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HIGH-BACK-PRESSURE LIQUID CHROMATOGRAPHY

II. DEVELOPMENT OF A POST-COLUMN-CONTROLLED FLOW SYSTEM

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SUMMARY

A liquid chromatograph including three pumps for use in micro-high-performance liquid chromatography was examined. The flow-rate of the mobile phase was controlled by a constant-flow pump located downstream of the detector. Mobile phases were supplied from two pumps, a constant-flow and a constant-pressure pump, and mixed through a T-piece. The mixing ratio could be varied stepwise by changing the flow-rate of the forward constant-flow pump. An inlet pressure required was applied in excess by the constant-pressure pump, consisting of a gas-tight syringe and a weight. This flow system permitted both stepwise gradient elution and isocratic elution. Some typical stepwise gradient separations are demonstrated.

INTRODUCTION

In modern high-performance liquid chromatography (HPLC), solvent gradient elution is a highly promising technique that reduces the analysis time or improves the selectivity. At present, various types of chromatographs equipped with gradient system are commercially available, with which both complex ternary and binary-solvent gradient elution can easily be performed.

On the other hand, micro-HPLC lacks a gradient system at present; when gradient elution is necessary simple gradient elution techniques^{1,2} have been adopted as follows. For stepwise gradient elution, an eluent that had been prepared previously was stored in a capillary tube prior to a chromatographic run and was forwarded on to the column by feeding the last solvent from a syringe-type pump². For linear or continuous gradient elution, the eluent was stored in a capillary tube while being prepared¹. These techniques are simple but require expert manipulation.

Recently, high-back-pressure liquid chromatography has been developed³ and the employment of low-boiling solvents such as butane or propane as the mobile phase in micro-HPLC has been examined. The chromatograph was a closed system and the pressure was kept sufficiently high to exceed the vapor pressure of the now liquid mobile phase by a back-pressure pump in order to prevent vaporization of the mobile phase in the whole system. This technique was also effective for operation at

elevated temperature⁴. These advances have been possible owing to the development of a micro valve injector.

The above closed-system chromatograph was modified and a new flow system employing three pumps was developed in this work, with which both stepwise gradient elution and isocratic elution were performed *in situ*.

EXPERIMENTAL

The liquid chromatograph developed in this work included a constant-pressure pump, two constant-flow pumps, a T-piece, three-way valves (JASCO, Japan Spectroscopic Co., Tokyo, Japan), a micro valve injector (JASCO), a micro column, a UVIDEC-100 UV spectrophotometer (JASCO) and a pressure gauge (JASCO). The apparatus is illustrated in Fig. 1.

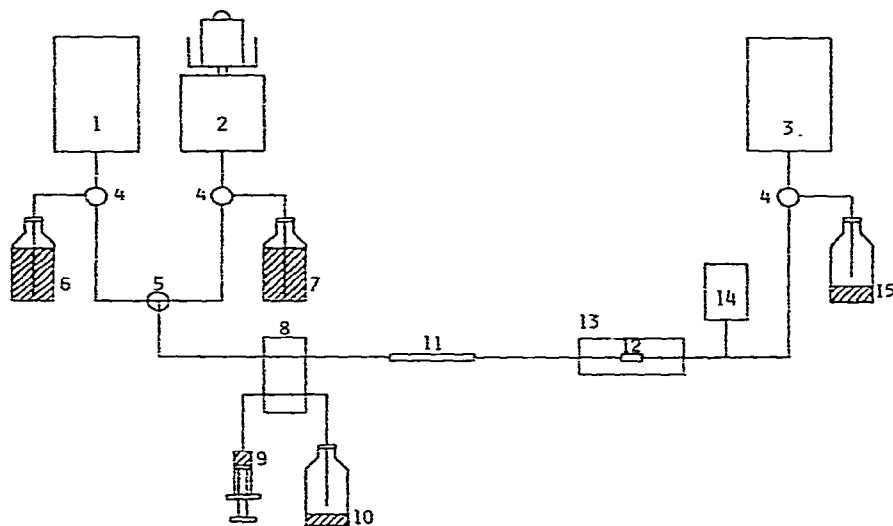


Fig. 1. Diagram of the apparatus. 1 = Constant-flow pump (Familic 100N); 2 = constant-pressure pump; 3 = constant-flow pump (Micro Feeder); 4 = three-way valve; 5 = T-piece; 6 = solvent reservoir; 7 = micro valve injector; 8 = sample; 9 = waste reservoir; 10 = column; 11 = micro flow cell; 12 = UV detector; 13 = pressure gauge; 14 = waste reservoir.

