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Synthesis of sulfo/vinyl biphasic silica hybrid monolithic capillary column and its application to on-column preconcentration for capillary electrochromatography

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ABSTRACT

A novel method to synthesize sulfo/vinyl biphasic silica hybrid monolithic column in one step was developed for on-column preconcentration. In this method, sulfo-based segment is located at the inlet of capillary column, which acts as preconcentration column. It is synthesized by polymerization of 3-sulfopropyl methacrylate potassium salt (SPMA) and vinyltrimethoxysilane (VTMS) with tetramethoxysilane (TOMS). Close to the preconcentration column, a vinyl functionalized segment is formed and serves as separation column. It is synthesized by polymerization of only VTMS with TOMS. Vinyl groups on vinyl functionalized segment are modified with ligand containing sulfhydryl group, such as octadecanethiol (C_{18}-SH for short), 6-mercapto-1-hexanol (HO-C_6-SH for short), via thiol-ene click reaction. The interface between the two segments is seamless and without any dead volume. The applicability of this system is demonstrated by successful separation of closely related amines including p-phenylenediamine, aniline, p-toluidine, N-methyl aniline, N,N′-dimethylaniline, and diphenylamine. Good separation and enrichment are obtained. The proposed system is also successfully applied to complex biological samples, such as peptide, diluted BSA hydrolysate, and the results indicate that the system has a capability for preconcentration of low abundance peptides.

1. Introduction

In the last decades, capillary electrophoresis (CE) equipped with conventional UV detector has become an alternative to traditional high-performance liquid chromatography (HPLC) for using in chemical and biochemical fields due to its major merit of high separation efficiency. Nevertheless, the biggest challenge of this technology was detection limit, resulting from short optical path length and small injection volume. Recently, several approaches were adopted to overcome this drawback: (i) modifying detection cell to increase the optical path length [1], (ii) using more sensitive detectors such as mass spectrometer (MS) [2–8], laser induced fluorescence (LIF) detector [9,10], and (iii) adopting on-line sample preconcentration approaches [2,3,11–27]. In all methods mentioned above, on-line sample preconcentration approach is a more promising option for routine laboratory, because further investment or equipment modification is not needed.

In the on-line sample preconcentration strategy, on-line SPE methods enable multiple volumes of sample to be injected because the analytes are adsorbed onto a stationary phase, which is positioned on the inlet end of the capillary, thus longer enough capillary can be used for separation. So far, many kinds of SPE matrix [28] have been proposed to couple with CE. However, compared to conventional CE, there is no improvement in CE separation itself. In recent years, an improvement was made to overcome this drawback. A stationary-phase was integrated in the separation segment, thus the column consists of two contiguous segments with different stationary phase, the inlet segment is a short segment for preconcentration and the next is a longer segment for separation. Therefore, the analytes eluted from the enrichment segment are subsequently separated in the separation segment of the capillary according to capillary electrochromatography (CEC) principle. It is possible in this way to attain better separation efficiency values when compared to conventional CE separation. Tony Tegeler described a biphasic packing capillary column in which the concentrating segment was packed with 5 μm Zorbax C_8 silica and the separation segment was packed with 5 μm laboratory-made ODS Nucleosil silica. Concentrations of 10^{-8}–10^{-9} M were determined with a z-cell equipped UV detector [1]. Zou's group reported a biphasic polymer monolithic capillary

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column for on-line preconcentration and separation of peptides, a cationic exchange monolith was in situ synthesized at the inlet of column which acted as enrichment medium, it was followed by a reverse monolith which served as separation medium. Good separation performance of this biphasic column was demonstrated [29]. However, the biphasic polymer monolithic column was synthesized in two steps and the procedure was complicated, in addition, the problems of swelling and shrinking during exposure to organic solvent were hardly to overcome, thus further improvement is needed.

