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Polyfunctional low-capacity cation-exchange packing material for the separation of underivatized amino acids

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ABSTRACT

A polyfunctional low-capacity cation-exchange column-packing material was developed for the simultaneous separation and determination of underivatized 20 amino acids including 16 proteinogenic ones using a binary gradient HPLC. The new packing material was prepared by sulfoacylating a highly crosslinked macroreticular poly(styrene-divinylbenzene) copolymer of 3 μm in diameter commercially available for size-exclusion chromatography. The polyfunctionality could derive from unintentional carboxy groups innately present in the base polymers probably came from polymerization initiators in addition to the intentionally introduced sulfopropionyl groups, which was certified and evaluated by measuring dynamic capacity curves of the cation exchanger. Sixteen underivatized proteinogenic amino acids were separated in 23 min using a binary high-pressure gradient elution system with two liquids of 1 mmol L^{-1} H_3PO_4 and 20 mmol L^{-1} NaH_2PO_4/30% (v/v) CH_3CN, which led to the cycle time of 25 min. In the case of separation of 20 amino acids, the cycle time was 27 min. The developed low-capacity cation-exchange chromatography with post-column fluorescence detection was sufficiently quantitative, providing linear calibration lines ranged through almost three digit for all analytes. The detection limits were calculated as nmol L^{-1} order of magnitude with 20-μL injection. The method was applicable to the direct analysis of urinary amino acids of diagnostic markers for inborn errors of metabolism.

1. Introduction

It is a long time since cation-exchange chromatography of underivatized amino acids has been automated [1,2]; and in those days, it had taken nearly 24 h for the separation of proteinogenic amino acids. The advent of high-performance liquid chromatography (HPLC) in the 1970's has dramatically reduced the chromatographic run time. In recent years, proteinogenic amino acids can be separated typically in 1 h by a cation-exchange chromatography system, as is well-known fact, because of the progresses in instruments and packing materials. Nowadays commercially available cation-exchange columns are packed with high-capacity materials (typically of mmol mL^{-1} order) in general, so that high-concentration eluents, for example 0.2-mol L^{-1} citrate buffer solutions, are necessary, in which the post-column derivatization detection using either ninhydrin [3,4] or *o*-phthalaldehyde [5] is exclusively utilized.

On the other hands, reversed-phase chromatography preceded by pre-column derivatization of amino acids can provide more high-speed separations using an ultra-high pressure HPLC system coming out in this century. For example, the reversed-phase separations of AQC (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate) [6] derivatives have become popular in recent years [7–10], in which chromatographic run times are significantly reduced comparing to those by the conventional cation-exchange chromatography. However, such pre-column derivatization is more prone to suffer any matrix effects, for example, the reaction efficiency can depend on the sample matrix; and accuracy in quantitation is rather poor comparing to the cation-exchange methods. In addition, the separation between biological amines and amino acids may be difficult by means of the reversed-phase mode.

Recently, liquid chromatography/mass spectrometry (LC/MS) and LC/MS/MS methods have been introduced for the analysis of underivatized amino acids, based on the reversed-phase HPLC [11–15]. In many LC/MS cases, however, the precedent liquid chromatographic separations have been failed or insufficient, which is recovered by the following extracted-ion chromatogram analyses. Such a hyphenated method may be cost-ineffective at present.

A so-called amino-acid analyzer based on the traditional cation-exchange chromatography has still been essential often

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in bio-analytical researches and food sciences. Such an apparatus is a highly specialized HPLC system equipped with a high-capacity cation-exchange column and a highly complicated pH-gradient elution system. Therefore, the equipment is less flexible to use and may make the running cost high in addition to its high purchase cost. In recent years, the authors are developing a low-capacity cation-exchange chromatography of underivatized amino acids using a conventional binary gradient HPLC system [16,17], in expectation of flexibility and cost-performance for the amino-acid analysis. Since the amino-acid selective low-capacity cation-exchange resin has been successfully created by sulfoacylating [18,19] a highly cross-linked macroreticular poly(styrene-divinylbenzene), PS-DVB, co-polymer of 5 μm in diameter that is the packing material of a size-exclusion chromatography (SEC) column of Tosoh (Tokyo, Japan) TSKgel HHR series. Such a cation-exchange column (exchange capacity, ca. 30 μmol mL^{-1}) has enabled to separate the proteinogenic amino acids in 50 min with an acceptable resolution using a 20-mmol L^{-1} phosphate eluent [16,17], allowing to use an ultraviolet detection because of the UV-transparent mobile phase; and it has facilitated the simultaneous determination of urinary creatinine and aromatic amino acids [20,21]. This can involve the analytical and technical advantages for the screening and/or chemical diagnosis of inborn errors of metabolism [22,23]. However, the separation between serine and glycine has been still unsatisfactory [17].