Two kinds of solutions were supplied from two pumps, a constant-pressure and a constant-flow pump, located upstream of the column and mixed through a T-piece. The flow-rates of the mobile phase were controlled by another constant-flow pump located downstream of the detector. The inlet pressure required to feed the mobile phase was applied by the constant-pressure pump, consisting of a gas-tight syringe [1710-N (100 μ l) (Hamilton, Reno, NV, U.S.A.), MS-GAN 025 (250 μ l) (Terumo Co., Tokyo, Japan) or MS-GAN 050 (500 μ l) (Terumo)] and a weight. The pressure was controlled so as to exceed the pressure drops across the separation column and capillary connecting tubing by changing the weight. The forward constant-flow pump was a Familic 100N (JASCO), altering the flow-rate stepwise instantaneously. The flow-rates depend on the dimensions of the gas-tight syringe em-

ployed. When using an MS-GAN 025 250- μl gas-tight syringe, flow-rates of 0.5–14.5 $\mu\text{l}/\text{min}$ can be selected at 0.5 $\mu\text{l}/\text{min}$ intervals. The backward constant-flow pump was a Micro Feeder (Azumadenki Kogyo Co., Tokyo, Japan) equipped with an MS-GAN 050 gas-tight syringe, generating ten different flow-rates between 0.69 and 16.7 $\mu\text{l}/\text{min}$ stepwise. The mixing ratio of two solutions was varied by changing the flow-rate of the forward constant-flow pump, with which stepwise gradient elution was performed, keeping flow-rate of the mobile phase constant.

A home-made T-piece was employed as the mixing joint, consisting of stainless-steel tubing of 0.33 mm I.D. and 0.63 mm O.D. connected by silver solder as shown in Fig. 2. Prior to silver-soldering, one of the stainless-steel tubes was filed and a hole was opened in the middle of the tube so as to fit the cross-section of the other tube. Both tubes were packed with silica gel in order to prevent the silver solder entering the tubes during soldering. The connecting volume between the separation column and the T-piece was about 18 μl .

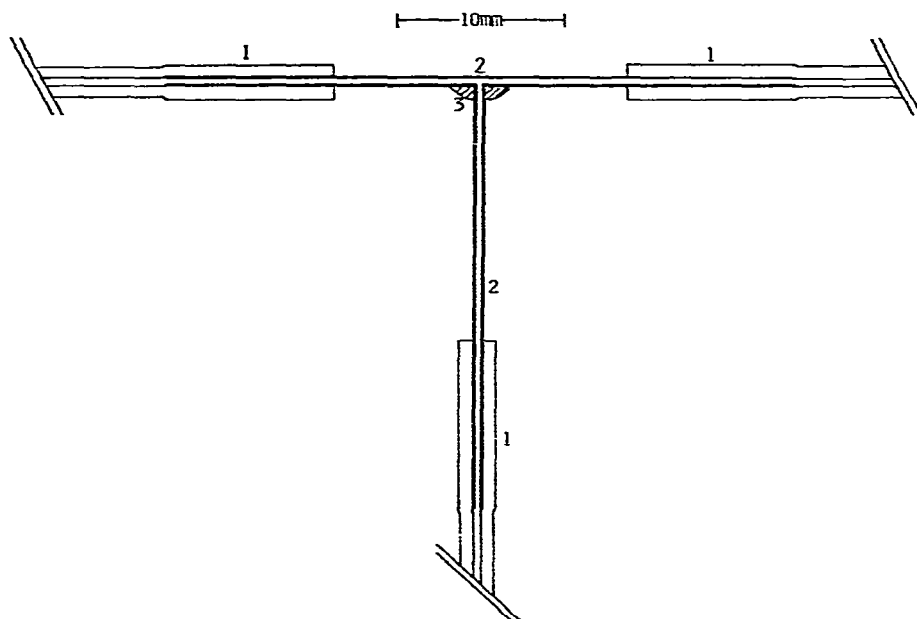


Fig. 2. Schematic diagram of T-piece. 1 = PTFE tubing (0.5 mm I.D., 2 mm O.D.); 2 = stainless-steel tubing (0.33 mm I.D., 0.63 mm O.D.); 3 = silver solder.

