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COMPARISON OF METHODS OF SAMPLE INRODUCTION DURING SCALE-UP OF LIQUID CHROMATOGRAPHY

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SUMMARY

The comparison of band spreading caused by various methods of sample introduction, depending on the mobile phase flow-rate, is presented. The experiments were carried out under conditions typical for pilot research on preparative separation and under conditions most often encountered when preparative columns are used. Modifications of the shape of the sample loop, introduction of a sample by an extra pump, and methods equivalent to application of an extra pump were taken into account. A method of modification of a standard sampling valve is proposed, which allows repetitive introduction of a sample in the form of rectangular pulses. Application of an extra pump or methods equivalent to application of an extra pump results in significant advantages, especially during pilot research of the stage of selection of preparative separation conditions by means of an analytical column.

INTRODUCTION

Band spreading during introduction of a sample is one of the extra-column effects resulting in deterioration of the efficiency of separation attainable in practice, and a decrease of the resolution of the substances in a column[1–3]. Numerous papers have dealt with the problem, and it can be considered solved in the case of analytical liquid chromatography (LC) when columns of diameter 2–6 mm are used. However, preparative chromatography is a source of other difficulties, when it is often necessary to inject large volumes of the sample V_i, exceeding at times the volume of the column V_c, while any increase of the column diameter (d_c) or length (L_c) results in an increase of the column volume and the flow-rate (W) of the mobile phase. Treating all the variables as a degree of the change of scale between analytical and preparative columns (*e.g.* $L = L_{cp}/L_{ca}$; $W = W_p/W_a$; $V = V_{cp}/V_{ca}$; $I = V_{ip}/V_{ia}$; $d = d_{cp}/d_{ca}$), the following relationships can be written for $L \cong 1$:

$$I \cong V = Ld^2 \quad (1)$$

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and

$$W \cong d^2 \tag{2}$$

where the subscripts p and a, denote preparative and analytical column, respectively.

Particular attention should be paid to ensuring that the distribution of an injected sample in the injection zone is as close to rectangular as possible under conditions of large sample volumes compared with the volume of the column[4,5,7]. It is important at the same time that the extra-column effects related to injection of the sample should be identical on analytical and preparative scales. Only then can the conditions for separation established by means of an analytical column (pilot research) be transferred to a preparative column (real scale). Such a transfer should be based on partly modified rules found in the literature[4–6,8]. Here, a method for calculation of concentration profiles of sample peaks relative to the volume of sample injected into the preparative column[7] is described, as well as a method for calculating the optimal injection volume in preparative LC[8].

Three methods of sample introduction during preparative seperation can be distinguished among the existing methods for their particular usefulness:

(1) Application of multi-port sampling valves equipped with open[6,9,10] or packed[1] sample loops;

(2) Application of an extra pump forcing the sample to the column while the eluent-forcing pump is switched off[4,6,10];

(3) Application of containers connected parallel to the main pump: the sample is forced to the column by compressed inert gas[11–13].

It can be intuitively anticipated that the second and third methods should be the best ones to meet the requirement of a rectangular injection zone. Application of the third method usually results in longer injection times, since the injection is carried out under a pressure lower than that during separation, and at the same time the viscosity of the injected sample is usually higher than the viscosity of the eluent. The results of some experiments indicate that under unfavourable geometrical conditions the above anticipations are not necessarily true[10].

On the other hand, application of open sample loops is the most readily available method when laboratory liquid chromatographs are used for preparative purposes. However, large deviations of the shape of the injection zone from rectangular distribution, especially in the descending part, should be taken into account[1,6]. Work by Taylor[14], Coq *et al.*[1] and Hoffmann and Halasz[15,16] has led to qualitative conclusions concerning the dependence of the variance of the distribution of concentration in the injection zone σ_{vi}^2 on the diameter d of the sample loop, its length L and the liquid flow-rate W. However, even when the viscosity η and other physicochemical properties of the eluent are similar to those of the injected sample, quantitative calculations of σ_{vi}^2 lead to results consistent with the experiments only under specific conditions[1]. Further theoretical studies are still necessary in this field. Hence, practically significant results can be achieved more readily by experimental studies. The paper presents comparison of known and modified (by the authors) methods of sample introduction under conditions typical for pilot research and real scale preparative LC separations.