The main objective of this work was to increase the column efficiency and to reduce the analytical run time for the low-capacity cation-exchange chromatography of underivatized amino acids. This could simply lead to an idea to use a smaller particles, so that a 3-μm PS-DVB packing materials obtained from a commercial SEC column, or Tosoh TSKgel SuperH series, were functionalized by sulfoacylating in low capacity. Since this 3-μm hydrophobic resin of less than 3-nm micropore, well controlled for the SEC separations, is structurally hard and uniform in size because of its macroreticular nature [18], further improvements in chromatographic resolution was expected. Although such smaller particles were simply expected to provide higher resolution, it was found that they could provide another excellent selectivity to amino-acid cations effected by the endogenous base-gel property. This paper presents a newly developed 3-μm ϕ polyfunctional low-capacity cation-exchange resin highly selective to amino-acid cations, which can reduce the chromatographic run time less than half of those taken by usual cation-exchange separations. The polyfunctional property of the resin is also characterized by measuring the dynamic cation-exchange capacity curves [24].

2. Experimental

2.1. Chemicals and materials

Analytes: L-Alanine (Ala, A), L-arginine (Arg, R), L-aspartic acid (Asp, D), L-glutamic acid (Glu, E), glycine (Gly, G), L-histidine (His, H), L-isoleucine (Ile, I), L-leucine (Leu, L), L-lysine (Lys, K), L-methionine (Met, M), L-phenylalanine (Phe, F), L-proline (Pro, P), L-serine (Ser, S), L-threonine (Thr, T), L-tryptophan (Trp, W), L-tyrosine (Tyr, Y), and L-valine (Val, V) were purchased from Wako (Osaka, Japan). α-Aminobutyric acid (α-ABA), β-alanine (β-Ala), L-citrulline (Cit), and taurine (Tau) were from Wako. One-letter or three-letter symbols were used in the Tables and Figures to represent amino acids.

Synthetic agents: 3-Chloropropionyl chloride (98%) was purchased from Aldrich Japan (Tokyo, Japan). Dichloromethane of synthetic grade, aluminum chloride (99.9%), and tetrahydrofuran (THF), methanol, dimethylsulfide, hydrochloric acid, and sodium sulfite of guaranteed grade were from Wako. All reagents were used as purchased.

Eluent components: Acetonitrile of HPLC grade was purchased from Aldrich; and phosphoric acid of biochemistry grade and sodium dihydrogenphosphate of guaranteed grade were from Wako. Ultra-pure water was obtained by passing tap-water through a Nihon Millipore (Tokyo, Japan) Direct-Q UV water purification system just before use.

Post-column reaction agents: Boric acid and sodium hydroxide for amino-acid analyzer, ethanol of guaranteed grade, *o*-phthalaldehyde (OPA) and 2-mercaptoethanol of biochemistry grade, and polyoxyethylene(23)laurylether (corresponding to Brij® 35) of analytical grade were purchased from Wako. The reaction mixture was prepared by dissolving 0.2 g of OPA, 10 mL of ethanol, 1 mL of 2-mercaptoethanol, 4.2 g of NaOH, 6.2 g of H_3BO_3, and 9 mL of 10%(w/w) Brij® 35 into water, which was finally adjusted to 1.0 L. The mixture light-shielded was stored in a refrigerator after use; and was best for use within five days.

Urine samples: Five urine samples from patients with phenylketonuria (PKU) and 10 control urine samples from healthy newborns were furnished by Shimoshizu National Hospital and Sanatorium and Yokohama City University Hospital, respectively. Each urine sample was isolated as its basic fraction and allowed 10-times dilution to the intact urine sample, which was further diluted 10 or 20 times in the case of fluorescence detection. The preparative chromatographic procedure has been described in our previous paper [25].

Base polymer: A hyper-crosslinked macroreticular PS-DVB co-polymer packed in Tosoh TSKgel SuperH2500 SEC column (6.0 mm I.D. × 150 mm; particle diameter 3 μm in average; pore diameter 3 nm; exclusion molecular mass 2×10^4, according to the manufacturer's open data) was taken out from the column after washing with methanol sufficiently, and was allowed dryness for sulfo-functionalization.

2.2. Analytical column

A newly created low-capacity cation-exchange resin was slurry packed into a stain-less steel column of 4.6 mm I.D. × 70 mm using a 5 mmol L^{-1} H_3PO_4 as the suspending and packing solvents under a backpressure of nearly 25 MPa, which was named as SP-TMR3. The working cation-exchange capacity under the chromatographic condition was approximately 15 μmol per column or 13 μmol per mL-wet resin. The synthetic procedure is described below.

The 3-μm low-capacity cation-exchange resin was prepared through an improved sulfoacylation procedure more efficient than earlier [16,18]. This consists of three-step reactions as follows: (i) A 0.8-g portion of the dry PS-DVB polymer was suspended in 25 mL of CH_2Cl_2 in a 300-mL round-bottom flask under sonication; a 0.0376 mL (0.39 mmol) of $ClCH_2CH_2COCl$ was added to the suspension; and the mixture was stirred for 15 min at room temperature. Subsequently, stoichiometric amount 0.052 g (0.39 mmol) of anhydrous $AlCl_3$ powder well grinded using an agate mortar was thrown into the stirring mixture in one go, and then it was left standing for 10 min at room temperature (Friedel–Crafts acylation). The reaction was stopped by adding 100 mL of THF. The resultant resin was filtered through a hydrophilic PTFE membrane filter (1-μm pore, 45-mm circle, Advantec, Tokyo, Japan) under a reduced pressure by machine-aspirating, and was then washed by passing 100 mL each of 1/1 (v/v) THF/H_2O, 2 mol L^{-1} HCl, H_2O, and CH_3OH in the order. (ii) The acylated resin was suspended in a mixture of 15 mL $(CH_3)_2S$ and 20 mL CH_3OH, and the suspension was stirred gently for 6 h at room temperature. The resin suspension was subjected

to a suction filtration, and the resin was washed by passing methanol. (iii) The resultant sulfonium-formed resin was suspended in 100 mL of 1 mol L^{-1} Na_2SO_3 and stirred for 1 h over a water bath at 80 °C. After this, the resultant sulfoacylated resin was filtered off and washed sufficiently with water, and then with methanol on a suction filtration device. The final wet resin was allowed to be dry at room temperature.

