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MICROBORE PACKED-COLUMN ANION CHROMATOGRAPHY USING A UV DETECTOR

SOUJI ROKUSHIKA*, ZONG YIN QIU, ZHUO LIAN SUN* and HIROYUKI HATANO
Department of Chemistry, Faculty of Science, Kyoto University, Sakyo-ku, Kyoto 606 (Japan)
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SUMMARY

A micro packed-column anion chromatograph with a UV detector has been constructed and the applicability of the system is demonstrated. A flexible fused-silica capillary (0.19 mm I.D.) was used as a chromatographic tube and a surface-agglomerated and bonded low ion-exchange capacity anion-exchange resin, developed for ion chromatography, was packed into an ion-exchange column. Separations of inorganic ions, nucleobases, nucleotides and organic acids were achieved using this system.

INTRODUCTION

Ion-exchange chromatography has been widely used as a potential separation technique for various compounds which are ionizable or which have the ability to interact with the matrix of the ion exchanger, such as amino acids, nucleic acid related compounds and inorganic ions.

To obtain good resolution, various packing materials (small-diameter totally porous ion-exchange gels, pelicular or chemically bonded silica gels) have been developed as high-performance liquid chromatography (HPLC) packings. However, silica gel-based ion exchangers, especially pellicular-type packings, have not always worked satisfactorily owing to their instability in extreme pH media and consequent poor reproducibility of results.

Recently a surface-agglomerated anion-exchange resin of low ion-exchange capacity composing a latex layer of a strong anion-exchange resin and a surface-sulphonated polymer-bead core, has been introduced by Small *et al.*[1] as a packing material for ion chromatography, a relatively novel technique for the analysis of ionizable inorganic and organic compounds. Hanaoka *et al.*[2] developed a stable surface-agglomerated and bonded anion-exchange resin for the same purpose. Recently micro packed-column liquid chromatography has been developed[3,4]. Micro packed-column ion chromatography with a micro hollow-fibre suppressor system has been

* Present address: Analytical Research Center, Department of Chemical Engineering, Dalian Institute of Technology, Dalian, Liaoning, China.

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reported previously[5]. In this paper the applicability of the micro packed anion-exchange column to the chromatographic separation of various compounds using a UV detector is described.

MATERIALS AND METHODS

The instrument consisted of a Milton Roy minipump (LDC, Riviera Beach, FL, U.S.A.), a JASCO UVIDEC 100-II UV detector, a Model ML 422 micro loop injector, the sample volume of which was fixed at 0.05 μl, and a pulse damper fitted with a pressure gauge (Japan Spectroscopic, Tokyo, Japan). The eluent flow was split before the sample injector to obtain a low flow-rate using an ordinary pump[6]. The major part of the eluent was returned to the eluent reservoir through a 1.6-mm O.D. stainless-steel resister tube (used to control the flow-rate of the eluent) into which a narrow-bore fused-silica tubing (10 cm × 50 μm I.D.) was inserted coaxially, the space between the tubes being filled with an epoxy resin. The flow-rate of the eluent was controlled by setting the flow-rate of the eluent pump to produce an appropriate pressure. The splitting ratio could be changed by changing the size of the resister tube. A flow diagram of the chromatograph is shown in Fig. 1.

The slurry of the surface-agglomerated and bonded strong anion-exchange resin YEW AX-1 (particle diameter 10 μm, ion-exchange capacity 25 μequiv./g; Yokogawa-Hokushin Electric, Tokyo) was packed into a fused-silica capillary (0.19 mm I.D.) under pressure. The pressure was increased gradually from 10 kg/cm^2 to *ca.* 250 kg/cm^2 using a JASCO A 700 pump in the constant-pressure mode.

Fused-silica tubings were obtained from Scientific Glass Engineering (North Melbourne, Australia). All chemicals were purchased from Nakarai Chemicals (Kyoto, Japan).