In this work, we describe a novel method to synthesize biphasic silica hybrid monolithic column by one step. As shown in Fig. 1, a sulfo-based silica hybrid monolith (segment A) and a vinyl functionalized silica hybrid monolith (segment B) are simultaneously synthesized in the capillary. The interface between segment A and B is seamless and without any dead volume because the whole column is synthesized in one step. Herein, the biphasic silica hybrid monolithic column is used in on-line preconcentration and separation of amines. Effective enrichment is obtained in concentration segment, and high selectivity and separation efficiency is achieved in the separation segment. The proposed system is also successfully applied to complex biological samples, such as peptide, diluted BSA hydrolysate, and the results indicate that the system has a capability for preconcentration of low abundance peptides.

2. Experimental

2.1. Reagents and chemicals

All water was distilled and deionized using a Milli-Q synthesis A10 water purification system. (Millipore Inc., Milford, MA), and degassed by ultrasonic for 5 min prior to use. Tetramethoxysilane (TMOS, 99%), 2,2′-azobis (2-methylpropionamidine) dihydrochloride (V50, 99%), vinyltrimethoxysilane (VTMS, 98%) and fluorescein isothiocyanate (FITC, 98%) were obtained from Aldrich (Oakville, ON, Canada). p-Phenylenediamine (99%), aniline (99%), p-toluidine (99%), N-methyl aniline (99%), N,N′-dimethylaniline (99%), diphenylamine (99%), 6-mercapto-1-hexanol (99%), octadecanethiol (98%), 2-acrylamide-2-methyl-propanesulfonic acid (AMPS, 99%), 3-sulfopropyl methacrylate potassium salt (SPMA, 99%) and poly(ethylene glycol) (PEG, Mn = 20,000) were purchased from Aladdin (Shanghai, China). Fused-silica capillary with 75 μm i.d. and 375 μm o.d was purchased from Reafine Chromatography Ltd. (Yongnian, China). All other reagents were of analytical grade and were used as received.

2.2. Biphasic hybrid monolithic capillary column preparation

Preparation of the monolithic CEC columns involved the following procedures. First, pretreatment of the inner fused-silica surface. The fused-silica capillary was pretreated by rinsing with