2.3. Instrumentation

The binary gradient HPLC system consisted of a Shimadzu (Kyoto, Japan) DGU-20A3 degasser, two Shimadzu LC-10AD*VP* solvent delivery pumps equipped with a static mixer for two liquids, a Rheodyne (Cotati, CA) Model 7725i syringe-loading sample injector with a 100-μL sample loop, a Shimadzu CTO-20A column oven, a Waters (Milford, MA) 474 scanning fluorescence detector, and a Hitachi (Tokyo, Japan) D-2500 chromatographic integrator. In addition, a Shimadzu SPD-10A UV-VIS spectrophotometric detector was used for the optimization study for convenience.

The post-column OPA derivatization system consisted of a Hitachi (Tokyo, Japan) L-7110 pump for the delivery of a reaction agent and a Sugai (Wakayama, Japan) U-620 oven as a reaction chamber. A reaction coil made of a stainless-steel tubing of 0.5 mm I.D. × 2 m was placed in the chamber and was connected with the chromatographic line and the agent line via a T-joint.

The data acquisition system was constructed as follows: The analog output signals from the detector were digitized *via* an Advantest (Tokyo, Japan) R6441A digital multimeter through an RS232C interface, and the chromatographic data were acquired by using a personal computer installed with a self-made data acquisition program written with Visual BASIC working under Microsoft-Windows® environments. A set of chromatographic data stored as "dat" file was transferred to a "csv" file executable for Microsoft-Excel® to draw a chromatogram.

The capacity analyzing system consisted of a Hitachi L-7110 pump (1.0 mL min^{-1}) connected with an Upchurch Scientific (Oak Harbor, WA, USA) 6-port solvent selection valve, a Sugai U-620 column oven (40 °C), and a Tosoh CM-8000 conductivity detector [24]. The analog-output signals from the detector were digitized *via* an ADCMT (Hiki, Saitama, Japan) 7351E digital multimeter, and the conductivity changes were recorded through a USB interface on a Windows® computer with an acquisition program, 7351_u_demo05 written with Microsoft-Excel® VBA downloadable from the ADCMT Web site. The method can provide the real-time working capacity of the target cation-exchange stationary phase during the chromatographic run. The column effluent pH was measured using a HORIBA (Kyoto, Japan) F-24 pH/Ion meter with a glass electrode (Model 6367-10D).

2.4. Chromatographic conditions

The high-pressure binary gradient elution system was established by mixing two solvents A: 1 mmol L^{-1} H_3PO_4 and B: 20 mmol L^{-1} NaH_2PO_4/30%(v/v) CH_3CN. The time programs to change the A/B delivery ratio are listed in Table 1. Since the numbers of analytes were different between the two detection systems used, the time programs were individually optimized. Flow rates of the mobile phase and the post-column derivatization mixture were both 0.5 mL min^{-1}. Temperatures of the column oven and the reaction chamber were 40 and 65 °C, respectively. Excitation and emission wavelengths of the fluorescence detector were set at 340 and 430 nm, respectively. In the case of UV detection, the wavelength was set at 210 nm. The injection volume was 20 μL throughout the experiments.

Table 1
Binary gradient time programs for 17 amino acids with UV detection and 20 amino acids with fluorescence detection.

UV detection			Fluorescence detection		
Time (min)	Delivery ratio (%)		Time (min)	Delivery ratio (%)	
	Solvent A	Solvent B		Solvent A	Solvent B
0	100	0	0	100	0
10.00	65	35	10.00	60	40
12.00	30	70	12.00	60	40
20.00	30	70	13.00	30	70
20.01	100	0	22.00	30	70
30.00	100	0	22.01	100	0
			30.00	100	0

Solvent A: 1 mmol L^{-1} H_3PO_4
Solvent B: 20 mmol L^{-1} NaH_2PO_4/30%(v/v) CH_3CN.

Table 2
Conditioning agents for the column equilibration at different pH.

Conditioning agent	Column effluent pH [a]
50 mM H_3PO_4/5 mMNaH_2PO_4/100 mM $NaNO_3$	1.75
50 mM H_3PO_4/50 mMNaH_2PO_4/100 mM $NaNO_3$	1.99
10 mM H_3PO_4/100 mMNaH_2PO_4	3.04
100 mMNaH_2PO_4	4.66
100 mM NaH_2PO_4/10 mM Na_2HPO_4	5.81
50 mM NaH_2PO_4/50 mM Na_2HPO_4	6.83
5 mM NaH_2PO_4/50 mM Na_2HPO_4	7.90
50 mM Na_2HPO_4	8.96
50 mM $NaHCO_3$/50 mM Na_2CO_3	10.13
50 mM Na_2CO_3	11.30
33 mM Na_3PO_4	12.10

[a] Corresponds to the averaged pH of the column effluent between 20 and 30 min after a start of conditioning. Each value can slightly change from column to column to be examined.

