMN

Column: length 55 mm, I.D. 4 mm; sorbent, Separon Si C_{18}, d_p = 6 μm. Solutes: resorcinol (k = 0.05), 4-aminoazobenzene (k = 1), 2-aminoazotoluene (k = 2) and N,N-dimethyl-4-aminoazobenzene (k = 3.7). Electrochemical detector, cell volume V_c = 7 nl. A = 17.89 (average experimental value). ΔC = $C - E(k)d_p^2/D_m$.

k	$C \cdot 10^3$ (sec)	$E(k)d_p^2/D_m \cdot 10^3$ (sec)	$\Delta C \cdot 10^3$ (sec)	H_{min}^{theor} (μm)	u_{opt}^{theor} (mm sec^{-1})
0.05	3.77	0.45	3.32	20.30	0.63
1.0	1.42	1.69	−0.27	21.34	1.02
2.0	2.07	2.73	−0.66	22.29	0.85
3.7	2.41	2.95	−0.54	22.13	0.79

This value is valid provided that the band broadening appears only in the column. The agreement between experiment and the theory (small values of ΔC in Table I) can be considered as proof of the fact that in the given arrangement extra-column contributions do not affect the height equivalent to a theoretical plate. The experimental arrangement is thus suitable for the work at higher velocities of the mobile phase and hence for the work with short analysis times. For the sake of comparison we connected the column with identical parameters with a commercial spectrophotometer detector with a cell volume of 8 μl. The result of the measurements is shown in Fig. 2. The coefficient C decreases with increasing capacity ratio. It approaches
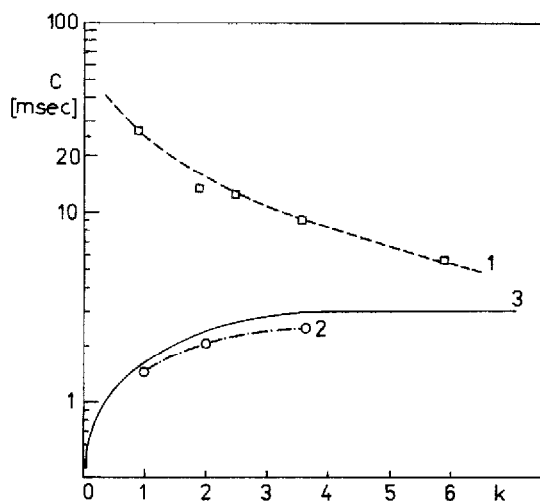

Fig. 2. Influence of the capacity ratio, k, on C for particles with d_p = 6 μm (Separon Si C_{18}). Column: length 55 mm, I.D. 4 mm. 1, UV detector, V_c = 8 μl; 2, electrochemical detector, $V_c \approx$ 7 nl; 3, theoretical dependence, $C = E(k) d_p^2/D_m$.

the theoretical value only at sufficiently high values of k, where the volumetric variance of the solute leaving the column[24],

$$\sigma_v^2 = (\pi d_p^2)^2 \, Lh$$

is great enough due to a rising function $h = f(k)$ with a constant column length, L.

To evaluate the column packed with the sorbent of particle size 3.2 μm, we proceeded in an analogous manner. We worked with a material (Separon SIX) the surface of which was modified with octyl and octadecyl groups (Table II). An opposite dependence on the capacity ratio, k, to that corresponding to the theoretical one was found for C. The character of this dependence, as is obvious from Fig. 3, is analogous to the dependence found for the sorbent of particle diameter $d_p = 6$ μm and the spectrophotometric detector.

TABLE II

COMPARISON OF EXPERIMENTAL AND CALCULATED VALUES OF PARAMETERS IN EQN. 1 FOR THE 30-mm COLUMN

Column: length 30 mm, I.D. 4 mm; sorbent, Separon SIX C_8, C_{18}, $d_p = 3.2$ μm. Electrochemical detector, $V_c = 7$ nl. Solutes: see Table I.

Sorbent	k	$C \cdot 10^3$ (sec)	$E(k)d_p^2/D_m \cdot 10^3$ (sec)	$\Delta C \cdot 10^3$ (sec)	H_{min} (μm)	u_{opt} (mm sec^{-1})
RP 8 ($A = 4.68^\star$)	0.1	4.38	0.13	4.25	9.80	0.58
	0.5	3.01	0.29	2.72	8.93	0.70
	0.7	3.60	0.34	3.26	9.32	0.65
	1.0	3.17	0.42	2.75	9.04	0.69
RP 18 ($A = 6.74^\star$)	0.3	2.39	0.21	2.18	10.52	0.79
	1.3	1.79	0.49	1.30	10.02	0.91
	2.2	1.33	0.62	0.71	9.55	1.06
	3.2	1.17	0.71	0.45	9.39	1.13

$\star$ Average experimental value.

