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STUDY OF THE PERFORMANCE OF CATION-EXCHANGE COLUMNS IN OPEN-TUBULAR MICROCAPILLARY LIQUID CHROMATOGRAPHY

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SUMMARY

Aromatic and aliphatic cation-exchange columns have been prepared for open-tubular microcapillary liquid chromatography. The performance of these columns was evaluated by using a UV spectrophotometer as the detector and nucleosides as samples. The dependence of solute retention on the pH and salt concentration of the mobile phase or column temperature was examined. A difference in selectivity between aromatic and aliphatic cation-exchange columns was observed, and is ascribed to the difference in matrices. The ion-exchange capacity per unit column length was about $2 \cdot 10^{-8}$ equiv./m.

INTRODUCTION

During the last five years a number of studies of the use of small-bore columns in high-performance liquid chromatography (HPLC) have been published. The consumption of both solvents and packing was found to be reduced and the low flow-rates facilitated the direct combination to a mass spectrometer.

Open-tubular microcapillary columns have also been observed to have higher efficiencies in terms of theoretical plate numbers compared with conventional HPLC columns. We have prepared various types of open-tubular microcapillary columns and examined their chromatographic performances: columns physically coated with silicone grease[1], β,β'-oxydipropionitrile[2] and polyethylene glycols[2]; solid columns[3,4]; chemically bonded octadecylsilane columns[5,6] and polystyrene columns[7]. Good results were obtained on the various columns and the applicability of the columns was substantiated. The results were also supported theoretically by Knox and co-workers[8,9].

The theoretical plate height (H or HETP) was determined from the equation proposed by Golay[10]

$$H = \frac{2D_m}{u} + \frac{(11k'^2 + 6k' + 1)\, d_c^2}{96\,(1 + k')^2\, D_m} \cdot u + \frac{2k'd^2}{3\,(1 + k')^2\, D_s} \cdot u \qquad (1)$$

where D_m and D_s are the diffusion coefficients of a solute in the mobile and the

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stationary phase, u is the linear velocity of the mobile phase, k' is the capacity factor, d_c is the column diameter and d is the thickness (or depth) of the stationary phase, respectively. Eqn. 1 indicates that d_c and d should be minimized in order to attain a low H value. Since the contribution of the first term in eqn. 1 (a longitudinal diffusion term) is usually small, the desired chromatographic system is one which results in large D_m and D_s values.

We have found[2] that the contribution of the mass transfer resistance in the mobile phase (the second term in eqn. 1) to H was predominant for physically coated columns of 30–60 μm I.D. Thus, the stationary phase can be considered to exist as a thin layer having low resistance to mass transfer.

The above results encouraged us to develop narrower-bore and/or longer columns which can generate larger numbers of theoretical plates. However, it is difficult to prepare columns having such dimensions, especially chemically bonded columns.

Recently, a preparation technique for chemically bonded stationary phase has been developed and chemically bonded octadecylsilane (ODS) columns (5–20 m × 30–50 μm I.D.) were prepared[6]. The conditions, such as temperature of pre-treatment with an alkaline solution, reaction temperature and time and diluents of silane reagents, were examined in detail. The reactions between silane reagents and silanol groups were promoted by heating for longer than 4 h at 150°C. In the case of the above ODS columns, the contribution of mass transfer resistance in the mobile phase was predominant. Thus, it is expected that narrower-bore columns can be prepared.

This method can be applied to the preparation of various kinds of stationary phases, and we now describe the preparation of chemically bonded cation-exchange columns. Both aromatic and aliphatic cation-exchange columns have been prepared and some parameters which affect solute retention have been examined. The use of aliphatic cation-exchange columns in HPLC has previously been reported[11,12].

EXPERIMENTAL

The reagents were purchased from Wako (Osaka, Japan), unless noted otherwise.

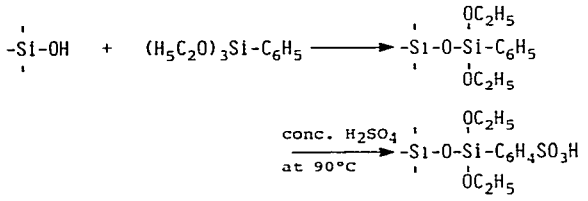
A liquid chromatograph was assembled from a micro feeder (Azumadenki Kogyo, Tokyo, Japan) and a gas-tight syringe as a pump, a UV spectrophotometer UVIDEC-100 (Japan Spectroscopic, Tokyo, Japan) with a modified flow cell and a home-made column oven comprising an asbestos board equipped with a heater and a fan. The temperature was adjusted by a slide rheostat which altered the applied voltage.