EXPERIMENTAL

Reagents

Heptane, methanol and demineralized water, and the following separated substances: benzene, naphthalene, and methyl, ethyl, and *n*-propyl esters of 4-hydroxybenzoic acid, were used.

Chromatographic columns

LiChrosorb C_{18} columns, d_p = 5, 7, or 10 μm, 120 × 4 mm I.D., 250 × 4 mm I.D., and 120 × 4 mm I.D.

Instruments

Two pumps (Model 64.00 Knauer), with increased capacity (1–100 ml/min) equipped with preparative heads; sampling valves types RH 7125 and RH 7010 with changeable sample loops (produced by Rheodyne); UV spectrophotometric detector (type 87.00 Knauer), equipped with a cell of optical pathlength 0.4 mm; *Y*–t strip-chart recorder. Schematic drawings of the two types of sample loop used (volume 0.5 and 2 ml) are shown in Fig. 1. The sample loop designated by "a" in Fig. 1 was connected to a sampling valve by short segments of a tube 1.6 mm O.D. × 1 mm I.D. A schematic diagram of a sample container built in the form of a "pseudo-syringe", as well as alternative ways of connecting it to sampling valves, are depicted in Fig. 2.

Procedure

The shape of a curve of the concentration distribution of benzene in the injection zone was recorded. Injection devices were connected directly to the UV detector through a capillary. Methanol was used to remove benzene from the injection devices. The injection devices mentioned were subsequently used during separation of substances in chromatographic columns.

Sampling was performed by three methods: using an additional pump; using a six-port valve provided with sample loops shown in Fig. 1; using a six-port valve equipped with a "pseudo-syringe". The sampling device in Fig. 2a, equipped with a 15-ml "pseudo-syringe" (12 mm I.D. tube), was mainly used. However, the devices presented in Fig. 2b, a′, and b′, equipped with identical "pseudo-syringes" connected to Rheodyne sampling valves (frequently employed in laboratories), were also tried. The results obtained in this case were similar to those described below. In one of the

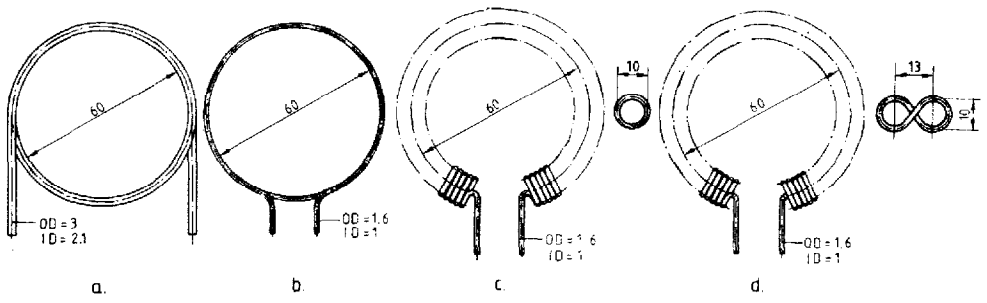

Fig. 1. Sample loops used (dimensions in millimetres).

earlier experimental series a version of the device presented in Fig. 2a, equipped with a 240-ml "pseudo-syringe" (24 mm I.D. tube; connecting tubes and channels in multi-port valve *ca.* 2 mm I.D.), was also successfully used with flow-rate of *ca.* 0.5 l/min[15]. Backward motion of the piston of the 15-ml "pseudo-syringe" (sucking of a sample) was accomplished manually, whereas backward motion of the 240-ml "pseudo-syringe" was forced gravitationally by a weight.