A small volume (0.02 μl) of sample solution was loaded by employing a micro valve injector. The reproducibility of the sample amounts injected was good, as reported previously⁵.

A fused silica tube (0.35 mm I.D.) (Gasukuro Kogyo Co., Tokyo, Japan) was used as the micro column and was packed with ODS SC-01 silica (5 μm) (JASCO).

The flow cell of the UV detector should withstand high pressures. The flow cell shown in Fig. 3 was resistant to about 40 atm. A fused silica tube of 57 μm I.D. and 0.31 mm O.D. (Scientific Glass Engineering, Victoria, Australia) was inserted in a

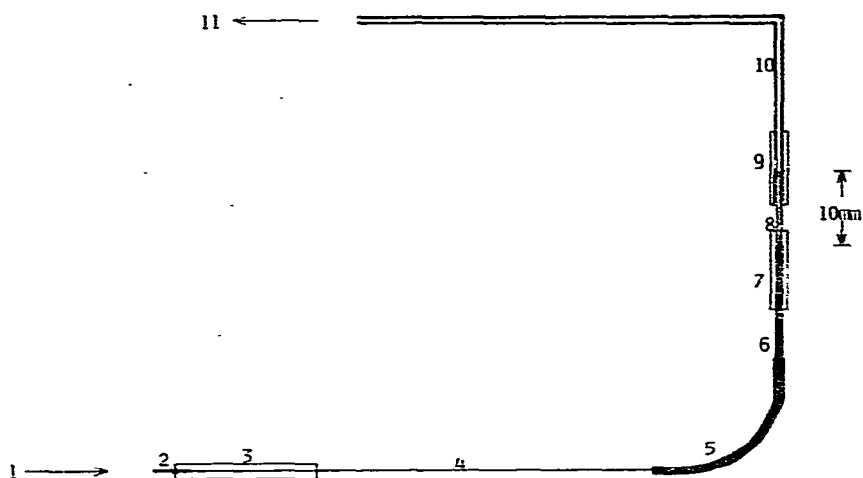


Fig. 3. Schematic diagram of the modified micro flow cell for UV detector. 1 = Eluent; 2 = stainless-steel tubing (0.13 mm I.D., 0.31 mm O.D., 4 mm long); 3 = PTFE tubing (0.2 mm I.D., 2 mm O.D.); 4 = fused-silica tubing (57 μ m I.D., 0.31 mm O.D., 9 cm long); 5 = stainless-steel tubing (0.41 mm I.D., 0.71 mm O.D., ca. 2.5 cm long); 6 = stainless-steel tubing (0.33 mm I.D., 0.63 mm O.D., ca. 1.5 cm long); 7 = PTFE tubing (0.5 mm I.D., 2 mm O.D.); 8 = quartz tubing (0.3 mm I.D., 0.6 mm O.D.); 9 = PTFE tubing (1 mm I.D., 2 mm O.D.); 10 = PTFE tubing (0.5 mm I.D., 1 mm O.D.); 11 = to downstream constant-flow pump.

stainless-steel tube of 0.33 mm I.D. and 0.63 mm O.D. with adhesive. The slit was 0.3 mm wide and 1.5 mm long. The detection volume and dead volume between the column and the flow cell were 0.1 and 0.6 μ l, respectively.

RESULTS AND DISCUSSION

The pressure attained by the constant-pressure pump depended on both the weight and the dimensions of the gas-tight syringe employed, as shown in Fig. 4. The smaller the inner volume of the gas-tight syringe, the larger was the pressure attained with constant weight. Nearly linear relationships between weight and pressure attained were observed. Each pressure attained was about 80% of the calculated value, owing to the friction between the plunger and the inner wall of the gas-tight syringe, as shown in Table I. In addition, the gas-tight syringes employed withstood about 70 atm pressure.

The flow-rate of the mobile phase could be controlled by the downstream constant-flow pump if a pressure exceeding the pressure drop across the system, including the column and capillary connecting tubing, was applied by the constant-pressure pump. Otherwise, bubbles were generated in the detector. It was desirable to keep the pressure of the downstream constant-flow pump at 10–30 atm, nearly the same as that of the detector. On the other hand, if leakage of the mobile phase occurred at the connections between the column and the downstream constant-flow pump, the mobile phase composition changed, leading to variations in the retention time of the solute.