Soda-lime glass capillary tubing (30–60 μm I.D.) was prepared with a glass drawing machine, as described previously[2], and treated with a 1 N sodium hydroxide aqueous solution at 20–50°C for 2 days. Subsequent to pre-treatment, the capillary tubings were washed with pure methanol or hydrochloric acid or with a mixture of these compounds and then dried in a stream of helium at 120°C for 5 h. The capillary was filled with a toluene solution of phenyltriethoxysilane or 2-mercaptoethyltriethoxysilane (Tokyo Chemical Industry, Tokyo, Japan) and the reaction was promoted at elevated temperature (*ca.* 140°C). Alternatively, the capillary was coated with a 20% (v/v) phenyltriethoxysilane solution in dichloromethane by the dynamic coating method, as previously reported[1,2,5]. Solvents employed were dried on a column

packed with 4-Å molecular sieves (8–12 mesh; Yoneyama Yakuhin kogyo, Osaka, Japan). Ion-exchange groups were introduced into the capillaries by treatment with concentrated sulphuric acid at 90°C for 4 h (for phenylsilyl groups) or with 1 *N* sulphuric acid containing 2% (w/w) potassium permanganate at room temperature (for 2-mercaptoethylsilyl groups). After the reaction, the columns were washed with deionized water in the former case and with 0.1 *M* oxalic acid and deionized water in the latter case.

Reaction models are given in Fig. 1. Silane reagents are shown as reacting mono-functionally with silanol groups, but in practice, some reagents react bi-functionally.

(A) Aromatic cation-exchange column



(B) Aliphatic cation-exchange column

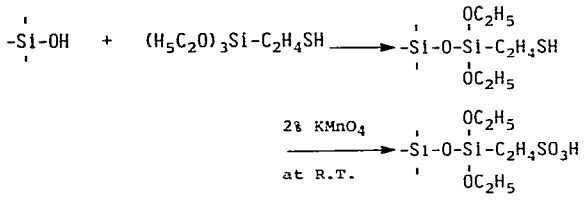


Fig. 1. Reaction models for cation-exchange columns.

A support coated open-tubular column was also prepared in order to compare the properties of the chemically bonded stationary phases with commercially available packings, *e.g.*, Shodex 125S (sodium polystyrene sulphonate, 12.5 μm; Showa Denko K.K., Tokyo, Japan). A soda-lime capillary, which had previously been treated with an alkaline solution, was filled with an aqueous suspension of the packing material (ground to particles less than 1 μm). It was then heated at a rate of 4°C/min from 30 to 130°C before being kept at 130°C overnight. Finally, the capillary was washed with deionized water.

Ion-exchange capacities were measured by the following method. A sodium chloride solution (0.1 *M*, 50–100 μl) was passed through the prepared cation-exchange column (H^+) converting it completely into the sodium form. The effluent contained hydrochloric acid in an amount equivalent to the capacity of the column, which was then determined by titration with sodium hydroxide solution (10^{-3} *M*).

The performance of cation-exchange columns was estimated by employing nucleosides (Kohjin, Tokyo, Japan) as test samples and formic acid–ammonium formate as the mobile phase. The pH of the mobile phase was adjusted with formic acid.

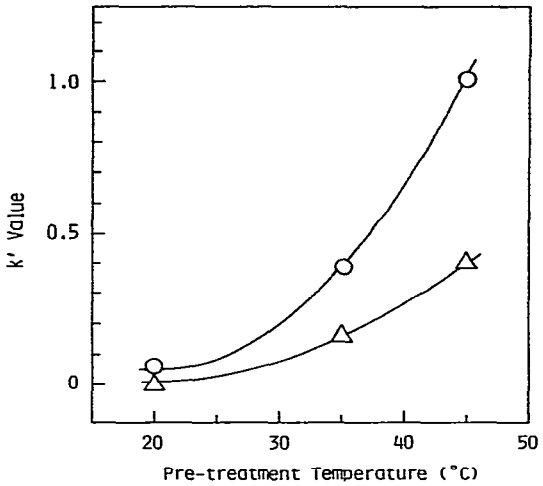


Fig. 2. Effect of pre-treatment temperature on retention for phenylsilane column. Stationary phase: phenylsilane, reacted for 15 h at 170°C. Mobile phase: acetonitrile–water (2:8 v/v). Samples: △, naphthalene; ○, biphenyl.