To evaluate the influence of the extra-column volume on the dependence of the height equivalent to a theoretical plate on the velocity, we increased the volume between the electrode of the electrochemical detector and the column.. We shifted the electrode (Fig. 1) to the distance of 4 and later 8 mm from the outlet of the chromatographic column. The coefficients of eqn. 1, measured with a volume behind the column created in this way, are presented in Table III. It is obvious that as the extra-column volume between the column and the detector increases, H_{min}, u_{opt} and A increase, as expected. However, in all instances, we observed a simultaneous decrease in C. In view of the fact that this coefficient affects the speed of analysis to a considerable extent, we decided to investigate the consequences of an increase in C on the speed of analysis. We started from a well known equation for the time of the analysis, t_a, derived from the relationship for the separation of two components corresponding to a separation coefficient, R_S, at the given capacity ratios of the substances, k_1, k_2, and relative retention $k_2/k_1 = r_{2,1}$:
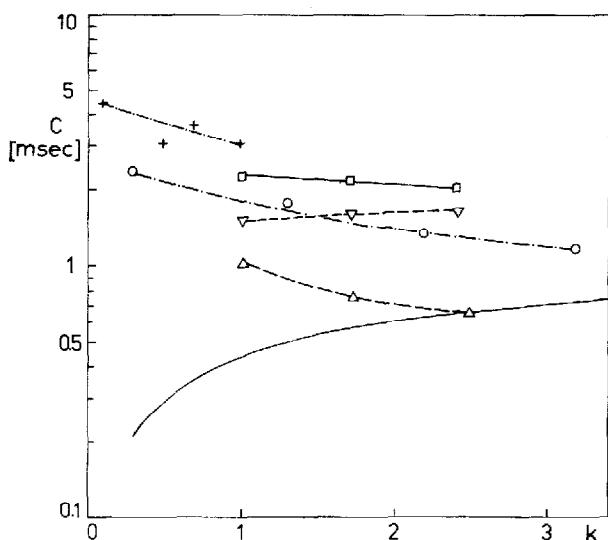
Fig. 3. Influence of the capacity ratio, k, on C for particles with d_p = 3.2 μm (Separon SIX C$_8$, C$_{18}$). Column: length 30 mm, I.D. 4 mm; electrochemical detector. First measurement: V_c = 7 nl; +, Separon SIX C$_8$; O, Separon SIX C$_{18}$. Second measurement: Separon SIX C$_{18}$; $\square$, V_c = 7 nl; ∇, V_c = 10 μl; $\triangle$, V_c = 20 μl. Lower full curve, theoretical dependence, $C = E(k)\, d_p^2\, /D_m$.

$$t_a = \frac{R_S^2\,(1 + k_2)^3\,r_{2,1}^2}{(r_{2,1} - 1)^2\,k_2^2} \cdot (C + A/u) \tag{4}$$

Values of C and A were taken from Table III and were used for the respective pair of k values. We assumed that, owing to the increase in the extra-column contribution, C would increase similarly to the case of the detector with a larger cell for the columns

TABLE III

COMPARISON OF EXPERIMENTAL AND CALCULATED VALUES OF PARAMETERS IN EQN. 1 FOR THE 30-mm COLUMN WITH DIFFERENT EXTRA-COLUMN VOLUMES

Column: length 30 mm, I.D. 4 mm; sorbent, Separon SIX C$_{18}$, d_p = 3.2 μm. Electrochemical detector, V_c = 7 nl, 10 μl and 20 μl. Solutes: 4-aminoazobenzene, 2-aminoazotoluene and N,N-dimethyl-4-aminoazobenzene.

Conditions	k	$C \cdot 10^3$ (sec)	$E(k)d_p^2/D_m \cdot 10^3$ (sec)	$\Delta C \cdot 10^3$ (sec)	H_{min} (μm)	u_{opt} (mm sec^{-1})
(A) V_c = 7 nl, A = 6.52*	1.05	2.35	0.44	1.91	10.27	0.80
	1.75	2.27	0.57	1.70	10.21	0.82
	2.46	2.02	0.66	1.36	10.00	0.86
(B) V_c = 10 μl, A = 8.14*	1.05	1.51	0.44	1.07	11.15	1.00
	1.75	1.68	0.57	1.11	11.31	0.95
	2.46	1.61	0.66	0.95	10.52	1.26
(C) V_c = 20 μl, A = 10.9*	1.05	1.01	0.44	0.57	13.36	1.22
	1.75	0.76	0.57	0.19	13.84	1.01
	2.46	0.61	0.66	−0.05	12.81	1.57

* Average experimental value.