A schematic diagram of a micro packed column and flow cell is shown in Fig. 2. A porous PTFE frit was introduced at the end of the fused-silica tubing. A part of the end of the surface polyimide coating of the fused-silica tubing was burnt out to prepare the micro flow cell which was connected to the end of the column with short narrow-bore fused-silica tubing. The outside of the flow cell was covered by a

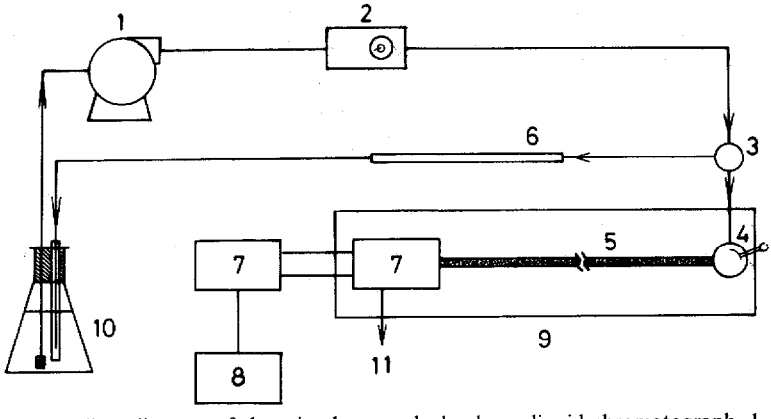


Fig. 1. Flow diagram of the microbore packed-column liquid chromatograph. 1 = Eluent pump; 2 = pressure gauge and dumper; 3 = splitter "T"; 4 = micro injector; 5 = microbore packed column; 6 = resister tube; 7 = UV detector; 8 = recorder; 9 = thermostatted oven; 10 = eluent reservoir; 11 = drain.

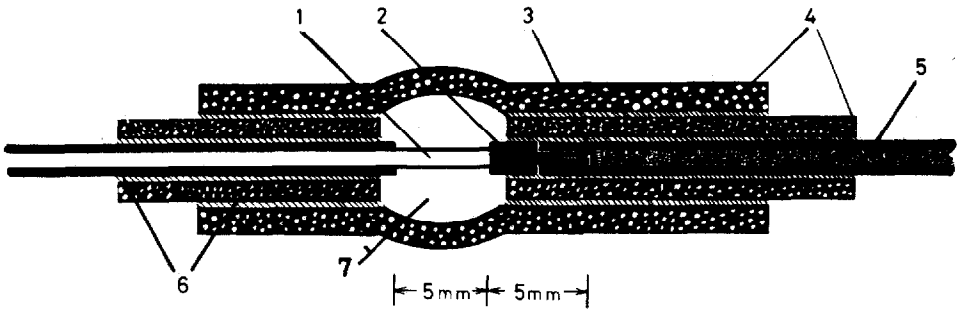


Fig. 2. Schematic diagram of a microbore packed column and UV flow cell. 1 = Flow-cell; 2 = fused-silica tube connector; 3 = porous PTFE frit; 4 = stainless-steel tube; 5 = microbore packed column; 6 = epoxy resin; 7 = cell window.

stainless-steel tube protector which had windows for the light path and was glued using epoxy resin. A slit, width *ca.* 0.1 mm, composed of two thin stainless-steel razor blades, was placed behind the cylindrical micro flow-cell.

To maintain a constant temperature during chromatography, a temperature-controlled oven was built into which were placed all parts after the eluent splitter, including the micro injector, the micro packed column and the micro flow-cell with the optical unit of the UVIDEC 100 II UV detector. This detector was composed of two parts, a control unit and an optical unit, each unit being built into the case separately and connected to each other with wires. The optical unit was relatively temperature-stable and worked well in the oven from ambient temperature to 45°C.

RESULTS AND DISCUSSION

Ion chromatography provides a sensitive and rapid method for the separation of inorganic and organic ions. Ion chromatography is presently an almost unparalleled method, especially for the analysis of inorganic anions such as halogens, nitrites, nitrates, phosphates, and sulphate ions in dilute aqueous solution using a conductivity detector.