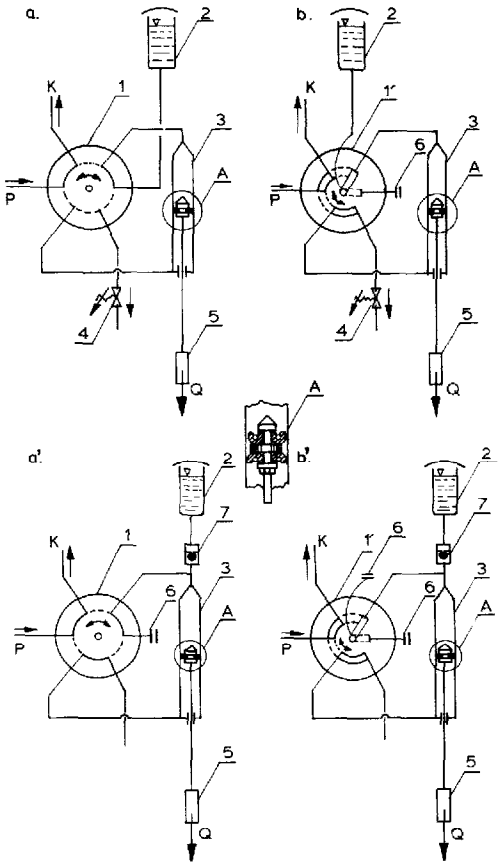

Fig. 2. Alternative ways of connecting a "pseudo-syringe" to Rheodyne sampling valves: a,a' = type RH 7010; b,b' = type RH 7125; a,b = suction of the sample through the sampling valve; a',b' = suction of the sample with by-passing of the sampling valve (check valve 7). Designations: 1 = sampling valve; 2 = injected solution container; 3 = "pseudo-syringe"; 4 = check valve; 5 = piston holder or weight; 6 = blind; P = from the pump; K = to the column; A = construction of the piston of the "pseudo-syringe"; Q = force applied.

A body of a "pseudo-syringe" from Fig. 2 was made of a piece of stainless-steel tubing with the internal wall surface carefully polished. Two typical sealing rings of a micropump piston of a Spectra-Physics chromatograph were employed as piston washers of a 15-ml "pseudo-syringe", which permitted operation in the 0–40 MPa pressure range. On the other hand, a 240-ml "pseudo-syringe" was operated at 0–6

MPa. So far, no damage to these devices has been observed, even though they have been used for over 500 sampling operations during 6 months. It follows from Fig. 2 that there are two operating stages of a sampling valve equipped with a "pseudo-syringe".

(1) Filling a "pseudo-syringe" with the sample solution (from a container), which is accomplished in the "load" position of a sampling valve by manual or gravitational shifting of a piston to the lower position;

(2) Sampling a solution of separated mixture onto a column, which is accomplished in the "inject" position of a valve. Then the mobile phase is forced from a chromatographic pump into a "pseudo-syringe" and, pushing the piston, drives the sample solution into an LC column. The sample volume is determined by measurement of the movement time of the "pseudo-syringe" piston or, better, by measurement of the liquid volume leaving the column during the sampling step. Consecutive filling of a "pseudo-syringe" with the solution was performed when the liquid volume over the piston was insufficient for successive sampling.

The eluent pump was switched off during sample injection carried out by means of an extra pump.

RESULTS AND DISCUSSION

Fig. 3. presents the concentration profiles of benzene recorded at various flow-rates of eluent (methanol), obtained by various 2-ml sample loops presented in Fig. 1 and the "pseudo-syringe" presented in Fig. 2a. Fig. 4 presents a comparison of the concentration profiles obtained using the 2-ml sample loops in Fig. 1a and d with the profiles obtained using an extra pump with the same capacity as the eluent pump. The results as well as the data presented in Table I, lead to the following conclusions:

(1) Very unfavourable concentration profiles of benzene in the injection zone were obtained with 2.1-mm I.D. sample loops at flow-rates less than 20 ml/min. The higher the flow-rate of the eluent, the smaller the differences between the properties of the 2.1-mm and 1-mm I.D. loops. Disruption of elution of the sample contained

