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CONTINUOUS GRADIENT ELUTION IN MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A continuous gradient elution method for micro high-performance liquid chromatography was developed. Gradient eluent was supplied from a mixing vessel, into which another solution was continuously fed by a micro syringe-type pump. The gradient profiles observed agreed with calculated plots. Some typical continuous gradient separations are described.

INTRODUCTION

Solvent gradient elution is the most popular mode of gradient elution in high-performance liquid chromatography (HPLC). It is a highly promising technique that reduces the analysis time or improves the selectivity. A binary or ternary solvent gradient is commonly employed in computerized HPLC.

Micro HPLC(MHPLC) has attracted interest in the 6 years since it was first reported[1,2]. However, at present, a gradient system for MHPLC is not commercially available. When gradient elution was necessary in MHPLC, a continuous or stepwise gradient of eluent was stored in a capillary tubing prior to a chromatographic run and was forwarded to the column by means of a syringe-type pump[2,3]. It is somewhat difficult to maintain exact gradient profiles with this technique, owing to the diffusion of the eluent in the capillary.

Recently, we developed a post-column controlled flow system using two constant-flow and one constant-pressure pumps, which enabled stepwise gradient elution *in situ*[4]. A gradient was achieved by changing the ratio of the flow-rates of a constant-flow and a constant-pressure pump. In the present study a continuous gradient elution method for MHPLC using a single pump and a simple mixing chamber was developed and applied to some typical separations.

The simple one-chamber gradient device for HPLC has already been used by many workers[5–7]. In its simplest form it produces only an exponential gradient in which the early portion approaches a linear gradient. Let us consider that a solution enters into the mixing vessel and the solution in the mixing vessel in steadily stirred, as shown in Fig. 1. The solution which enters the vessel is mixed instantly with the

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original solution in the vessel and must displace an equal volume of liquid from the vessel. If the incoming solution differs from the original solution in composition, the composition of the outgoing solution varies exponentially.

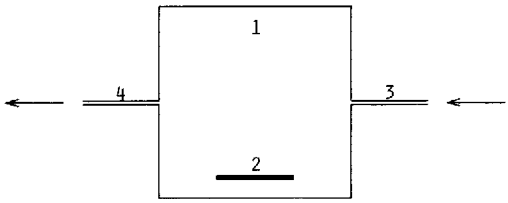

Fig. 1. Flow diagram for continuous gradient elution. 1 = Mixing vessel; 2 = stirring rod; 3 = inlet; 4 = oulet.

If we assume that changes in volume can be neglected when different solutions are mixed, the gradient solution flows out at the same rate as the inlet flow-rate. Then the variation of the concentration of the outgoing solution with time is expressed as

$$x = a - (a - x_0) \exp\left(-\frac{v}{V_0} \cdot t\right) \tag{1}$$

where a is the concentration of the incoming solution, x_0 is the initial concentration of the solution in the mixing vessel, v is the flow-rate, V_0 is the volume of the mixing vessel and t is the time, respectively. Eqn. 1 shows that the ratio of the flow-rate to the volume of the mixing vessel determines the gradient profile. Since flow-rates employed in MHPLC are 1–10 μl/min, most exponential gradient profiles can be attained by means of mixing vessels with inner volumes of 7–2000 μl.