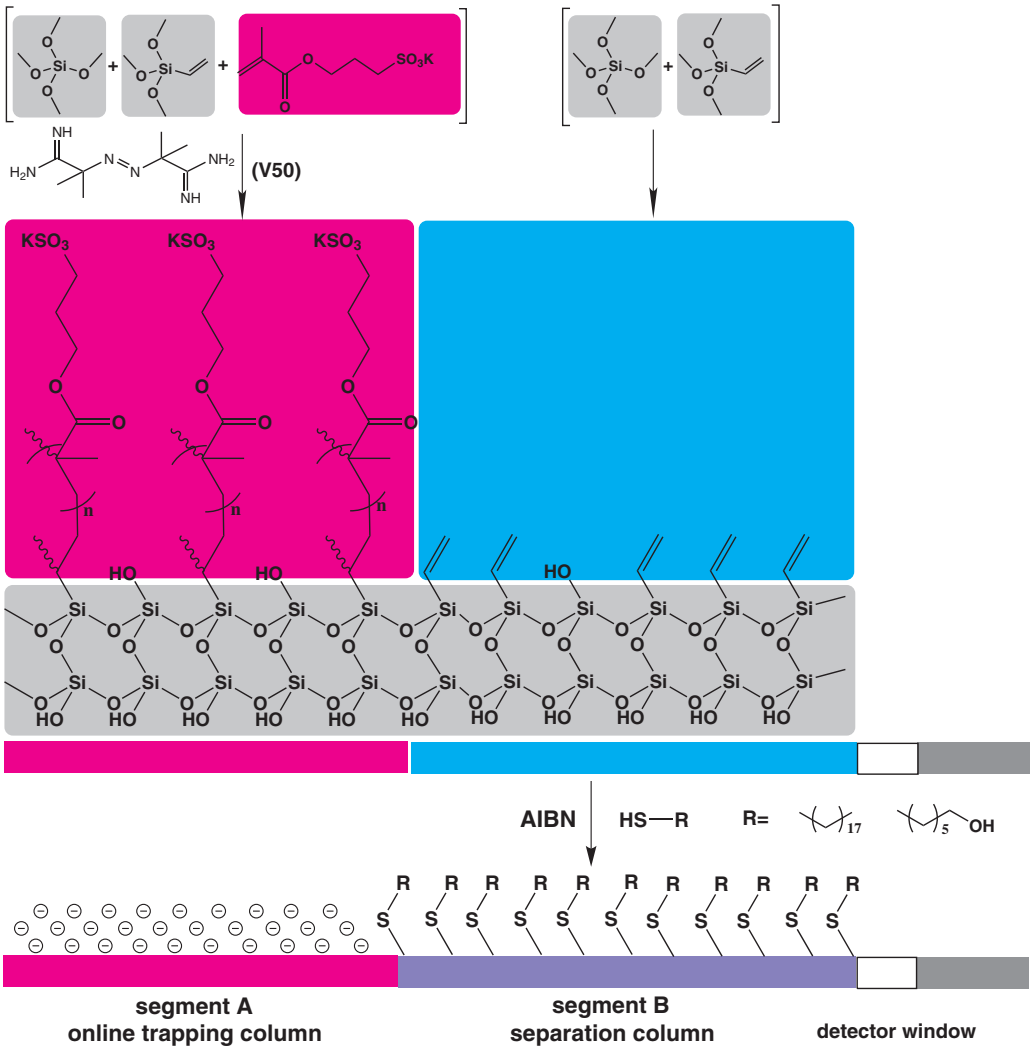

Fig. 1. Scheme for the preparation of the biphasic organic-silica hybrid monolith in segment A and segment B.

1.0 M NaOH for 12 h, water for 30 min, 1.0 M HCl for 12 h, and water for another 30 min, respectively, and then dried by nitrogen gas at room temperature. Second, preparation of the sol–gel pre-condesation solution and filling the capillary. The sol–gel pre-condesation solution A was prepared by mixing of acetic acid (0.01 M, 1.27 mL), PEG (20,000 MW, 0.135 g), urea (0.175 g), TMOS (0.45 mL), VTMS (0.15 mL) and SPMA (70 mg) in a 10 mL flask, and the sol–gel pre-condesation solution B was prepared by mixing of acetic acid (0.01 M, 1.27 mL), PEG (20,000 MW, 0.135 g), urea (0.175 g), TMOS (0.45 mL), VTMS (0.15 mL) in a 10 mL flask. These two mixtures were stirred at 0 °C for 4 h to form a homogeneous solution, respectively. V50 (2 mg) was added into solution A, and then they were sonicated at 0 °C for 5 min, and manually introduced into the pretreated capillary with 40 cm length using a syringe. Introduction length for sol–gel pre-condesation solution B was 23 cm and introduction length for sol–gel pre-condesation solution A was 5 cm. Third, both ends of the capillary were sealed with rubber, and the condensation reaction was carried out at 47 °C for 12 h. Fourth, the obtained biphasic hybrid monolithic column was then flushed with water and then methanol to remove the PEG and other residuals.

2.3. Fluorescein derivatization on the surface of biphasic hybrid monolithic column

Firstly, biphasic hybrid monolithic column was rinsed with derivative solution containing 20 mM cysteamine and 2 mM AIBN for 3 h, then both ends of the capillary were sealed with rubber, and the derivatization reaction was carried out at 60 °C for 12 h. The derived biphasic hybrid monolithic columns were then flushed with methanol to remove unreacted residuals. Secondly, the derived biphasic hybrid monolithic column was rinsed with 2 mg/mL FITC solution for 3 h and then removed unreacted FITC with water.

2.4. Modification on biphasic hybrid monolithic column

Biphasic hybrid monolithic column was rinsed with derivative solution containing 20 mM 6-mercapto-1-hexanol (or octadecanethiol) and 2 mM AIBN for 3 h, then both ends of the capillary were sealed with rubber, and the derivatization reaction was carried out at 60 °C for 12 h. The biphasic hybrid monolithic column was then rinsed with methanol to remove unreacted residuals. With both ends sealed by silicon rubber, the biphasic hybrid monolithic column was stored at 4 °C in darkness before use.