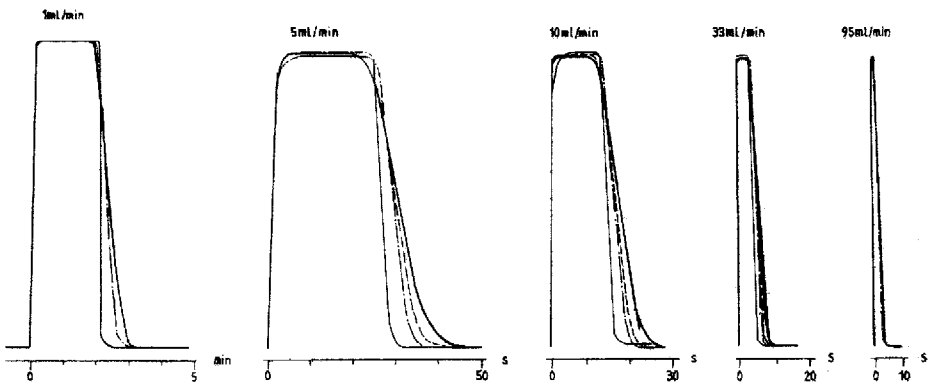

Fig. 3. Profiles of benzene concentration at the outlet of the sample loops presented in Fig. 1 (2 ml): —— = b; — — = c; –·–· = d; —— = application of a "pseudo-syringe" presented in Fig. 2a. Detector, UV 260 nm 2.56 a.u.f.s.