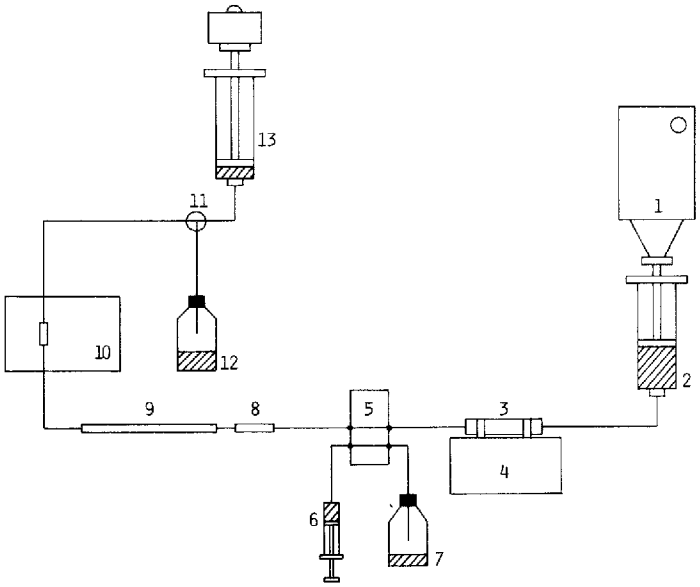

Fig. 2. Apparatus for continuous gradient elution. 1 = Micro feeder; 2 = gas-tight syringe; 3 = mixing vessel; 4 = vibrator; 5 = injector; 6 = sample; 7 = waste reservoir; 8 = pre-column; 9 = separation column; 10 = UV detector; 11 = three-way valve; 12 = waste reservoir; 13 = back-pressure pump.

EXPERIMENTAL

The apparatus employed in this work is illustrated in Fig. 2. The liquid chromatograph was assembled from a Micro Feeder (Azumadenki Kogyo, Tokyo, Japan) equipped with a gas-tight syringe as a pump, a home-made mixing vessel, vibrator, a micro valve injector (0.02 μl; Jasco, Japan Spectroscopic, Tokyo, Japan), a pre-column, a separation column, a UV spectrophotometer UVIDEC-100 (Jasco) equipped with a home-made micro flow cell and a back-pressure pump.

The mixing vessel comprised a stainless-steel or a modified gas-tight syringe without plunger. The latter type is illustrated in Fig. 3. A gas-tight syringe was modified and both ends of the syringe were mounted with needles. A stainless-steel stirring rod (0.63 mm O.D.) was placed in the mixing vessel in order to stir the solution effectively by vibration. It was easy to produce this syringe type of mixing vessel with various volumes, *e.g.*, 40–2000 μl. Connections between the mixing vessel and the injector were formed from stainless-steel capillary tubings (10 cm × 0.13 mm I.D. and 6 cm × 0.17 mm I.D.). The former tubing was coiled to absorb the vibration.

The pre-column comprised a PTFE tubing packed with Develosil ODS 15/30 (15–30 μm; Nomura Chemical, Seto, Japan) and the separation column was composed of fused silica tubing (Gasukuro Kogyo, Tokyo, Japan), packed with silica ODS SC-01 (5 μm, Jasco). Fused silica tubing was selected as the column material since it was found to exhibit high column efficiencies in previous studies[8,9].

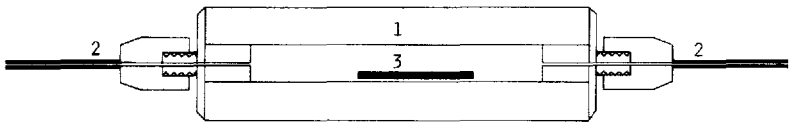

Fig. 3. Schematic diagram of a syringe-type mixing vessel. 1 = Gas-tight syringe; 2 = needle; 3 = stainless-steel rod, *ca.* 10 × 0.63 mm O.D.

Reagents were purchased from Wako (Osaka, Japan) and Tokyo Chemical Industry (Tokyo, Japan).

RESULTS AND DISCUSSION

Mixing vessels with inner volumes of around 40, 100 and 400 μl were made and gradient separations of typical standard samples were examined by using these vessels.

Fig. 4 shows gradient profiles obtained by using a 109-μl mixing vessel. An acetonitrile solution containing 0.3% (v/v) of benzene was the initial solution and pure acetonitrile was supplied from a pump at various flow-rates. Gradient profiles could be observed by measuring the UV absorbance of the outgoing solution. The open symbols in Fig. 4 represent calculated points. The good agreement between the calculated and found values is illustrated.

The reproducibility of the retention volume of solutes was examined by using a 39-μl mixing vessel and phthalates as test solutes. Fig. 5 shows the gradient separation achieved, the relative standard deviation of the retention volume of each solute being around 1%, less than that obtained by stepwise gradient elution[4].