2.5. Capacity analysis

The cation-exchange capacity of the stationary phase active on the chromatographic run, depending on the dynamic equilibrium pH of the mobile phase, was measured to evaluate the functional groups of the 3-μm ϕ low-capacity cation-exchange resin. An instrumental method to estimate the dynamic exchange capacity (DEC) of a low-capacity cation-exchange column has been constructed in our latest study [24]. Since, however, the duration time of the conditioning period to establish the Na^+-form cation-exchange sites was too long due to low ionic strength of the conditioning agents used at that time, the procedure was reexamined to promote the efficiency and accuracy. The reconstructed conditioning agents and the column equilibration pH are listed in Table 2, in which each pH is actual value of the column effluent between 20 and 30 min after start conditioning, and the values are slightly variable from measurement to measurement. A recommendable procedure is outlined as follows: The target column was connected and placed in the oven. Each conditioning agent was passed through the column at a flow rate of 1 mL min^{-1} for 30 min at 40 °C; and then the residual Na^+ ions in the column were washed away by passing carbonate-free water at 1 mL min^{-1} for 30 min, until the detector would exhibit a bottom conductivity value. Subsequently, a 10 mmol L^{-1} HNO_3 (regenerant) was passed through the Na^+-form column until almost all Na^+ ions replaced by H^+ ions came out from the column, which could exhibit a steep change in conductivity from those corresponding to 10 mmol L^{-1} $NaNO_3$ (ca. 1200 μS cm^{-1}) to those of 10 mmol L^{-1} HNO_3 (ca. 4000 μS cm^{-1}). The calculating method for the capacity was detailed in our literature cited [24].

3. Results and discussion

3.1. Capacity control

The cation-exchange capacity of the 3-μm packing material was controllable even in the present low-capacity level by changing the amount of the acylation agent, 3-chloropropionyl chloride, as well as in high-capacity levels shown by Seubert and Klingenberg [18]. Fig. 1 shows the dependence of exchange capacity on the amount of the acylation agent at pH 4, all measured with 100 mM $NaNO_3$/10 mM HNO_3 as conditioning-agent/regenerant pair [24]. Because this conditioning agent has no buffering capacity, the equilibrium pH observed is apparent value. Under such a condition, the cation-exchange capacity estimated is almost derived from sulfo groups. A quadric correlation (r^2=1.0000) was observed between them. This was a good guiding principle to prepare a desired low-capacity cation-exchange resin.

3.2. Polyfunctional property

It is well known that the cation-exchange capacity of sulfo-functionalized cation-exchange resin is constant over the wide range of the eluent pH, or is independent of the surrounding pH because of its strong-acid nature. However, such a common knowledge may not hold in the low-capacity materials. Namely, the DEC of the present 3-μm low-capacity cation-exchange resin considerably depended on the column equilibration pH. Fig. 2 shows dynamic capacity curves on pH for the SP-TMR3 resin and the intact PS-DVB gel from the SuperH column. The significant increases in DEC were observed in both according to the increase in the equilibrium pH (see Table 2). It was in a sense a surprising fact that the SEC polymer used as the starting gel could have a significant cation-exchange property. In this case, the DEC of SP-TMR3 found around pH 2–3 should correspond to the posteriorly introduced sulfopropionyl groups. Such an additional capacity was well correlated with the difference in the dynamic capacity curves between the two materials. The increases in DEC observed in both materials can derive from weakly dissociable functional groups such as carboxy groups typically. In addition, the dynamic capacity curves both have inflection points around pH 6, which clearly indicates the presence of carboxy groups having pK_a 6 [26]. This is inexplicable with a side reaction of Friedel–Crafts acylation of PS-DVB using ω-chloropropionyl chloride producing carboxy groups with a few percent possibility [18]. Such unexpected carboxy-functionality may originate from the base polymer of high cross-linkage, probably due to the residual benzoyl peroxide used as a radical-reaction initiator.

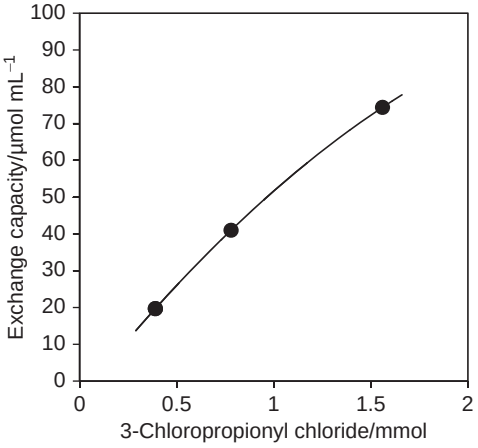

Fig. 1. Dependence of cation-exchange capacity on the amount of 3-chloropropionyl chloride.