RESULTS AND DISCUSSION

The treatment of soda-lime glass capillaries with a 1 *N* sodium hydroxide aqueous solution effectively increases their surface areas. Fig. 2 shows the effect of pre-treatment temperature on solutes retention for a phenylsilane column. In this case, a 20% silane solution in dichloromethane was coated by the dynamic coating method, as described earlier[5]. The retention of solutes increases with increasing pre-treatment temperature, *i.e.*, more phenylsilyl groups are introduced on the glass surface at higher temperatures.

Tables I and II show the variations of solute retention with sulphonation for the aromatic and aliphatic cation-exchange columns, respectively. On both cation-exchange columns, retention of aromatic hydrocarbons decreased after sulphonation, while that of nucleosides increased. This is ascribed to the decrease of hydrophobicity, which results in the introduction of ion-exchange groups. Nucleosides were

TABLE I

VARIATION OF SOLUTE RETENTION WITH SULPHONATION ON THE AROMATIC CATION-EXCHANGE COLUMN

Mobile phase	*Solute*	*k'*	
		$-C_6H_5$	$-C_6H_4SO_3H$
Acetonitrile–water (3:7)	Biphenyl	0.35	0
	Pyrene	0.69	0
$2 \cdot 10^{-3}$ *M* Ammonium formate, pH 2.7	Uridine	0	0
	Guanosine	0	0.06
	Adenosine	0.08	0.46
	Cytidine	0.19	0.43

TABLE II

VARIATION OF SOLUTE RETENTION WITH SULPHONATION ON THE ALIPHATIC CATION-EXCHANGE COLUMN

Mobile phase	*Solute*	*k'*	
		$-C_2H_4SH$	$-C_2H_4SO_3H$
Acetonitrile–water (2:8 v/v)	Biphenyl	0.25	–
	Pyrene	0.80	0.18
1×10^{-3} *M* Ammonium formate, pH = 3.4	Uridine	0	0
	Guanosine	0.03	0.06
	Adenosine	0.26	0.93
	Cytidine	0.19	0.98

retained on phenylsilane and 2-mercaptoethylsilane columns, leading to a difference in selectivity between the aromatic and aliphatic cation-exchange columns.

In ion-exchange HPLC, solute retention depends on various parameters such as the pH and the ionic strength of the mobile phase, concentration of organic solvent, column temperature, etc. Fig. 3A and 3B illustrates the influence of pH of the mobile phase on solute retention for support coated and chemically bonded aromatic cation-exchange columns, respectively. Similar pH dependences were observed, confirming that the chemically bonded columns possessed ion-exchange properties. The *k'* values and elution order of adenosine and cytidine were sensitive to pH, while the retention of guanosine increased gradually with decreasing pH. However, uridine was not retained. Four nucleosides could be separated at low pH (*e.g.*, 2.2).

Fig. 3C illustrates the dependence of solute retention on pH of the mobile phase for the aliphatic cation-exchange column. The behaviour of adenosine and cytidine was different from that on the aromatic cation-exchange columns. The two