2.5. Morphology characterization of the biphasic hybrid monolithic capillary column

Microscope and scanning electron microscope (SEM) were used for the characterization of monolithic capillary column. Microscopic pictures were taken with an inverted microscope Leica DMI4000B equipped with a Leica DFC480 camera (Leica Camera, Germany). Scanning electron micrographs were obtained using a JEOL JSM-840 scanning electron microscope (JEOL, Tokyo, Japan), operated at 15 kV and a filament current of 60 mA. The samples were acquired from segments of biphasic hybrid monolithic column initially cut into equal lengths (0.5 cm) and positioned longitudinally within a retractable aluminum stage. These samples were used to depict the surface view of the sol–gel coating from a longitudinal segment of the open tubular column. This stage, with the mounted capillary segments, was then placed into a Balzers SCD 050 sputter coating chamber and coated with a gold/palladium alloy at 40 mA for 60 s to avert subsequent charging.

2.6. Permeability test measurements

Permeability test measurements are carried out with LC 20A HPLC system (Shimadzu, Tokyo, Japan). Monolithic column was connected to pump with a converter, and then the pressure was collected under flow rate of 0.01 mL/min.

2.7. BSA digestion

BSA (10 mg/mL) was dissolved in 1 mL 50 mM Tris–HCl (pH 8.1) buffer containing 8 M urea. Then, 100 μL of 100 mM dithiothreitol (DTT) was added, and the tube was incubated at 50 °C for 20 min. The solution was allowed to cool to room temperature, and then 100 μL of 100 mM iodoacetamide was added. The obtained solution was incubated at room temperature in the dark for 20 min, followed by dilution with 50 mM Tris–HCl (pH 8.1) buffer to decrease the urea concentration below 1 M, and then trypsin was added with weight ratio of trypsin to protein at 1/25 and incubated at 37 °C overnight. Finally, the samples were stored at −20 °C before use [30].

2.8. Enrichment and CE procedure

All enrichment and separation procedures were performed on a Beckman P/ACE MDQ system (Beckman, Fullerton, CA, USA). Biphasic hybrid monolithic capillary column was installed in a P/ACE 2000 or 5000 capillary cartridge. The total length of the capillary column was 36.5 cm with the length from the detection window to the outlet being 8.5 cm. The effective length of the column was 28 cm. We used 20 mM KH_2PO_4–Na_2HPO_4 buffer (pH 7.17) containing 15% acetonitrile as the running buffer. Prior to operation, the biphasic hybrid monolithic column was conditioned by rinsing with running buffer followed by electrokinetic conditioning at 15 kV for 20 min.

3. Results and discussion

3.1. Preparation of biphasic hybrid monolithic capillary column

As shown in Fig. 1, two segments were contained in the biphasic hybrid monolithic column, the synthesis of VTMS monolith in segment B could refer to Ref. [31]. However, the synthesis of anion hybrid monolith in segment A was not reported. Zou's group previously reported the synthesis of organic–inorganic hybrid monolithic column by one-pot method [32], cationic monomer and initiator were added to VTMS-TOMS-sol–gel pre-condensation solution, polymer functional groups were thus introduced into the monolith by simultaneous condensation and radical polymerization reaction. In our work, anion hybrid monolith was synthesized referring to this method by adding anionic monomers to VTMS-TOMS-sol–gel pre-condensation solution. Several kinds of anionic monomers were tested, including acrylic acid, AMPS and SPMA, the result showed that a larger amount of SPMA, but not AMPS or acrylic acid could be added. This may be related to the fact that SPMA is a neutral strong electrolyte, which can promote the process of phase separation in the synthesis of monolith, and AMPS and acrylic acid may inhibit phase separation in gel process. Accordingly, SPMA was selected as the anionic monomer for synthesis of anion hybrid monolith in segment A.