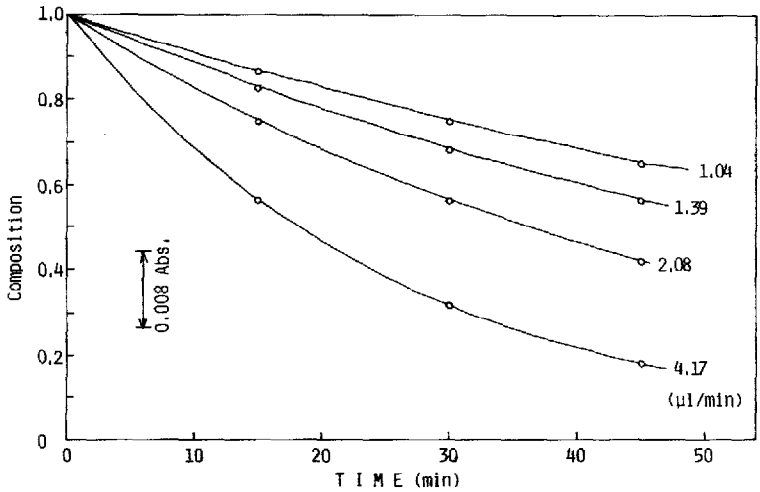

Fig. 4. Gradient profiles obtained by continuous gradient elution. Volume of mixing vessel: 109 µl. Initial solution: acetonitrile containing 0.3% (v/v) benzene. Incoming solution: pure acetonitrile. Wavelength of UV detection: 254 nm. Open symbols represent calculated points.

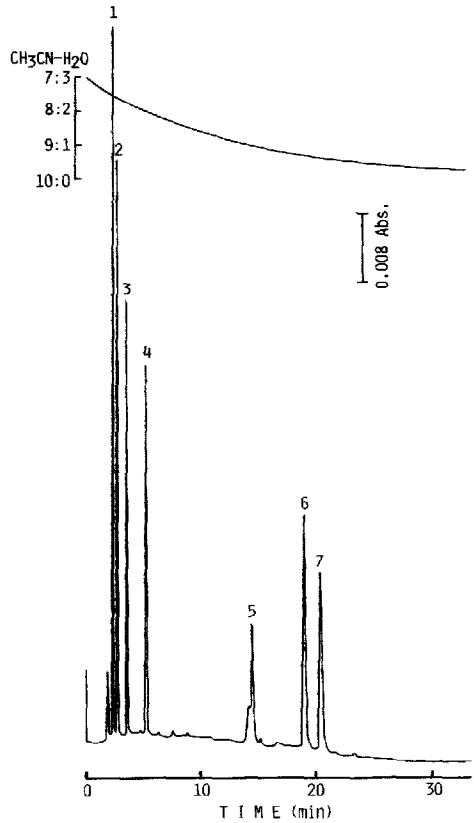

Fig. 5. Gradient separation of phthalates. Column: Silica ODS SC-01, 10 cm × 0.26 mm I.D. Mobile phase: acetonitrile–water. Flow-rate: 2.78 µl/min. Volume of mixing vessel: 39 µl. Sample: 1 = Dimethyl phthalate; 2 = diethyl phthalate; 3 = diisopropyl phthalate; 4 = di-*n*-butyl phthalate; 5 = diheptyl phthalate; 6 = di-2-ethylhexyl phthalate; 7 = dinonyl phthalate. Injection volume: 0.02 µl. Wavelength of UV detection: 235 nm.