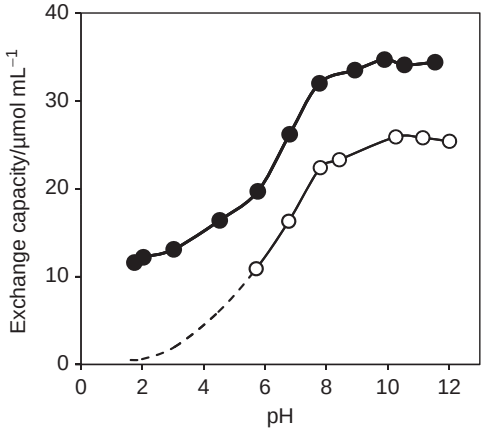

Fig. 2. Dynamic capacity curves over the column equilibration pH for the present SP-TMR3 resin (●) and the SuperH base polymer (○). The exchange capacity was expressed by μmol per wet volume of the resin. The broken line was depicted by an estimation because there was some difficulty in capacity measurements under a condition less than pH 3 in the case of weak-acid materials.

The polyfunctional property of the stationary phase can lead to multiple interaction with the analyte amino-acid cations in the mobile phase, involving major electrostatic interaction (via sulfo and dissociated carboxy charged sites), hydrophilic interaction (undissociated hydroxy and carboxy groups), hydrophobic interaction (polymer backbone, propionyl spacer arm), π–π interaction (benzene ring), hydrogen bonding interaction (hydroxy, carboxy, and propionyl oxygen), and so on. Since the key to success in low-capacity cation-exchange chromatography of amino acids depends on the quality of separation among the acidic and neutral-hydrophilic amino acids, i.e., Asp, Ser, Gly, Thr, Glu, and Ala. The endogenic carboxy groups and/or some other active sites as mentioned above in addition to the intentional sulfopropionyl groups can multiply interact with the diverse functional groups of amino-acid cations, which may be factors of the high selectivity.

Although the capacity analyzing method is still time-consuming, the dynamic cation-exchange capacity active under a chromatographic condition can be accurately evaluated and characterized. This can become an essential technique in the development study for low-capacity ion-exchange materials.

3.3. Separation of acidic and neutral-hydrophilic amino acids

Seven amino acids of interest were isocratically chromatographed using a milli-molar order of phosphoric acid as eluent. Fig. 3 shows the relationships between the retention factor (k) of the analyte amino acid and the pH of eluent. The individual log k/pH plots represents nearly straight lines with slopes of $-y/x$, where x is the charge of eluting ion, H^+, and y is the charge of an analyte ion [27–29]. Since the horizontal axis is negative-logarithmic scale, in this case, the slope shows a positive value. The slopes and determination coefficients (r^2) calculated were as follows: Asp, 0.323 (0.982); Ser, 0.458 (0.993); Gly, 0.521 (0.991); Thr, 0.406 (0.993); Glu, 0.365 (0.988); Ala, 0.474 (0.992); and Pro, 0.308 (0.997). Since the monovalent H^+ acts as eluting ion, the averaged charge of the amino-acid cations, calculated to be 0.4-valent, is consistent with effective charges of the amino-acid cations estimated from their pK_1 values. In addition, the homologous amino-acid pairs, i.e., (Asp, Glu), (Ser, Thr), and (Gly, Ala), give similar slopes individually as can be seen in Fig. 3, which may reflect the difference in multi-interaction working between

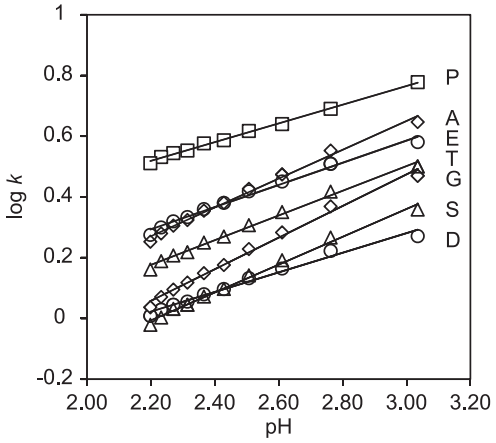

Fig. 3. Changes in retention factors of acidic and neutral-hydrophilic amino acids over the eluent pH by phosphoric acid.

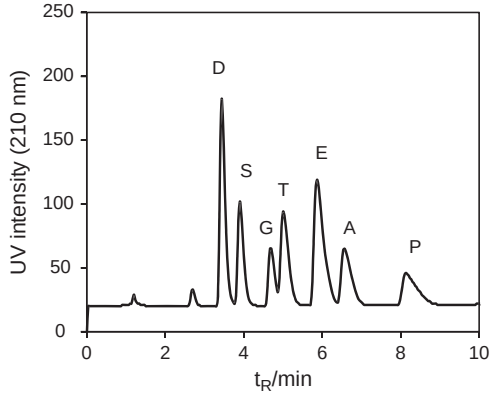

Fig. 4. A standard chromatogram of acidic and neutral-hydrophilic amino acids by 1 mmol L^{-1} H_3PO_4 with isocratic elution at a flow rate of 0.5 mL min^{-1}. Sample size: 20 μL × 1.0 mmol L^{-1} each. Detection: UV 210 nm.

analytes and stationary phase. The graph can suggest that 1 or 2 mmol L^{-1} H_3PO_4 (pH 2.8–3.0) is a good eluent.