A UV detector can also be used for the analysis of various inorganic ions[7]. Nitrite, nitrate and bromide ions absorb UV light at around 200 nm and hence these ions can respond to the UV detector[8–10]. Fig. 3 shows chromatograms of a mixture of these three inorganic anions and a much greater amount of sulphonate the amount of which was 33–70 times greater than for the other ions. Three wavelengths were used for the detection of these compounds. At both 195 and 210 nm, three positive peaks and a negative SO_4^- peak were obtained. The intensity of each peak and the relative intensity varied with the wavelength according to their absorption spectrum. The peak intensity of the Br^- ion decreased with increasing wavelength, and at 230 nm Br^- did not respond to the detector. NO_3^- showed almost the same peak height at both 195 and 210 nm and NO_2^- gave a maximum intensity at 210 nm. The largest negative peak of SO_4^{2-} was observed at a shorter wavelength; however, considering the amount of sample, the sensitivity was much lower than those of other three anions. The negative peak of the non-UV-absorbing compound may be explained by the change in the refractive index of the eluate rather than the eluent "matrix effect"

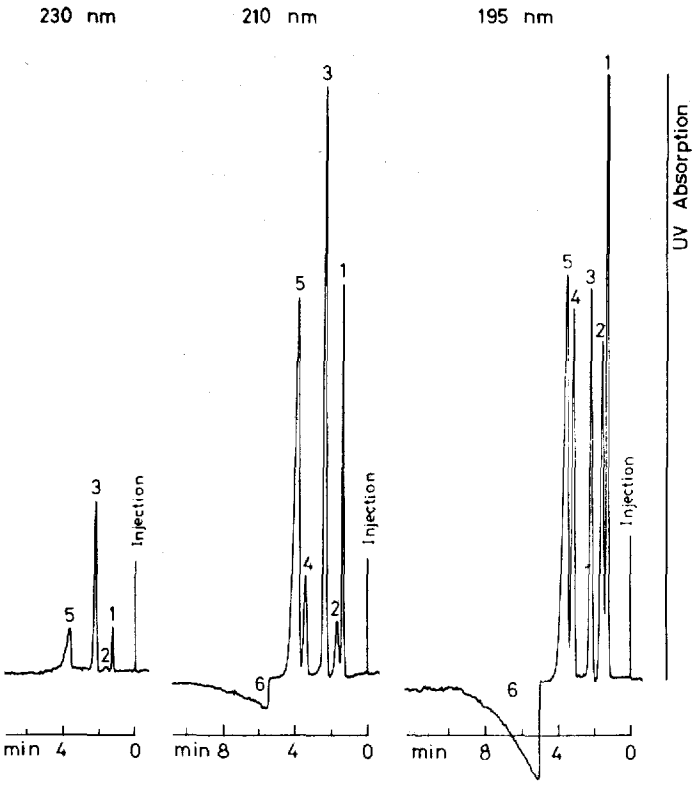


Fig. 3. Comparison of chromatograms of inorganic anions detected at three different wavelengths. Column, YEW AX-1 anion exchange resin 28 cm × 0.19 mm I.D.; eluent, 4 m*M* sodium hydrogen carbonate and 4 m*M* sodium carbonate; temperature, 40°C; pressure, 38 kg/cm^2; flow-rate, 4 μl/min; sample volume, 0.05 μl. Peaks: 1, 2 = impurity; 3 = NO_2^- (75 ppm); 4 = Br^- (50 ppm); 5 = NO_3^- (105 ppm); 6 = SO_4^{2-} (3480 ppm).

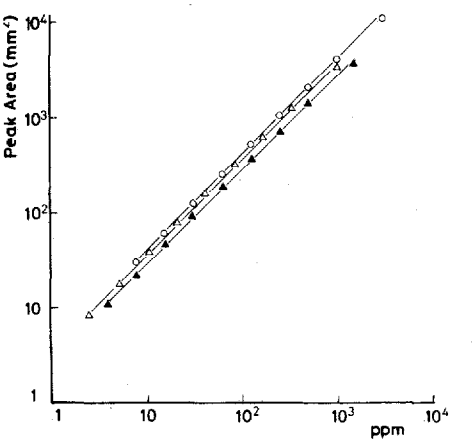


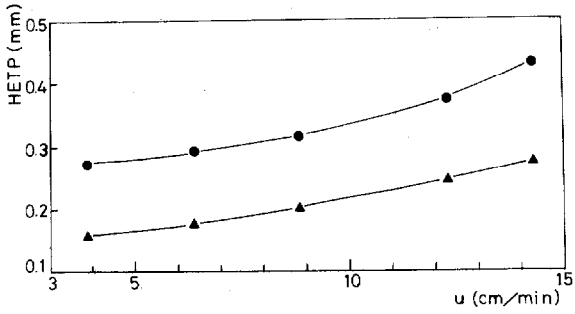
Fig. 4. Calibration curve for inorganic anions. Wavelength, 195 nm. Other conditions as in Fig. 3. Curves: NO_3^- (○), Br^- (△), NO_2^- (▲).