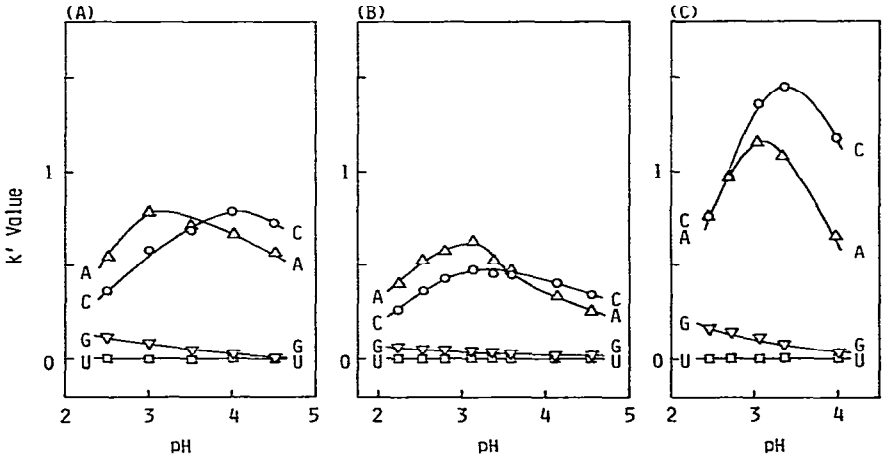


Fig. 3. *k'* values as a function of pH of the mobile phase. Columns: A, coated with Shodex HC-125S R-SO_3Na; B, chemically bonded aromatic column, $-C_6H_4SO_3H$; C, chemically bonded aliphatic column, $-C_2H_4SO_3H$. Mobile phases: ammonium formate, 5×10^{-3} *M* (A) or 2×10^{-3} *M* (B and C). Sample: U = uridine; G = guanosine; C = cytidine; A = adenosine.

nucleosides could not be separated at low pH (less than 3), but they could be separated at pH *ca.* 3.5. This difference in selectivity between the two kinds of stationary phases may be due to difference in the matrices.

Fig. 4 shows the effect of the concentration of a salt on the retention of cytidine. The smaller the salt concentration the larger is the k' value obtained, which is consistent with the results of conventional ion-exchange chromatography.

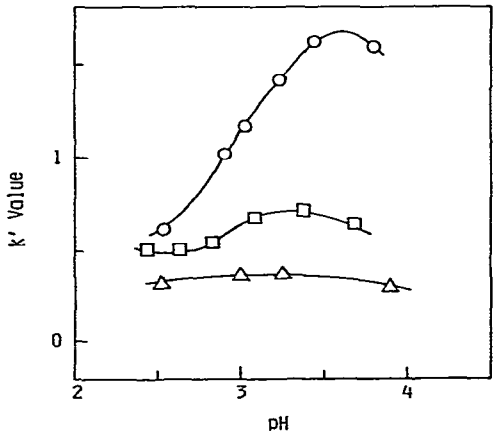


Fig. 4. k' value of cytidine as a function of the pH and ionic strength of the mobile phase on the aliphatic cation-exchange column (5.3 m × 52 μm I.D.). Mobile phases: ammonium formate, $1 \cdot 10^{-3}$ *M* (○), $5 \cdot 10^{-3}$ *M* (□), $1 \cdot 10^{-2}$ *M* (△). The pH was adjusted with formic acid.

Typical chromatograms obtained with chemically bonded cation-exchange capillary columns are shown in Fig. 5. Operating conditions were selected according to the above observations. Four nucleosides are satisfactorily separated on both columns. The peak shapes are symmetrical on the aliphatic cation-exchange column, but slight tailing is observed on the aromatic cation-exchange column.

The diffusion coefficients of solutes in the mobile phases employed in ion-exchange chromatography are small. Thus, it is preferable to operate at higher temperatures. In general, the column temperature affects both the column efficiency and solute retention. Fig. 6 shows the effect of column temperature on both parameters. For the non-retained solute (uridine), theoretical plate numbers increased with increasing temperature, whereas those for cytidine decreased at 75°C. The chromatographic peaks observed at higher temperature had pronounced leading edges for retained solutes, resulting in poor efficiency. The k' value of cytidine decreased with increasing column temperature and plots of k' value on a logarithm scale against reciprocal of absolute temperature were linear.

Baseline separation of nucleosides was achieved at 43°C, as shown in Fig. 7.

Eqn. 1 indicates that a lower H value can be attained with a smaller-bore capillary column. Fig. 8 shows the relationship between linear velocity, u, and HETP obtained with columns of different bores. Lower H values and lower slopes of the plots are observed for the smaller-bore column, in agreement with the preceding discussion.