Fig. 4 shows a chromatogram of the 7 analytes by an isocratic elution with 1 mmol L^{-1} H_3PO_4. The chromatographic resolution of the critical peak pair, i.e., between Gly and Thr, is 1.0. The eluting order of Asp, Ser, Gly, Thr, Glu, Ala, and Pro was very different from those of Ser, Gly, Asp, Thr, Ala, Glu, and Pro observed in our previous low-capacity cation-exchange chromatography [16]. Such a better selectivity to amino acids can derive from the diverse polyfunctionality and proper cation-exchange capacity of the SP-TMR3 material in combination with the optimum eluent composition and pH.

3.4. Separation of neutral-hydrophobic and basic amino acids

In order to fix the B-solvent, several candidates comprised of 10–30 mmol L^{-1} phosphate and 10–35%(v/v) acetonitrile were studied. An acceptable phosphate concentration with %(v/v) acetonitrile appropriate for the B-solvent was found to be 20 mmol L^{-1} NaH_2PO_4/30%(v/v) CH_3CN after repeated trial and error. Fig. 5 shows relationships between retention factors and the %-acetonitrile varied by changing the A/B ratio of the two solvents. Almost linear lines of log k over %-acetonitrile were observed. This can indicate that the retentions of the hydrophobic amino acids are dominated by reversed-phase interaction [30] and those of the basic amino acids are by both cation-exchange and reversed-phase interactions. The hydrophobic and basic

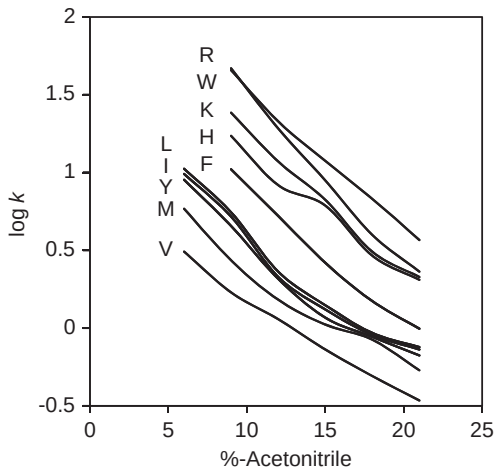

Fig. 5. Changes in retention factors of neutral-hydrophobic and basic amino acids over the organic contents of eluent.

amino acids were finally separated within 18 min using a step gradient elution, 0→3.0 min, A/B=70/30 and 3.0→20 min, A/B=40/60, where the apparent pH of the mobile phase increased from 3.5 to 4.0 during the gradient elution. This is big different from the eluting pH region in the conventional cation-exchange chromatography of amino acids.

3.5. Separation of underivatized amino acids

On the basis of the isocratic retention data of the analyte amino acids, the binary gradient program using two solvents A: 1 mmol L^{-1} H_3PO_4 and B: 20 mmol L^{-1} NaH_2PO_4/30%(v/v) CH_3CN was optimized for the separation through trial and error. Fig. 6a shows an optimized chromatogram of 17 proteinogenic amino acids with UV detection at 210 nm. The linear-gradient program used for the UV-detection mode is listed in Table 1. The delivery of 100% solvent A from 20 to 30 min is for the column regeneration. The peak appearing around 11 min is a system peak due to a sudden change in refractive index of mobile phase in a UV cell caused by mixing the two liquids [31]. The chromatographic run time was 23 min and the cycle time was 25 min. The resolution of a critical peak pair, Ile and Leu in this case, was 1.0, which seemed to be within acceptance.

Since aliphatic amino acids are low UV-active, the post-column OPA derivatization fluorescence detection was introduced to the system. Because four non-proteinogenic amino acids were added as the analytes in the case of fluorescence detection, a suitable gradient time program was designed for a better separation as given in Table 1. Cysteine or cystine would appear between Pro and Val, but its post-column detection was less sensitive in this system. Therefore, they were omitted from the analytes in addition to proline. Since, consequently, a wide chromatographic window was seen between Ala and Val (see Fig. 6a), several biologically important amino acids, i.e., taurine, citrulline, α-aminobutanoic acid, and β-alanine, were chromatographed together with the 16 proteinogenic amino acids. Fig. 6b shows an optimized chromatogram of the 20 amino acids with fluorescence detection. The chromatographic run time was 24 min and the cycle time was 27 min. Although the separation between Gly and Thr, or Ile and Leu looks insufficient, probably due to the additional dead volume between the column outlet and the detector cell, such technical problem will be solved in due course.