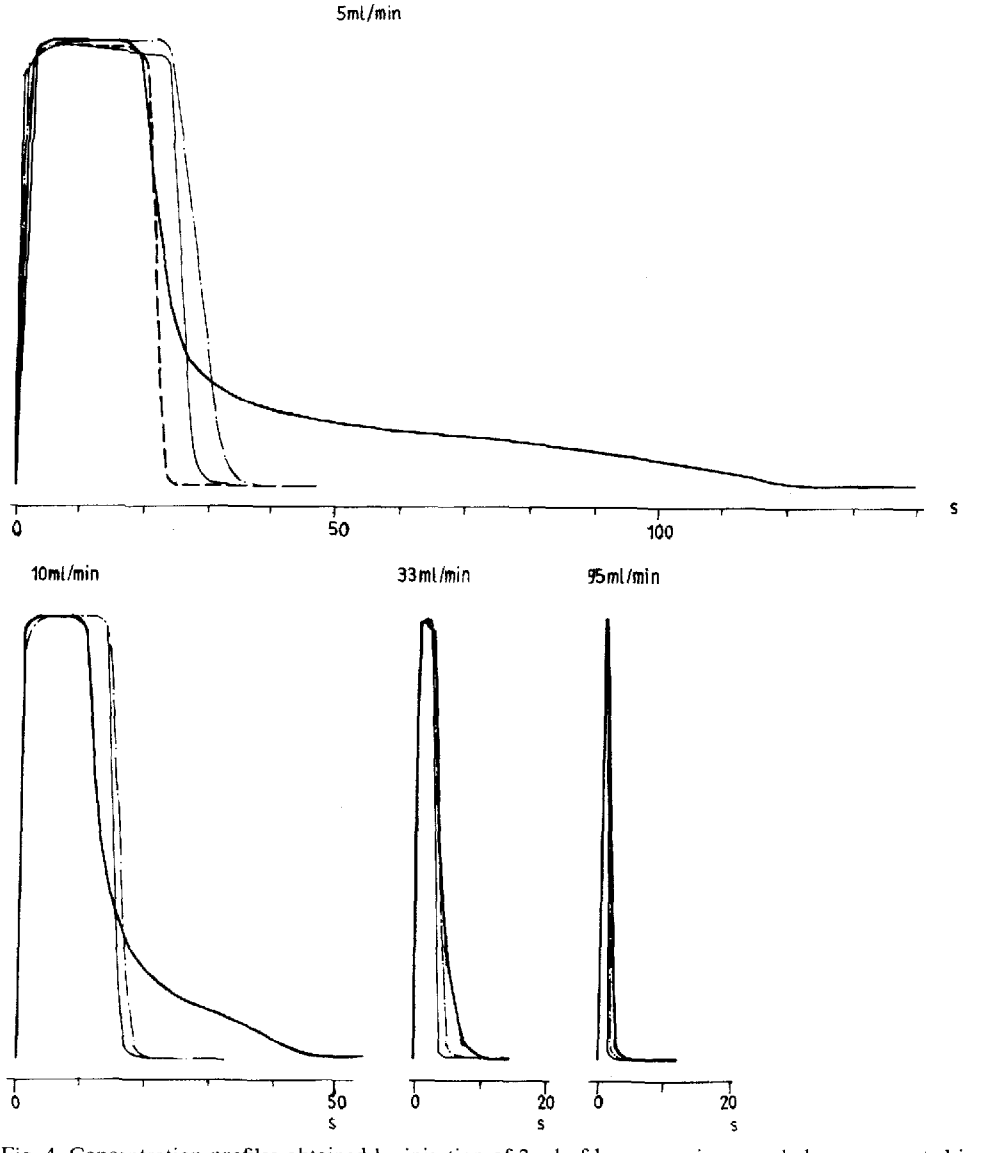

Fig. 4. Concentration profiles obtained by injection of 2 ml of benzene using sample loops presented in Fig. 1: —— = a; -·-· = d; or using an extra pump —— of various flow-rates of methanol used as an eluent; ----- detachment of sample loop "a" after a time corresponding to three-quarters of its volume. Conditions are the same as in Fig. 3.

in the sample loop when the volume of the eluted sample is about a quarter smaller than the volume of the 2.1-mm I.D. loop results in very advantageous concentration profiles in the injection zone. This conclusion is not new[1,8]: however, the previously published papers reported that only 50% of the volume of the loop was eluted.

(2) The decrease of the internal diameter of the loops from 2.1 to 1 mm, even

TABLE I

DEGREE OF BROADENING OF THE INJECTION ZONE AT THE BASELINE USING SAMPLE LOOPS COMPARED WITH THE WIDTH OF THE ZONE USING AN EXTRA PUMP (S/Sp)

Type of sample loop according to Fig. 1	*1 ml/min*	*5 ml/min*	*10 ml/min*	*33 ml/min*	*95 ml/min*
a	–	4.0	2.6	2.4	1.4
b	1.47	1.43	1.6	1.44	1.3
c	1.33	1.27	1.37	1.25	–
d	1.31	1.18	1.27	1.22	1.18

without any change of shape, is very advantageous (see Figs. 3 and 4), which is in agreement with theoretical studies[1,12]. However, in the case of low flow-rates, the differences between the 2.1-mm and 1-mm I.D. loops are of qualitative character and significantly exceed theoretical predictions of Coq *et al.*[1]. Additional improvements to the concentration distribution in the injection zone were achieved when the radius of the curvature of individual coils was decreased, and especially when the loop was coiled as shown in Fig. 1d. The relative significance of the effects described becomes greater as the flow-rate of the liquid eluting the sample from the sample loop decreases.

(3) The most advantageous solution, resulting in the shape of the concentration distribution curve being very close to rectangular (Figs. 3 and 4), was the application of an additional pump or a "pseudo-syringe" device presented in Fig. 2a, substituting for an additional pump during the injection.

The methods of modification of a sampling valve presented in Fig. 2 can hence assure an almost rectangular concentration profile in the injection zone, and introduction of the sample with a velocity identical with that applied during separation. The goal is achieved without application of an additional pump, and sample introduction can be carried out in the entire range of operating pressures of a chromatograph. Apart from rather insignificant technical complications, the solution described has another disadvantage: accurate washing of the space above the piston during changing of the injected solution is quite difficult. Hence, the modifications of a six-port sampling valve presented in Fig. 2 (and especially in Fig. 2a′ and b′) can be recommended in the case of frequent application of numerous cycles of repetitive separation of the same mixture of valuable substances. In other cases, forcing a sample into a column under the pressure of compressed helium can be more advantageous, even though the sampling pressure is lower than the operating pressure of a chromatograph, and hence the sampling time is long and the operation is more difficult to automate. The latter method of sample introduction to a preparative column probably results in the shape of the concentration profile being close to rectangular, provided that the diameter of the connection between the column and the liquid container is sufficiently small.

The results of the experiments described indicate that sample loops of 2 mm I.D. (or more) should be avoided, especially when low eluent flow-rates are used (less than 20 ml/min), owing to both a disadvantageous concentration distribution in the injection zone and high consumption of the separated solution during repeated filling

of the sample loop of large internal diameter (such a sample loop must be washed with a volume of the solution of separated substances a few times larger than the volume of the loop itself). High flow-rates during repeated filling of the sample loop are advantageous in such a case.