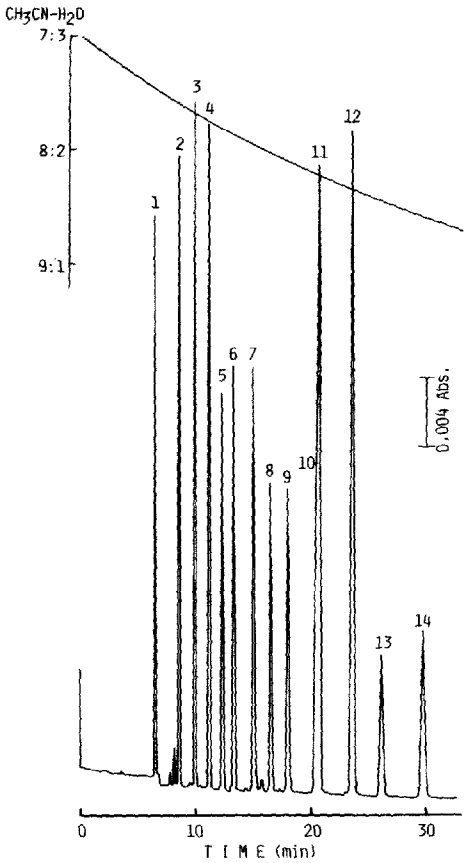

Fig. 6. Gradient separation of polynuclear aromatic hydrocarbons. Column: Silica ODS SC-01, 20 cm × 0.26 mm I.D. Mobile phase: acetonitrile–water. Flow-rate: 2.78 μl/min. Volume of mixing vessel: 109 μl. Sample: 1 = benzene; 2 = naphthalene; 3 = biphenyl; 4 = fluorene; 5 = phenanthrene; 6 = anthracene; 7 = fluoranthene; 8 = pyrene; 9 = *p*-terphenyl; 10 = chrysene; 11 = 9-phenylanthracene; 12 = 1,3,5-triphenylbenzene; 13 = perylene; 14 = 3,4-benzopyrene. Injection volume: 0.02 μl. Wavelength of UV detection: 254 nm.

This continuous gradient elution method was applied to some other separations. The separation of polynuclear aromatic hydrocarbons is demonstrated in Fig. 6. High resolution is achieved although chrysene and 9-phenylanthracene are not resolved. Fig. 7 shows the gradient separation of phenols.

Separations of organic constituents of water were then examined. Since they are commonly present at low levels in water, these constituents should be concentrated prior to the analysis. The off-line pre-column concentration technique was adopted, as presented previously[10]. Water samples were passed through a pre-column packed with Develosil ODS 15/30 and then the pre-column was connected on the top of the separation column.

The analysis of organic constituents of deionized water is shown in Fig. 8. The retention volume of the last large peak coincided with that of di-*n*-butyl phthalate. Fig. 9 demonstrates the analysis of distilled water extracts of powdered coal. A lot of

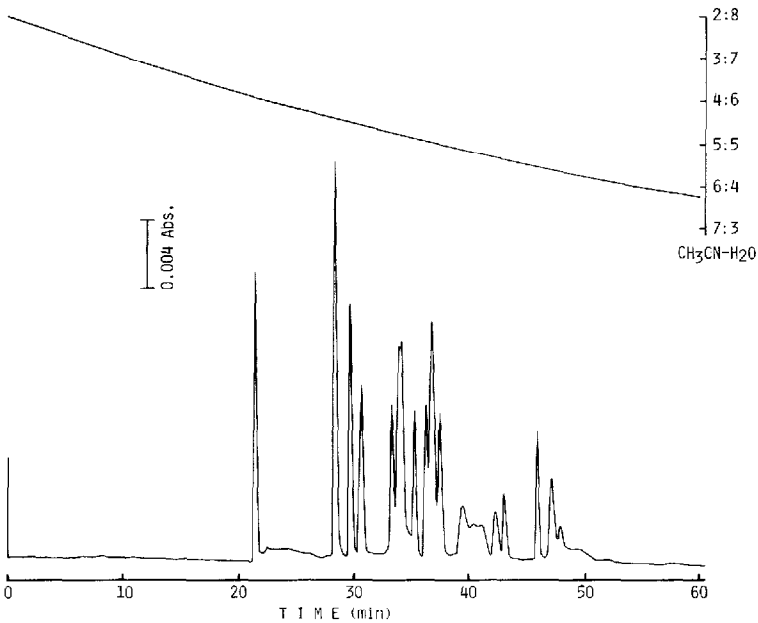

Fig. 7. Gradient separation of phenols. Column: Silica ODS SC-01, 20 cm × 0.26 mm I.D. Mobile phase: acetonitrile–water. Flow-rate: 1.39 μl/min. Sample: mixture of 27 phenols (phenol, chlorophenols, polychlorophenols, methylphenols and dimethylphenols). Injection volume: 0.02 μl. Wavelength of UV detection: 270 nm.