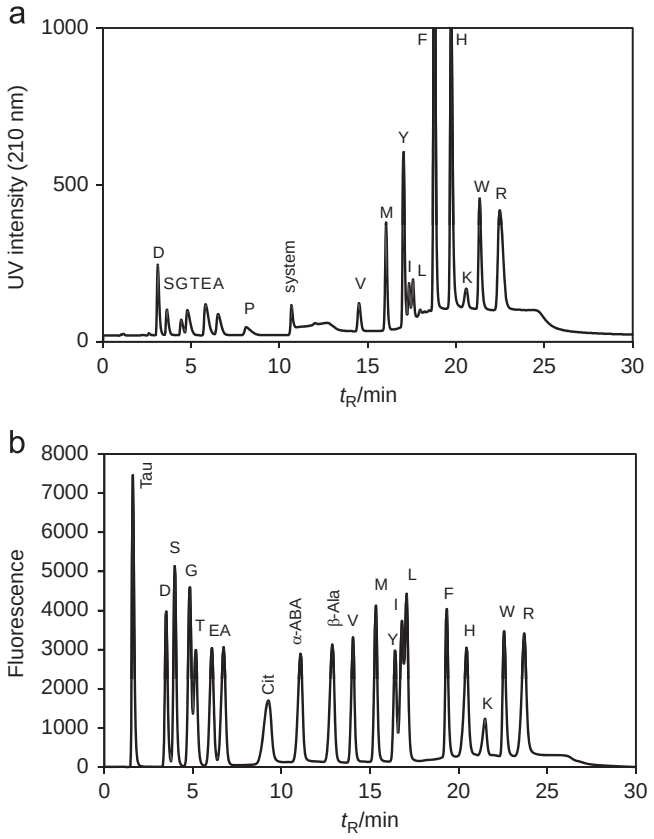

Fig. 6. Typical chromatograms of (a) 17 proteinogenic amino acids with UV (210 nm) detection and (b) 20 amino acids with post-column OPA fluorescence detection. Sample size for UV detection: 20 μL × 1.0 mmol L^{-1} for Val, Ile, Leu, Lys, Arg; 0.10 mmol L^{-1} for Met, Phe, His; and 0.010 mmol L^{-1} for Tyr, Trp. Sample size for fluorescence detection: 20 μL × 10 μmol L^{-1} each.

3.6. Quantitation study

Table 3 summarizes the quantitation data for the 20 amino acids by means of the low-capacity cation-exchange chromatography with post-column OPA fluorescence detection. The partly overlapping peaks such as seen in Fig. 6b were determined by vertically partitioning the peak-area responses for the peak pairs of interest, which could less affected the results. The retention times for all analytes were very repeatable with RSDs ($n=5$) less than 0.7%, which could imply that the chromatographic cycle time estimated was practically adequate. The peak area responses were also precise with RSDs ($n=5$) between 2.2 and 6.2%. Linear calibration line ranged through almost three-digit widths for each analyte with good r^2 values. In addition, the detection limit of each (in the case of 20-μL injection) was almost nmol L^{-1} order of magnitude calculated as 3.3σ/slope concentration. The present low-capacity amino-acid analysis system is highly precise and reliable.

3.7. Application example

As an example of real sample analyses, the developed amino-acid analysis method was applied to the determination of urinary amino acids as diagnostic markers [23] of inborn errors of amino-acid metabolism. Figs. 7a and b show typical chromatograms for urine samples from a healthy newborn and a patient with PKU, respectively, although the peak patterns look somewhat poor due to near end of column life. The diagnostic marker phenylalanine appeared clearly as a significantly intense peak (Fig. 7b) for PKU comparing to those for control (Fig. 7a). Table 4 summarizes the

Table 3
Quantitation data for 16 proteinogenic and additional 4 amino acids on the basis of low-capacity cation-exchange chromatography with post-column OPA fluorescence detection.

Analyte	Retention time [a]		Area response [b]	Test range (μmol L^{-1})	Linearity r^2	LOD [c] (μmol L^{-1})
	min ($n=5$)	%-RSD	%-RSD ($n=5$)			
Asp	3.56	0.42	2.98	0.05–20	0.9999	0.0048
Ser	4.07	0.48	2.42	0.02–20	0.9999	0.0017
Gly	4.95	0.64	5.01	0.05–20	0.9997	0.0083
Thr	5.27	0.52	2.99	0.05–20	0.9996	0.0050
Glu	6.12	0.64	3.76	0.05–20	0.9998	0.0062
Ala	6.90	0.65	2.31	0.05–20	0.9997	0.0038
Val	14.19	0.44	4.37	0.05–20	1.0000	0.0073
Met	15.43	0.31	2.62	0.05–20	0.9999	0.0039
Tyr	16.49	0.27	3.29	0.05–20	0.9995	0.0057
Ile	16.91	0.31	5.76	0.05–20	0.9996	0.0094
Leu	17.14	0.28	5.54	0.05–20	0.9994	0.0111
Phe	19.33	0.16	2.24	0.05–20	0.9998	0.0049
His	20.61	0.35	4.02	0.05–20	0.9997	0.0076
Lys	21.64	0.26	4.54	0.05–20	0.9997	0.0093
Trp	22.51	0.18	4.22	0.05–20	0.9998	0.0072
Arg	23.86	0.23	6.11	0.05–20	0.9994	0.0126
Tau	1.65	0.33	2.31	0.01–10	1.0000	0.0008
Cit	9.69	0.36	3.27	0.10–20	0.9996	0.0103
α-ABA	11.31	0.61	3.50	0.05–20	0.9998	0.0064
β-Ala	13.12	0.47	3.13	0.05–20	0.9998	0.0049

[a] Of the data on the sample size of 1 μmol L^{-1} × 20 μL.
[b] Of the data on the sample size of 1 μmol L^{-1} × 20 μL. Numerical values by integration unit were omitted.
[c] Calculated as 3.3σ/slope using the standard deviation (σ) of area data at the bottom of test range.