It should be noted that mixing of two solutions in a T-piece plays a significant

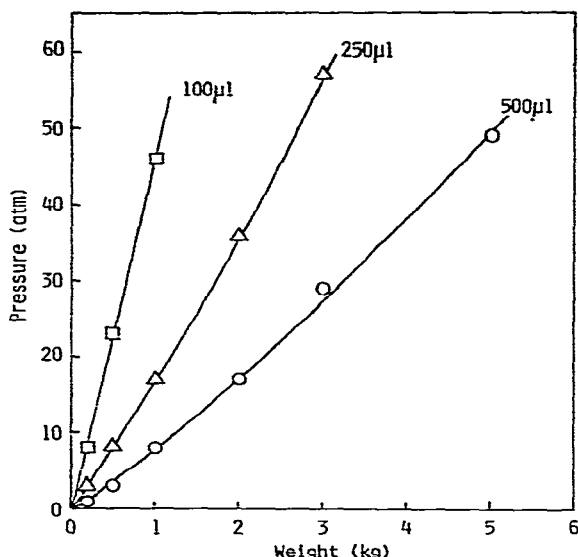


Fig. 4. Relationship between weight and attained pressure.

TABLE I
COMPARISON OF ATTAINED PRESSURES AND CALCULATED VALUES

Weight (kg)	Pressure (atm)					
	1710-N (100 μ l, 0.0167 cm^2)		MS-GAN 025 (250 μ l, 0.0417 cm^2)		MS-GAN 050 (500 μ l, 0.0833 cm^2)	
	Observed	Calculated	Observed	Calculated	Observed	Calculated
0.2	8	12	3	4.8	1	2.4
0.5	23	30	8	12	3	6
1	46	60	17	24	8	12
2			36	48	17	24
3			57	72	29	36
5					49	60

role in this new flow system. Fig. 5 illustrates baselines for stepwise gradient elution using the MS-GAN 025 as the constant-flow pump. Water and acetonitrile-water (1:1) were supplied from the constant-flow and the constant-pressure pump, respectively. The mixing direction is also indicated in Fig. 5. The upper trace was obtained by employing a T-piece packed with Develosil ODS (15–30 μ m) (Nomura Chemical Co., Seto-shi, Japan) in order to perform the mixing effectively. The noise of the baseline is slightly reduced compared with the lower trace, obtained by employing an unmodified T-piece. In both instances stable baselines were observed when the flow-rate of the constant-pressure pump (V_{cp}) was dominant. However, the drift or the noise of the baselines was noticeable when the flow-rate of the constant-flow pump

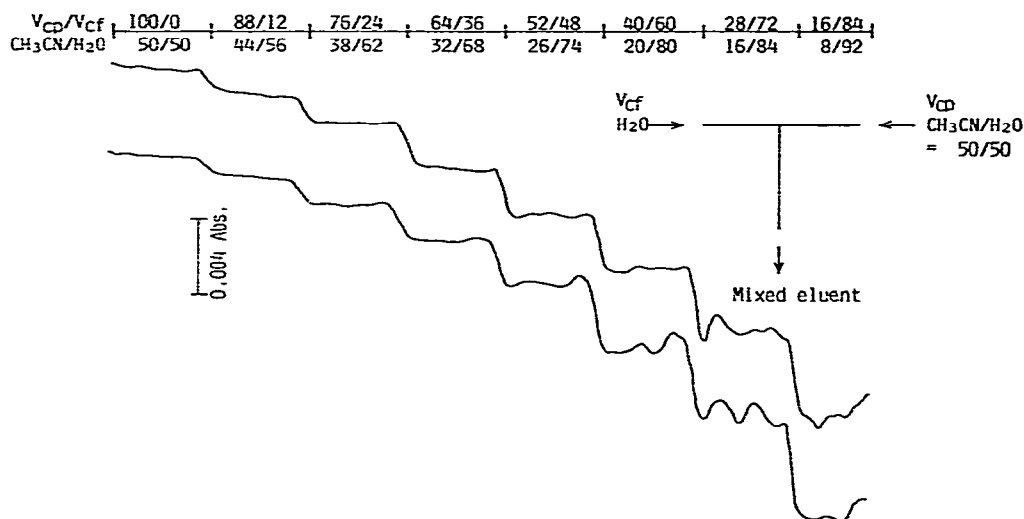


Fig. 5. Baselines for stepwise gradient elutions. Upper trace: obtained with a T-piece packed with Develosil ODS (15–30 μm). Lower trace: obtained with an unmodified T-piece. Wavelength of UV detection: 240 nm. V_{cp} : flow-rate of water with the constant-pressure pump. V_{cf} : flow-rate of acetonitrile-water (1:1) with the constant-flow pump.