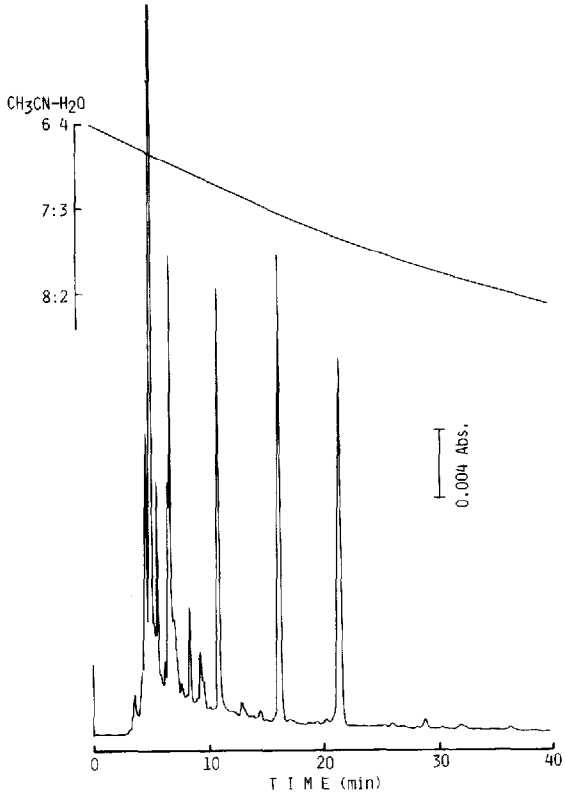

Fig. 8. Analysis of constituents of deionized water. Column: Silica ODS SC-01, 20 cm × 0.26 mm I.D. Mobile phase: acetonitrile–water. Pre-column: Develosil ODS 15/30, 10 × 0.2 mm I.D. Flow-rate: 2.08 μl/min. Volume of mixing vessel: 109 μl. Sample: 3 ml of deionized water. Wavelength of UV detection: 235 nm.

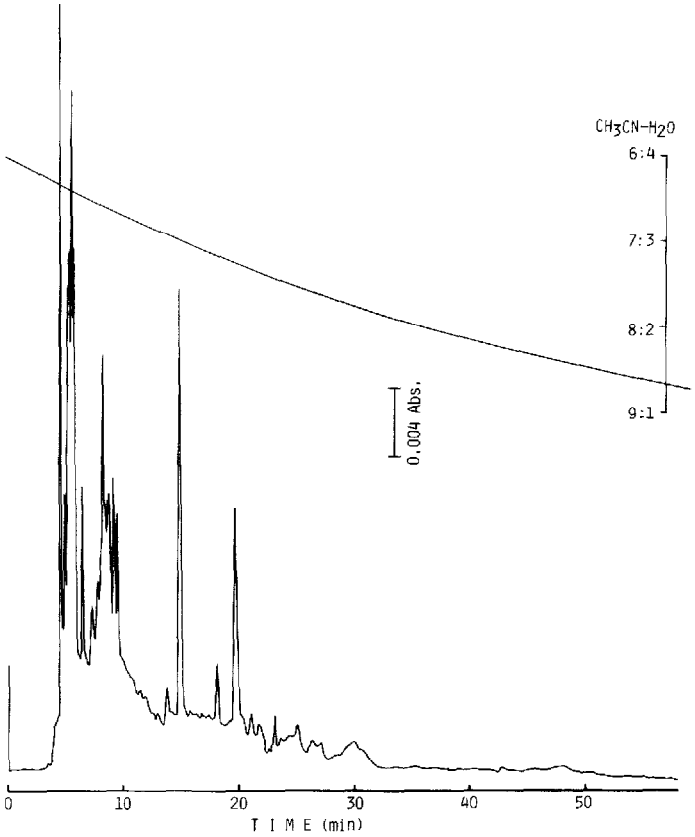

Fig. 9. Analysis of water extracts from coal. Operating conditions as in Fig. 8 except the sample. Sample: 2.5 ml of water in contact with powdered coal.

peaks appear in both Figs. 8 and 9, and a more complete resolution would be attained on a high-resolution column.

CONCLUSIONS

The continuous gradient elution developed was effective for the separation of components covering a wide range of polarity. The observed gradient profiles agreed with those calculated. This technique will be useful for gradient elution in MHPLC.

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