In practice, the most readily available way of assuring near rectangular profiles of sample concentration in the injection zone in most laboratories is elution of only a part of the contents of the sample loop. The maximum allowable injected volume and optimal conditions of repeated filling of the sample loop should then be determined by connecting the detector directly to the sampling valve. Figs. 5–7 present the results of verification of the above statements during separation of substances in a chromatographic column under conditions typical for pilot research in preparative applications of liquid chromatography. Under such conditions, the effects of injection are most apparent at low flow-rates.

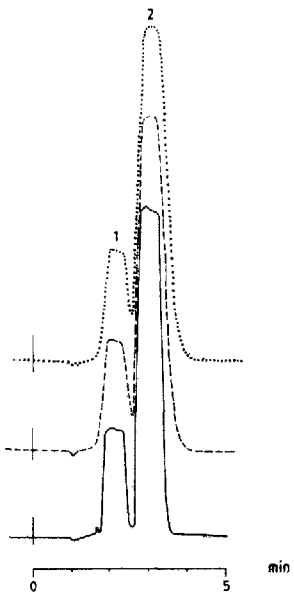

Fig. 5. Comparison of chromatograms obtained by various methods of injection of 0.5-ml solution of (1) benzene (2 mg/ml) and (2) naphthalene (0.2 mg/ml) into a 120 × 4 mm I.D. column packed with Nucleosil 7 C_{18}. Detection, UV 254 nm, 0.64 a.u.f.s.; mobile phase, methanol–water (8:2); flow-rate 1 ml/min. Curves: —— = injection by an extra pump; ···· = sample loop b; – – – = sample loop d (see Fig. 1).

On the basis of chromatograms presented in Figs. 5 and 6, obtained under typical volume-overloading conditions, it can be stated that the manner of injection of the sample does seriously influence both the shape and the width of the peaks and the degree of separation, both for 2 ml of the introduced sample (80% of volume of the column under the conditions presented in Fig. 5) and for 0.5 ml (*ca.* 40% of volume of the column under the conditions given in Fig. 6). Application of an extra pump for introduction of the sample leads to distinctly better separation of substances (R_s = 1.14 and 2.24) than application of sample loops (R_s = 0.78–0.82 and

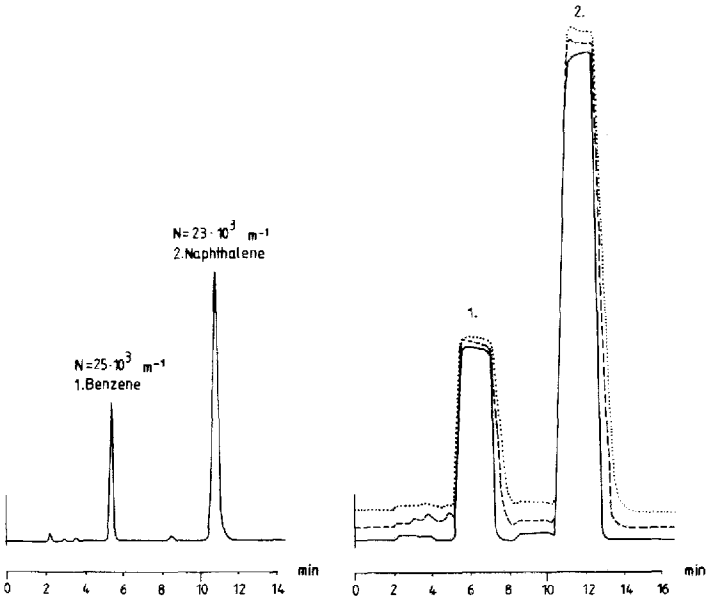

Fig. 6. Comparison of chromatograms obtained by various methods of introduction of solution of (1) benzene (2 mg/ml) and (2) naphthalene (0.2 mg/ml) using 2-ml sample loops to a 250 × 4 mm I.D. column packed with Eurochrom 10 C_{18}. Mobile phase, methanol–water (7:3). The analytical chromatogram is presented at the left side of the figure. For designations of curves see Fig. 5. Sample volumes: 20 μl (left); 2 ml (right).