with the sorbent of particle diameter 6 μm. For approximately the same range of capacity coefficients, we found C to be in the range 5–20 msec (Fig. 2). This range was only 2–3 msec for the electrochemical detector with a substantially smaller cell volume (*ca.* 7 nl). For the columns with a particle diameter of 3.2 μm and the same instrumental arrangement (with the electrochemical detector), we found a range of C values of only 1.2–2.4 msec. It should be mentioned that the decrease in C is not significant with regard to the increase in the speed of analysis with smaller k values, as also is obvious from Fig. 4. On increasing the volume in front of the detector electrode, we found an additional decrease in C down to the range 0.61–1.01 msec. With the expression in front of the final parentheses on the right-hand side of eqn. 4 kept constant, it seems that such an adaptation of the detector can lead to a reduction in the time of analysis. In view of the fact that both A and H_{min} increase simultaneously, a significant increase in the speed of analysis will occur only at higher mobile phase velocities, as demonstrated by Fig. 4.
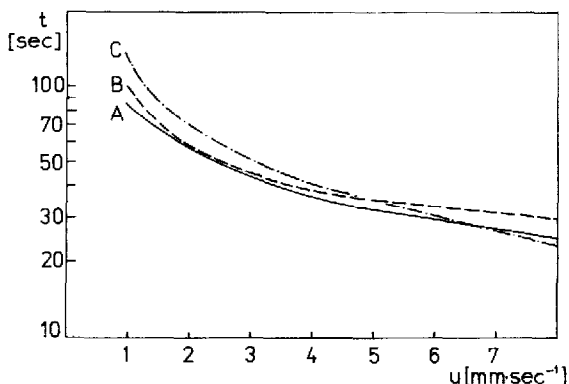

Fig. 4. Influence of the mobile phase velocity on the analysis time with varying cell volume. Column: length 30 mm, I.D. 4 mm, d_p = 3.2 μm; electrochemical detector. A, V_c = 7 nl; B, V_c = 10 μl; C, V_c = 20 μl.

With the use of sorbents with particle diameters of 2–3 μm dependences of H on u are often described with inexpressive minima and a wide range of mobile phase velocities at which the height equivalent to a theoretical plate deviates from the value of H_{min} only slightly. Using a sorbent with d_p = 2 μm, Dewaele and Verzele[4] found experimentally C = 0 for the range of linear velocities 1.4–4.2 mm sec^{-1}. On carrying out an experiment with the electrochemical detector with varying volumes of the detection cell, we found that this may be evoked or influenced by the extra-column volume.

Fig. 5 illustrates h *vs.* u dependences with the use of average constants determined experimentally and listed in Table III. It is obvious from Fig. 5 that the increase in the volume of the electrochemical detection cell is associated with the increase in the height equivalent to a theoretical plate in the range of optimal velocities of the mobile phase. When the volume increased from *ca.* 7 nl to 10 μl, H_{min} increased by 8%; with a volume increase to 20 μl under the same conditions, H_{min} increased by 24%. At higher velocities, the differences in the values of the height equivalent to
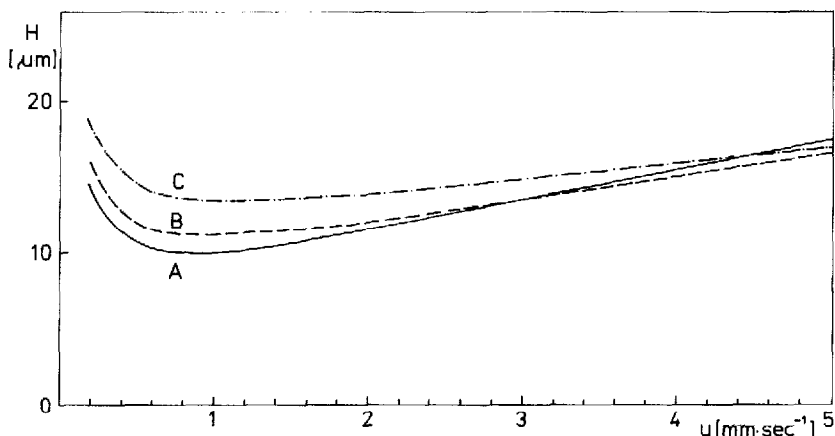
Fig. 5. *H* vs. *u* relationship for the column of length 30 mm, I.D. 4 mm, d_p = 3.2 μm. The course of curves was calculated on the basis of the average values from Table III. A, V_c = 7 nl; B, V_c = 10 μl; C, V_c = 20 μl.

a theoretical plate varied substantially. At a velocity of 5 mm sec^{-1} the height equivalent to a theoretical plate for a cell volume of 10 μl is 6% less than that determined with a 7-nl cell.