usually observed in ion chromatography which is caused by an ion-exchange equilibrium in the column[11]. When the solute band of eluted SO_4^{2-} arrives at the flow-cell, the UV light path should be deflected to alter the intensity of the light arriving at the sensor owing to the change in refractive index of the eluate. A pseudo-peak is then recorded in the chromatogram. At 230 nm, two small peaks for NO_2^- and NO_3^- were observed. These results imply the possibility of selectively detecting a minor UV-absorbing compound co-eluting with a major compound which cannot absorb UV light.

Calibration curves for inorganic anions were constructed by injecting a series of standard anion mixtures. Peak areas for three anions were measured at 195 nm and plotted against the concentration of each compound as shown in Fig. 4. A linear relationship was obtained for these compounds over more than two orders of magnitude, and by plotting peak height against the concentration, a linear relationship was also observed. However, when chromatography was carried out at ambient temperature, the curve for NO_3^- was not linear. This could be due to the tailing effect observed in the NO_3^- ion peak which was more prominent at the lower temperature than under the usual ion-chromatographic conditions of 40°C.

The relationship between height equivalent to a theoretical plate (HETP) and eluent flow-rate is plotted in Fig. 5. To a certain extent, column efficiency decreased with increasing flow-rate.

A chromatogram of dicarboxylic acids in sub-nanomole quantities is shown in Fig. 6. The elution order of these compounds is rather complex. A series of three acids, succinic, malonic, and oxalic, eluted in the order of decreasing number of carbon atoms linking the two carboxylic acid residues. The retention time of satur-



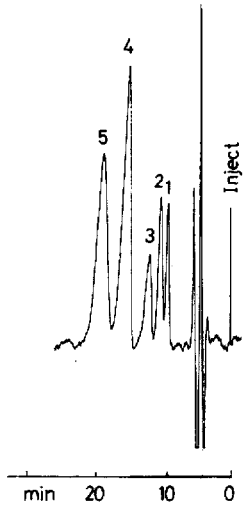


Fig. 5. Relationship between HETP and eluent flow-rate. Conditions as in Fig. 4. Curves: NO_3^- (●), NO_2^- (▲).

Fig. 6. Chromatogram of carboxylic acids. Column, YEW AX-1, 30 cm × 0.19 mm I.D.; flow-rate, 2.5 μl/min; pressure; 38 kg/cm²; temperature, ambient; eluent, 3.4 m*M* sodium hydrogen carbonate and 4.6 m*M* sodium carbonate; sample volume, 0.05 μl; wavelength, 195 nm. Peaks: 1 = succinic acid (0.5 nmol); 2 = malonic acid (0.5 nmol); 3 = tartaric acid (0.5 nmol); 4 = oxalic acid (0.5 nmol); 5 = fumaric acid (0.25 nmol).

ated-alkyl-chain dicarboxylic acids was at a minimum for succinic acid, and increased with the number of carbon atoms in the molecule, as previously reported[10]. Fumaric acid contains unsaturated carbon linkages in the molecule and thus shows strong UV absorbance, giving higher sensitivity than the saturated acids. This was more remarkable when the longer wavelength was used in the UV detector. On the same column, pairs of *cis* and *trans* isomers of unsaturated acids, mesaconic and citraconic acids and maleic and fumaric acids were separated well.

Nucleic acid-related compounds, nucleobases, nucleosides and nucleotides, have been separated on an anion-exchange column with UV detection[12–16], and recently these compounds were separated successfully by ion chromatography using a dual detector system comprised of conductivity and UV detector[17]. These compounds are of interest because of their biological importance. The applicability of the micro packed-column system to the chromatography of nucleotides and nucleobases was examined and chromatograms of mixtures containing 50 nmole of each compound are shown in Figs. 7 and 8. Dilute ammonium phosphate buffers were employed as eluents for both nucleotides and nucleobases. Fig. 7 shows that four nucleotides were separated completely within 12 min. The wavelength of the UV detector was tuned to 260 nm and the attenuation was set to the range for 0.04 absorbance units full scale (a.u.f.s.), the range used with a standard flow-cell. However, as the path length of this micro flow-cell was not determined, only relative sensitivity was obtained in the chromatogram. A chromatogram of a mixture of four nucleobases is shown in Fig. 8.