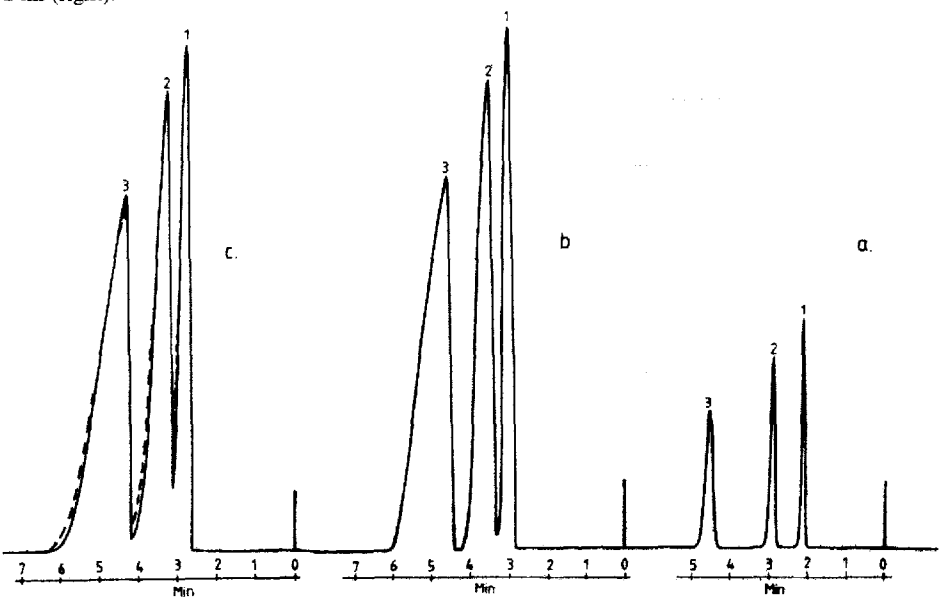

Fig. 7. Chromatograms obtained under conditions of (mass) overloading of a 250 × 4 mm I.D. column packed with Eurochrom 10 C_{18}. (a) Analytical test (UV 280 nm, 0.16 a.u.f.s.); sample 15 μl of a c/50 solution; (b) 2 ml of a c/50 solution injected with an extra pump or 40 μl of a c solution (loop 0.5-mm I.D.); (c) 2 ml of a c/50 solution injected with loop d and loop b (Fig. 1) (UV 280 nm, 1.28 a.u.f.s.). Conditions: mobile phase, methanol–water (6:4); flow-rate 2 ml/min. Separated substances: 1 = methyl, 2 = ethyl, 3 = propyl esters of 4-hydroxybenzoic acid at concentrations (c) of 0.11, 0.14 and 0.16 mg/ml.

1.5–1.65 in Figs. 5 and 6). Application of a sample loop of the shape depicted in Fig. 1d (chromatograms indicated by a dotted line) gives slightly better results (R_s = 0.82 and 1.65) than application of a loop presented in Fig. 1b (dashed line on the chromatograms presented in Figs. 4 and 6; R_s = 0.78 and 1.5).

The dependence of the degree of separation on the manner of sample introduction is still more significant under conditions of mass overloading of the column (Fig. 7). The chromatogram indicated by "b" in Fig. 7, obtained by application of an extra pump for injection of the sample, reveals complete separation of the substances, which was not achieved by application of both types of sample loop (chromatogram indicated by "c" in Fig. 7). However, also under such conditions, application of the loop shown in Fig. 1d (full line in chromatogram indicated by "c") is more advantageous than application of the loop shown in Fig. 1b (dotted line in chromatogram indicated by "c"). At the same time, chromatograms obtained by injection of 40 μl of a very concentrated solution of these substances (0.5 mm I.D. sampling loop) under the conditions given in Fig. 7 are identical with chromatogram 7b. This proves that chromatograms b and c in Fig. 7 were obtained under conditions of mass (concentration) overloading of the columns, and worse separation of the