With respect to changed values of constants of eqn. 1, the character of the dependence of *H* on *u* also varies. For illustration, values of *H* from Fig. 5 were converted with regard to H_{min} = 1 for three cases, A, B and C, and the results are shown in Fig. 6. If the mobile phase velocity at which the *H* values do not differ from the value H_{min} = 1 by more than 10% is selected as characteristic of the course of the curve, then we can see substantially different ranges of velocities for various instances. For the 7-nl cell the range is from 0.40 to 1.74 mm sec^{-1}, for the 10-μl cell
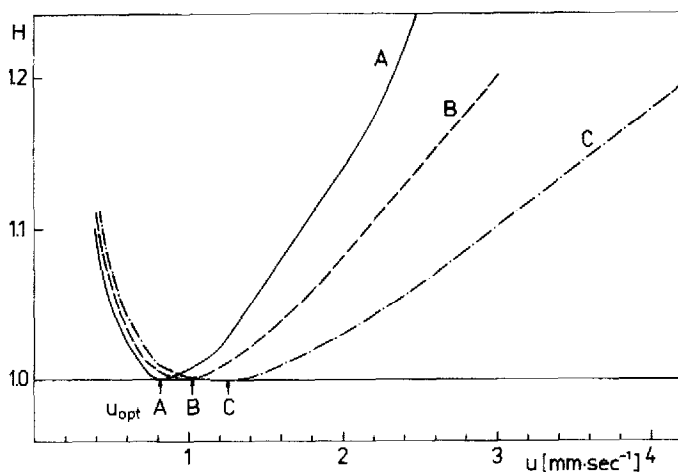

Fig. 6. H_{rel} vs. *u* relationship (H_{min} = 1). Conditions as in Fig. 5. A, V_c = 7 nl; B, V_c = 10 μl; C, V_c = 20 μl.
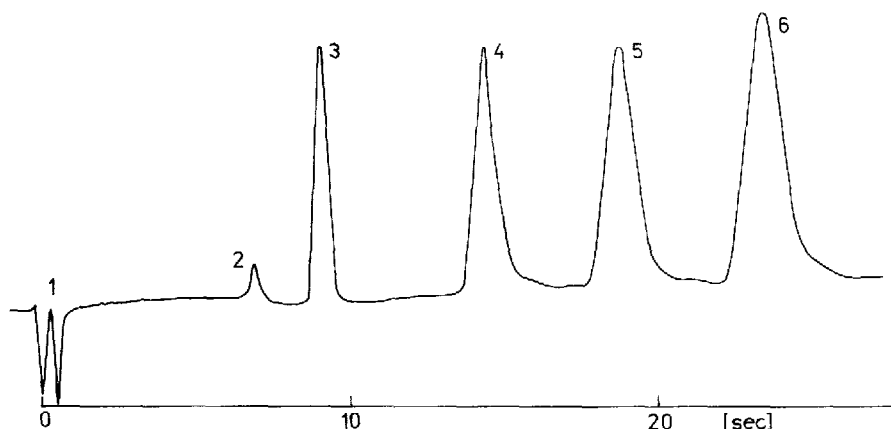
Fig. 7. Example of a rapid analysis of a standard mixture. Column: length 30 mm, I.D. 4 mm, Separon Si C_{18}, d_p = 3.2 μm. Mobile phase: acetonitrile–water (3:1) containing 0.1 M NaClO$_4$, u = 4.3 mm sec^{-1}, ΔP = 6.5 MPa, V_c = 20 μl. 1, Reaction of electrochemical detector to injection; 2, Na$_2$SO$_3$ (k = 0); 3, resorcinol; 4, 4-aminoazobenzene; 5, 2-aminoazotoluene; 6, N,N-dimethyl-4-aminoazobenzene.

from 0.45 to 2.18 mm sec^{-1} and for the 20 μl cell from 0.45 to 3.00 mm sec^{-1}. Simultaneously with this effect, we found that the optimal mobile phase velocity, u_{opt}, increased in the sequence A, B, C (Fig. 6) from 0.82 to 1.07 and 1.26 mm sec^{-1}, respectively.

It follows from the experiments performed that for the application of high-speed high-performance liquid chromatography (an example is shown in Fig. 7) there exists an optimal size of the detection cell. The magnitude of the volume obviously depends on the volumetric flow-rate of the mobile phase from the cell, the character of the liquid flow in the cell and, of course, the volumetric variance of the peak leaving the column. With the use of sorbents with particle sizes of about 3 μm and less, the extra-column volume is obviously an inseparable component determining the efficiency of the chromatographic system. Some phenomena, previously ascribed only to the value of the particle diameter of the sorbent used, are complex, involving both factors mentioned above.

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