The ion-exchange capacity is a significant characteristic of ion-exchange chro-

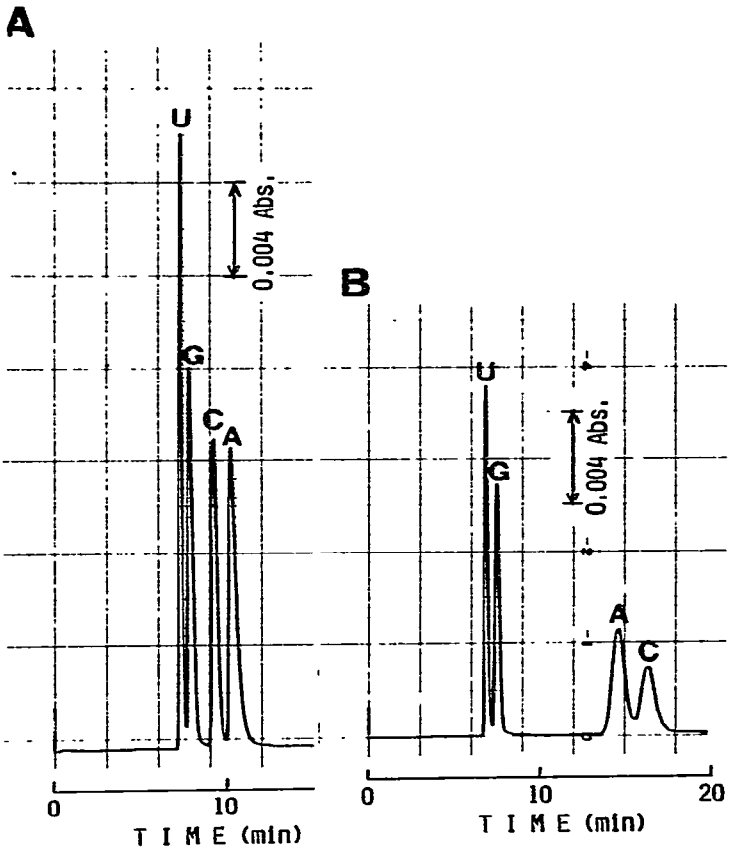


Fig. 5. Separations of nucleosides on cation-exchange columns at room temperature (*ca.* 20°C). Columns: A, 5.2 m × 44 μm I.D., $-C_6H_4SO_3H$; B, 5.3 m × 52 μm I.D., $-C_2H_4SO_3H$. Mobile phases: ammonium formate, $2 \cdot 10^{-3}$ *M*, pH 2.2 (A); $1 \cdot 10^{-3}$ *M*, pH 3.4 (B). Flow-rate: 1.1 μl/min (A); 1.7 μl/min (B). Sample: 12 ng uridine; 12 ng guanosine; 13 ng cytidine; 11 ng adenosine. Wavelength of UV detection: 260 nm.

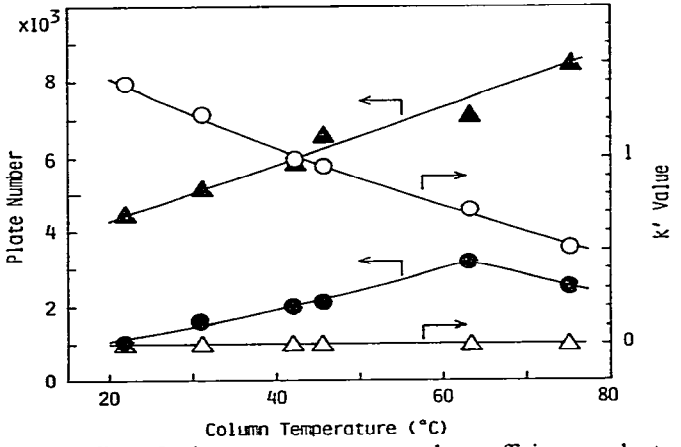
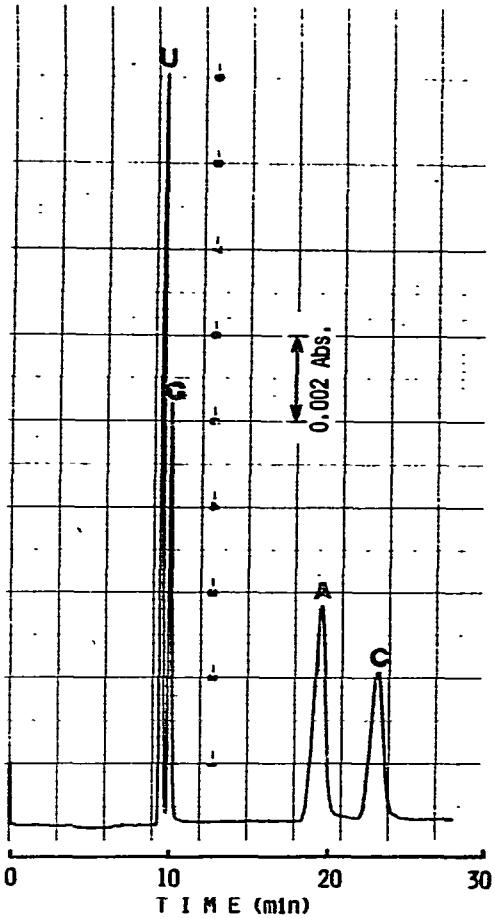


Fig. 6. Effect of column temperature on column efficiency and retention of nucleosides. Column: 5.1 m × 46 μm I.D., aliphatic cation-exchange. Mobile phase: $1 \cdot 10^{-3}$ *M* ammonium formate, pH 3.4; flow-rate 1.7 μl/min. Samples: △▲, uridine; ○ ●, cytidine.