First of all, the adding amount of SPMA was studied in detail, Fig. S1 shows the micrograph of anion hybrid monolith with varied amount of SPMA added. Uniform pore structure was obtained when the adding amount of SPMA was not very high, and the critical amount was found to be depending on the temperature, e.g., at 47 °C uniform pore structure was obtained when the adding amount of SPMA was 30–90 mg, but not 120 mg. While at 49 °C,

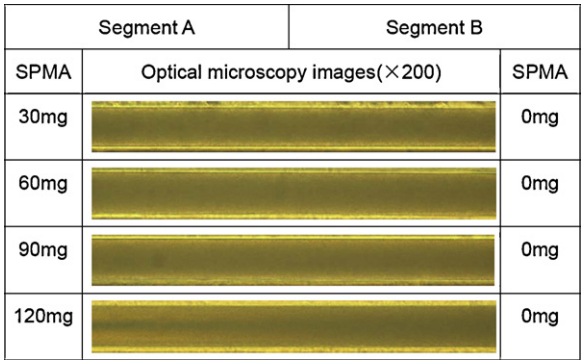

Fig. 2. Optical images of SPMA–VTMS/VTMS biphasic monolith with different amount of SPMA added.

it should not exceed 60 mg. This result implied that a lower temperature condition was favorable to increase the amount of SPMA, so the temperature of 47 °C was adopted in following experiments.

Because the two polymerization mixtures used in segment A and segment B were different, a colloid-type polymer would be easy to create by diffusion between these two polymerization mixtures, this may lead to blockage of the column, therefore, surface morphology and permeability of interface between segment A and segment B were investigated in detail, Fig. 2 shows the optical microscopy images of biphasic hybrid monolithic column with anion hybrid monolith and VTMS hybrid monolith synthesized simultaneously in the same capillary column. Uniform pore structure in both regions and seamless interface were obtained when the adding amount of SPMA was 30 mg, 60 mg or 90 mg, but not 120 mg, and a transition zone between segment A and segment B could be obviously observed when the adding amount of SPMA was 120 mg.

Further permeability test result is shown in Fig. S2. In pure water, the back pressure for biphasic hybrid monolithic column increases with the adding of SPMA. The back pressure was less than 6 MPa if the adding of SPMA was less than 60 mg. When the adding of SPMA increased to 90 mg, the back pressure dramatically increased to 10 MPa. In contrast to pure water, for organic solvent, e.g., acetonitrile, the back pressure kept low with varied amount of SPMA added. The reason for such result is probably related to the strong hydrophilicity of SPMA monomer, which shows significant swelling when exposed to water. In our experiment, an appropriate amount of 70 mg SPMA monomer was selected and the preparation of column was carried out at 47 °C.

Under the optimized conditions, the effect of the length of segment A was also investigated. It was found that the back pressure of the biphasic column increased dramatically to 9 MPa, when the length of segment A was longer than 7 cm, This indicates that upon increase of segment A length, the diffusion between the two prepolymerization solutions increases, and this led to a decrease in permeability of biphasic capillary monolithic column. Typically, lower permeability may result in interruption of the current during electrophoresis. So a 5 cm length of segment A was selected in following experiment.

In order to clearly show the interface and surface properties of these two segments of biphasic hybrid monolithic column, fluorescein isothiocyanate was used as a probe to study the surface of biphasic hybrid monolithic column on which cysteamine had been derived via thilo-ene reaction. C1 and C2 in Fig. 3 show the optical and fluorescence images of the same biphasic hybrid monolithic column. In segment B, strong fluorescence could be observed, in contrast, almost no fluorescence could be observed in segment A. While, in the interface of these two segments, the intensity of fluorescence increased from segment A to segment B. The result indicated that diffusion occurs in the border segment between the two absolutely different polymerization mixtures. However, the