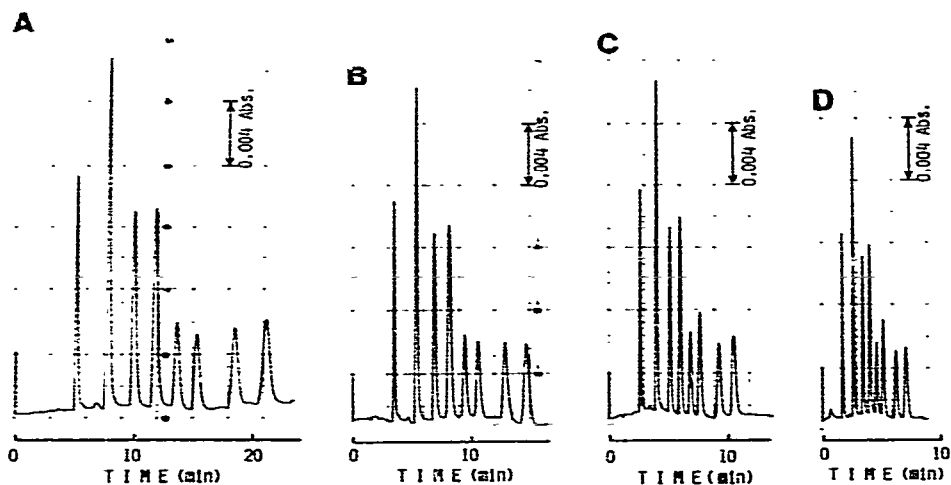


Fig. 6. Isocratic separations of aromatic hydrocarbons at various flow-rates. Column: 10.1 cm \times 0.35 mm I.D., packed with SC-01. Mobile phase: acetonitrile–water (64:36). Flow-rates: (A) 2.8 $\mu\text{l}/\text{min}$, $V_{cp} = 1.8 \mu\text{l}/\text{min}$ (acetonitrile) and $V_{cf} = 1.0 \mu\text{l}/\text{min}$ (water); (B) 4.2 $\mu\text{l}/\text{min}$, $V_{cp} = 2.7 \mu\text{l}/\text{min}$ and $V_{cf} = 1.5 \mu\text{l}/\text{min}$; (C) 5.6 $\mu\text{l}/\text{min}$, $V_{cp} = 3.6 \mu\text{l}/\text{min}$ and $V_{cf} = 2.0 \mu\text{l}/\text{min}$, and (D) 8.3 $\mu\text{l}/\text{min}$, $V_{cp} = 5.3 \mu\text{l}/\text{min}$ and $V_{cf} = 3.0 \mu\text{l}/\text{min}$. Samples: 1.8% (w/w) of benzene, 0.20% of naphthalene, 0.038% of biphenyl, 0.040% of fluorene, 0.0098% of phenanthrene, 0.0078% of anthracene, 0.040% of fluoranthene and 0.040% of pyrene, eluted in that order. Sample size: 0.02 μl . Wavelength of UV detection: 254 nm.

(V_{cf}) was dominant, which may be attributed to the pulsation of the constant-pressure pump. The baseline obtained with the 1710-N as the constant-flow pump was wavy owing to the pulsation of the constant-flow pump even when V_{cp} was larger than V_{cf} .

Fig. 6 demonstrates isocratic separations of aromatic hydrocarbons at various flow-rates. The total flow-rate of the mobile phase was varied by the downstream constant-flow pump. The flow-rate of the upstream constant-flow pump should be changed so as to keep the mobile phase composition constant. The baselines and peak shapes of the chromatograms were as good as those obtained with the one-pump system, indicating that the two solutions were mixed well. The reproducibility of the retention volume of each solute was good; the relative standard deviation of the retention volume of each solute for five measurements was 0.6–0.8%.

Column efficiencies obtained with this flow system are shown in Fig. 7. The dependence of the height equivalent to a theoretical plate (HETP) on the linear velocity of the mobile phase is small, which is characteristic of a fused silica flexible micro column⁶. The operating conditions are the same as those in Fig. 6.