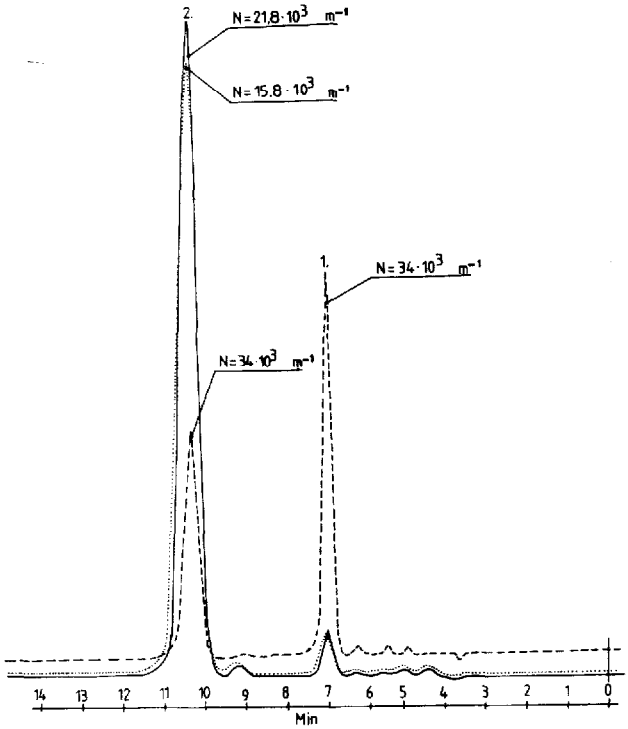

Fig. 8. Separation of 6 ml (——, ·····) and 20 μl (– – –) of a solution of (1) benzene and (2) naphthalene in a 120 × 32 mm I.D. preparative column packed with Nucleosil 7 C_{18}. Conditions: mobile phase, methanol–water (8:2); flow-rate, 19 ml/min; detection, UV 280 nm, 0.16 a.u.f.s. Curves: —— = injection with a pump, or loops b, c, d, of 0.5 mg/ml benzene and 0.2 mg/ml naphthalene; ····· = injection with loop a; – – – = analytical test of the column with 20-μl sample volume (130 mg/ml benzene and 4 mg/ml naphthalene).

substances in Fig. 7c, compared with Fig. 7b, results from a higher degree of broadening of the injected sample zone at the inlet of the column.

The chromatograms presented in Fig. 8, obtained by means of a 120 × 32 mm I.D. preparative column, indicate that it was only application of a 2-mm I.D. (6 ml) sample loop that slightly aggravated the efficiency of separation. No differences were found under such conditions between application of an additional pump and 1-mm I.D. sample loops presented in Fig. 1b, c, and d. This indicates that, under typical conditions of high flow-rates of mobile phase for preparative columns, the construction of the sample loop does not play so significant a role as during pilot research on analytical columns. The results obtained, together with the results presented in Figs. 3 and 4, indicate that the above conclusion is true, even though during the experiments presented in Fig. 8 the degree of volume overloading of the preparative column corresponding to conditions given in Fig. 5 was not achieved (owing to lack of sample loops of volume $V_i = 32^2/4^4 \times 0.5$ ml = 32 ml, fulfilling the relationship given in eqn. 1).

CONCLUSIONS

Application of an extra pump, or devices equivalent to an extra pump, *e.g.* as shown in Fig. 2, is the best way of introducing large volumes of samples into a chromatographic column, both during pilot research and during separation of substances in a preparative column.

The shape of the 1-mm I.D. sample loop presented in Fig. 1d leads to an improvement of the concentration distribution of the sample in the injection zone compared with sample loops used so far (Fig. 1b).

It is more difficult in practice to ensure a rectangular profile of the concentration distribution in the sampling zone under conditions of pilot preparative research using analytical columns than using real preparative columns of large diameters and high mobile flow-rates. This can render the application of the scale-up of LC rules difficult.

There still remains the problem of defining the derived characteristics of the pump of a preparative liquid chromatograph, so that the sample could be introduced at the suction side of the pump and be delivered to the column without significant loss of separation efficiency. Such a solution should be simple, reliable, and inexpensive simple to automate.

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