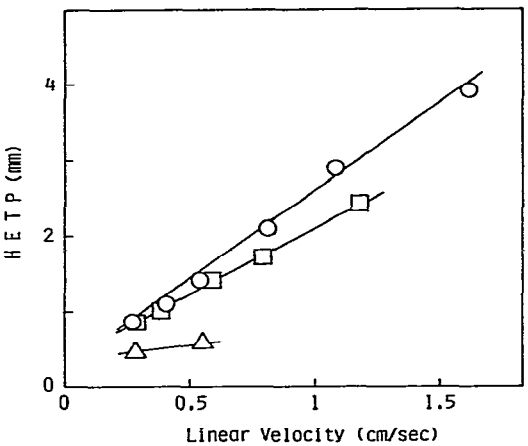


Fig. 7. Separation of nucleosides at 43°C, on the aliphatic cation-exchange column (6.0 m × 39 μm I.D.). Mobile phase: $2 \cdot 10^{-3}$ *M* ammonium formate, pH 3.4; flow-rate 0.83 μl/min. Sample: 6.9 ng uridine; 6.8 ng guanosine; 6.2 ng adenosine; 9.0 ng cytidine; eluted in that order. Wavelength of UV detection: 260 nm.

Fig. 8. Relationship between linear velocity and HETP. Columns ($-C_2H_4SO_3H$): ○, 4.2 m × 47 μm I.D.; □, 6.0 m × 39 μm I.D.; △, 5.8 m × 33 μm I.D. Sample: cytidine; k' 1.5 (○), 1.2 (□), 1.2 (△).

matography. Commercial ion-exchangers possess 2–5000 μequiv./g of ion-exchange capacity, depending upon the porosity and type of matrix. The cation-exchange capacities of some capillary columns prepared in this work are shown in Table III: $1 \cdot 10^{-8}$–$2 \cdot 10^{-8}$ equiv./m of cation-exchange capacity is obtained. For the second column the total cation-exchange capacity of $7.2 \cdot 10^{-8}$ equiv. corresponds to the number of sulphonic groups, $4.3 \cdot 10^{16}$, and the density of ion-exchange groups is calculated to be $7.3 \cdot 10^{15}$ groups per cm^2 when the glass surface is assumed to be bare (or smooth). Since it is accepted that there are $4 \cdot 10^{14}$–$8 \cdot 10^{14}$ silanol groups per cm^2 on silica gel, at most $4 \cdot 10^{14}$–$8 \cdot 10^{14}$ ion-exchange groups can be introduced of surface per cm^2.

The above results indicate that the second capillary column in Table III

TALBE III

CATION-EXCHANGE CAPACITIES

Capillaries were treated with 1 *N* sodium hydroxide aqueous solution for 2 days.

Pre-treatment temperature (°C)	*Stationary phase*	*I.D. (μm)*	*Length (m)*	*Volume (μl)*	*Ion-exchange capacity*		
					Total (equiv.)	*Per unit volume (equiv./μl)*	*Per unit length (equiv./m)*
35	$-C_6H_4SO_3H$	60	5.35	15.1	8.0×10^{-8}	5.3×10^{-9}	1.5×10^{-8}
50	$-C_2H_4SO_3H$	45	4.18	6.6	7.2×10^{-8}	1.1×10^{-8}	1.7×10^{-8}
48	$-C_2H_4SO_3H$	39	6.02	7.2	6.6×10^{-8}	9.2×10^{-9}	1.1×10^{-8}

possesses a larger surface area than that of bare glass, due to the pre-treatment with an alkaline solution: the surface area increased by a factor of at least 9–18 upon treatment with a 1 *N* sodium hydroxide aqueous solution for 2 days at 50°C.

CONCLUSION

Cation-exchange capillary columns prepared in this work showed similar characteristics to conventional ion-exchange columns. A difference in selectivity between aromatic and aliphatic cation-exchange columns was observed, which would serve to improve resolution. Operation at higher temperatures gave better results.

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