Fig. 8 shows isocratic and stepwise gradient separations of polynuclear aromatic hydrocarbons on an ODS column. In the latter instance the analysis time is reduced and the resolution of 1,3,5-triphenylbenzene and 3,4-benzopyrene is increased. Gradient elution was attained by changing the flow-rate of the upstream constant-flow pump containing water.

Stepwise gradient separations of phthalates and typical components of a cold medicine are shown in Figs. 9 and 10, respectively. The relative standard deviation of the retention of phthalates for ten measurements (Fig. 9) was a few percent. In both

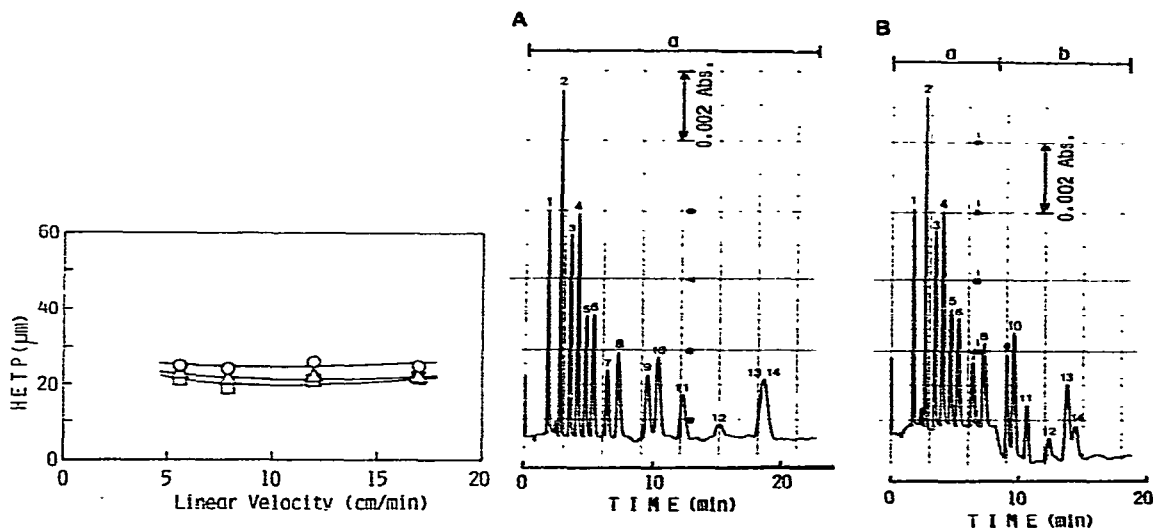


Fig. 7. Relationship between linear velocity and HETP. Operating conditions as in Fig. 6. Samples: ○, naphthalene; △, anthracene; and □, pyrene.

Fig. 8. Isocratic and stepwise gradient separations of polynuclear aromatic hydrocarbons. Column: 10.1 cm \times 0.35 mm I.D., packed with SC-01. Mobile phases: (A) acetonitrile-water (64:36) (a); (B) acetonitrile-water [64:36 (a) + 70:30 (b)]. Flow-rate: 8.3 μ l/min. Samples: 1 = 1.0% (w,w) of benzene; 2 = 0.097% of naphthalene; 3 = 0.019% of biphenyl; 4 = 0.022% of fluorene; 5 = 0.0076% of phenanthrene; 6 = 0.0058% of anthracene; 7 = 0.017% of fluoranthene; 8 = 0.020% of pyrene; 9 = 0.020% of *p*-terphenyl; 10 = 0.0074% of chrysene; 11 = 0.0061% of 9-phenylanthracene; 12 = 0.0025% of perylene; 13 = 0.011% of 1,3,5-triphenylbenzene; 14 = 0.0058% of 3,4-benzopyrene. Sample size: 0.02 μ l. Wavelength of UV detection: 254 nm. Temperature: 32°C.