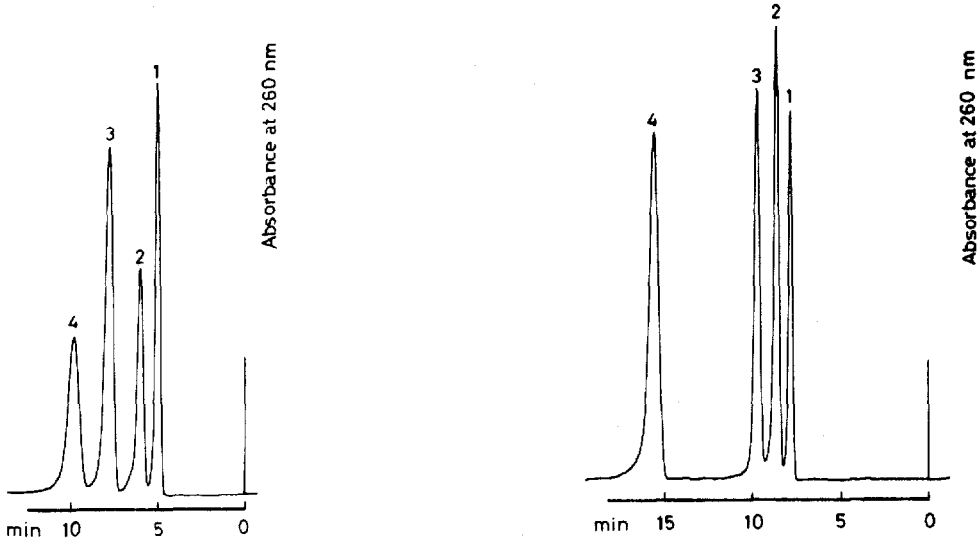


Fig. 7. Chromatogram of nucleotides. Column, YEW AX-1, 45 cm × 0.19 mm I.D.; eluent, 24 m*M* ammonium phosphate buffer (pH 8.95); flow-rate, 2.35 μl/min; pressure, 38 kg/cm²; temperature, 40°C; wavelength, 260 nm; attenuation, 0.04 a.u.f.s.; sample volume, 0.05 μl; sample concentration, 1 m*M* of each compound. Peaks: 1 = 5′-CMP; 2 = 5′-UMP; 3 = 5′-GMP; 4 = 5′-AMP.

Fig. 8. Chromatogram of nucleobases. Column, YEW AX-1, 45 cm × 0.19 mm I.D.; eluent, 24 m*M* ammonium phosphate buffer (pH 7.8); flow-rate, 3 μl/min; pressure, 45 kg/cm²; other conditions as in Fig. 7. Peaks: 1 = Cyt; 2 = Ura; 3 = Gua; 4 = Ade.