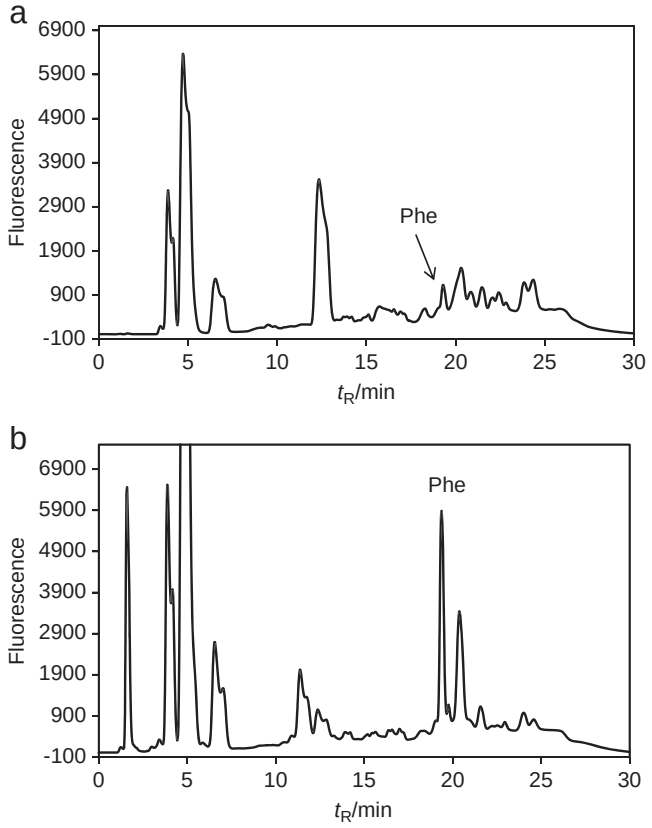

Fig. 7. Typical chromatograms for (a) control urine sample from a healthy newborn and (b) PKU urine sample from a newborn patient. The injected corresponded to 200-fold and 100-fold dilution of the intact urine samples, respectively. The peaks other than phenylalanine were not assigned.

Table 4
Concentrations of phenylalanine in control and PKU urine samples.

Sample	Marker Phe/mmol L^{-1}	Average	RSD
Control 1	0.027	0.027	0.015
Control 2	0.016		
Control 3	0.011		
Control 4	0.050		
Control 5	0.019		
Control 6	0.047		
Control 7	0.034		
Control 8	0.018		
Control 9	0.011		
Control 10 [a]	0.041		
PKU 1	0.552	0.458	0.323
PKU 2	0.971		
PKU 3 [b]	0.136		
PKU 4	0.317		
PKU 5	0.314		

[a] Chromatogram shown in Fig. 7a.
[b] Chromatogram shown in Fig. 7b.

phenylalanine concentrations found in 10 control and 5 PKU urine samples. The averaged concentration of the diagnostic marker is significantly higher than those of control. The method is applicable to many other inherited disorders of amino-acid metabolism (maker amino acid) such as citrullinemia (citrulline), tyrosinemia (tyrosine), and maple syrup urine disease (branched-chain amino acids) [23], and will provide a rapid profiling useful for the chemical diagnosis of the disease.

The developed method can provide more cost-effective results or information to clinical, pharmaceutical, and food chemistry demands rather than the expensive amino-acid analyzer now on the market.

4. Conclusion

A novel polyfunctional low-capacity cation-exchange packing material highly selective to amino-acid cations was successfully created by sulfopropionyl functionalization of the 3-μm ϕ hypercrosslinked macroreticular PS-DVB resin for the SEC separation. The polyfunctionality of the resin mainly consisted of the intentionally introduced sulfo groups and the unintentional carboxy groups residual in the base polymer. The new low-capacity column could reduce the chromatographic run time to half of those taken by conventional cation-exchange chromatography. In addition to this, the *capacity analyzer* was significantly useful to know and evaluate the functional characteristics of the low-capacity cation-exchange resin.

The low-capacity cation-exchange chromatography system can provide cost-effective analytical data because it consists of a simple and easy-operation equipment with an environment-friendly dilute mobile phase, which can accept a UV detection for UV-absorbing amino acids as occasion calls [20,21,31]. In addition, the column-regeneration takes not so long time because of the low-capacity stationary phase, leading to a short cycle time (ca. 25 min in this work), which seems to be an excellent technical merit. The self-packed column had enough lifetime, or the column performance was kept during this method development study.

It seems to be difficult to reduce the cycle time more in the conventional cation-exchange chromatography of amino acids. Contrary to this, the low-capacity cation-exchange method still has possibility in further improvement to reduce the cycle time, for example less than 15 min. Since, therefore, the analytical potential of the low-capacity cation-exchange materials is very high, further development studies are now under way to increase the chromatographic resolution and to reduce the chromatographic run time by intentionally controlling the polyfunctionality of the resin in synthesis; and the results will be presented in due course. It will be a promising method for many amino-acid treating researchers.

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