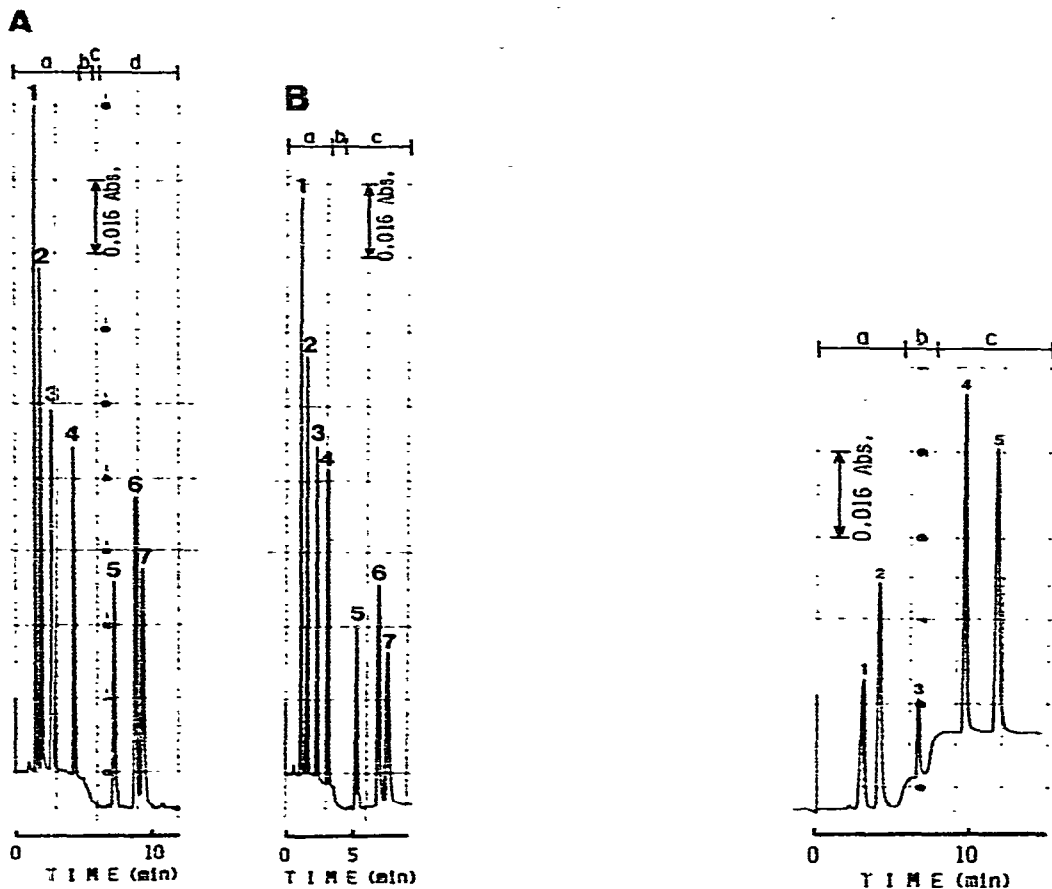


Fig. 9. Stepwise gradient separations of phthalates. Column: 10.3 cm \times 0.35 mm I.D., packed with SC-01. Mobile phases: (A) acetonitrile-water [64:36 (a) + 73:27 (b) + 82:18 (c) + 91:9 (d)]; (B) acetonitrile-water [64:36 (a) + 76:24 (b) + 88:12 (c)]. Flow-rates: (A) 5.6 μ l/min; (B) 9.3 μ l/min. Samples: 1 = 0.81% (w/w) of dimethyl; 2 = 0.76% of diethyl; 3 = 0.74% of diisopropyl; 4 = 0.78% of di-*n*-butyl; 5 = 1.09% of diheptyl; 6 = 1.38% of di-2-ethylhexyl; 7 = 1.46% of dinonyl phthalate. Sample size: 0.02 μ l. Wavelength of UV detection: 235 nm. Temperature: 29°C.

Fig. 10. Stepwise gradient separation of typical components in a cold medicine. Column: 10.1 cm \times 0.35 mm I.D., packed with SC-01. Mobile phases: acetonitrile-water [7.5:92.5 (a) + 15:85 (b) + 30:70 (c)], each containing 0.05% (w/v) of ammonium carbonate. Flow-rate: 4.2 μ l/min. Samples: 1 = 0.15% (w/v) of barbital; 2 = 0.14% of acetoaminophenol; 3 = 0.14% of caffeine; 4 = 0.14% of phenacetin; 5 = 0.13% of *p*-chloroacetanilide. Sample size: 0.02 μ l. Wavelength of UV detection: 240 nm. Temperature: 26°C.

instance a pressure of *ca.* 50 atm was applied by the constant-pressure pump. Stepwise gradient elutions are satisfactorily carried out with this new flow system as shown in Fig. 8B.

CONCLUSION

The new flow system employing three pumps permitted both stepwise gradient

elution and isocratic elution *in situ*, and it should be possible to perform continuous or linear gradient elution if a constant-flow pump altering the flow-rate continuously is developed for micro-HPLC.

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