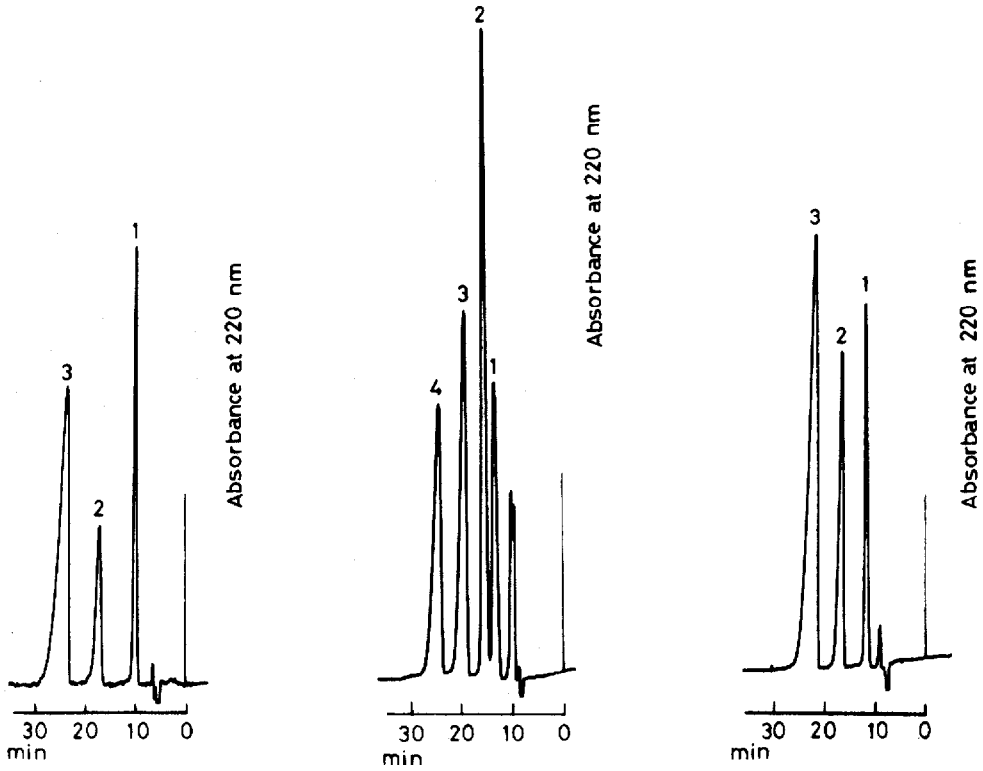


Fig. 9. Chromatogram of nitrobenzoic acids. Column, YEW AX-1; 45 cm × 0.19 mm; eluent, 8 m*M* sodium carbonate–methanol (5:3); flow-rate, 1.22 μl/min; pressure, 55 kg/cm²; wavelength, 220 nm; attenuation, 0.02 a.u.f.s.; sample volume, 0.05 μl; sample concentration, 0.66 m*M* of each compound. Peaks: 1 = *o*-nitrobenzoic acid; 2 = *m*-nitrobenzoic acid; 3 = *o*-hydroxybenzoic acid.

Fig. 10. Chromatogram of aminobenzoic acids. Eluent, 0.1 *M* sodium hydroxide–methanol (5:3); flow-rate 1.1 μl/min; pressure, 55 kg/cm²; attenuation, 0.08 a.u.f.s.; other conditions as in Fig. 9. Peaks: 1 = *p*-methylaminobenzoic acid (2.7 m*M*); 2 = *o*-aminobenzoic acid (0.9 m*M*); 3 = *p*-dimethylaminobenzoic acid; (3.8 m*M*); 4 = *m*-aminobenzoic acid (1.9 m*M*).

Fig. 11. Chromatogram of aminosulphonic acids. Conditions as in Fig. 10. Peaks: 1 = *p*-aminobenzenesulphonic acid (5.5 m*M*); 2 = benzenesulphonic acid (2.2 m*M*); 3 = *p*-methylbenzenesulphonic acid (5.5 m*M*).

Aromatic acids were adsorbed strongly on the anion-exchange resin column and were eluted very slowly by the diluted eluents usually used in ion chromatography. A mixture of 8 m*M* aqueous sodium carbonate and methanol was used for the separation of nitrobenzoic acid isomers. The *o*- and *m*-nitrobenzoic acids were completely separated from each other and from *o*-hydroxybenzoic acid as shown in Fig. 9, while the *ortho* and *para* isomers could not be separated for either compound.

Aminobenzoic acids can be separated using the same eluent. *p*-N-methylaminobenzoic acid eluted faster than *p*-aminobenzoic acid while *p*-N-dimethylaminobenzoic acid was slower. A smoother baseline but with the same retention times was obtained by using an alkaline methanol eluent. The *ortho* and *para* isomers of aminobenzoic acid were also eluted at the same retention time. A chromatogram of these

compounds is shown in Fig. 10. Sulphobenzoic acid and its derivatives were chromatographed using the alkaline methanol eluent (Fig. 11).

During recent years, micro-scale liquid chromatographic techniques have been rapidly developed by many workers. In this report anion-exchange chromatography with a microbore packed column has been introduced, the results proving that the system works as well for the separation of a variety of compounds as does conventional-size chromatographs. The column efficiency for the micro packed column is still a little lower than that of a conventional column packed with the same packing material. However, it was observed that the efficiency was improved considerably by using a narrower-bore fused-silica tube for the detector cell.

The miniaturized column requires only small amounts of packing material, sample and eluent. The amounts of these materials required were reduced, by a factor of compared to the conventional column system, about 1:500 based on the ratios of the cross-sectional areas of the columns.

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