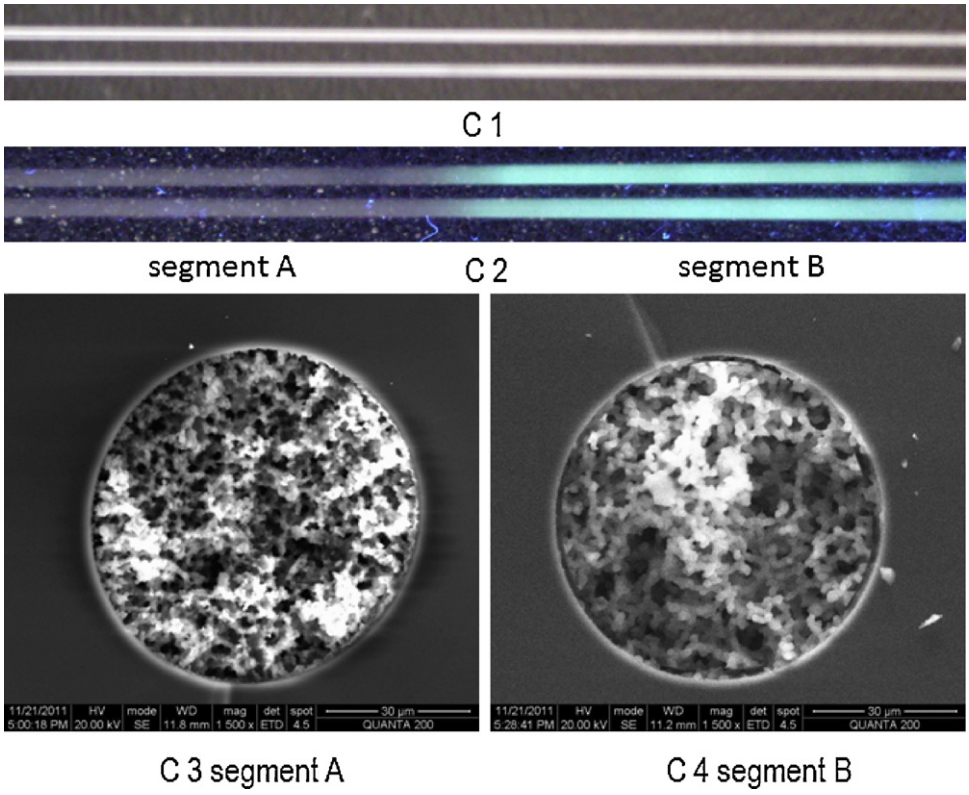

Fig. 3. Optical (C1), fluorescence (C2) and SEM (C3, C4) images of the biphasic monolithic column.

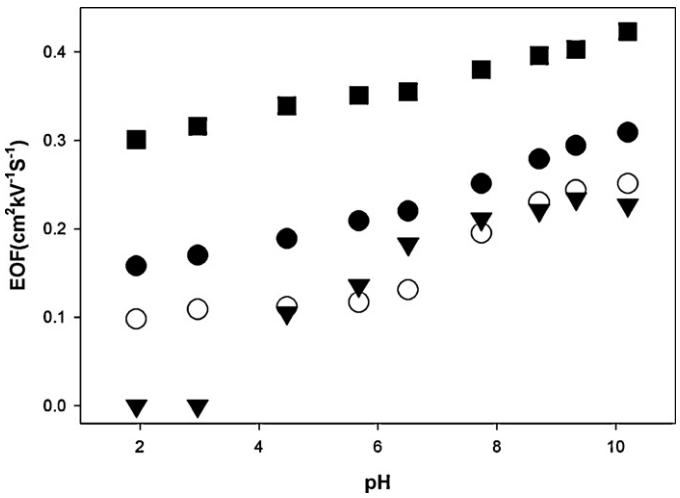

Fig. 4. The effect of pH value on the EOF of VTMS monolithic column (▼), SPMA–VTMS hybrid monolithic column (■-), SPMA–VTMS/VTMS biphasic hybrid monolithic column (●), and SPMA–VTMS/HO-C_6-S-VTMS biphasic hybrid monolithic column (○). Conditions: separation buffer, 10 mM phosprate buffer (H_3PO_4–NaH_2PO_4 for pH 2–5, NaH_2PO_4–Na_2HPO_4 for pH 6–8, and Na_2HPO_4–NaOH for pH 8–10) containing 30% ACN with various pH values (pH 2–10); void time marker of EOF, thiourea; applied voltage, 15 kV; and detection wavelength 203 nm.

difference between polymerization mixtures used in these two segments was so small that a colloid-type polymer was not formed between the two types of monoliths after reaction. Such continuous interface results in the elimination of dead volume of the column. In this study, further surface morphology investigation of monolith was carried out using SEM, C3 and C4 in Fig. 3 show the SEM images for segment A and segment B of biphasic hybrid monolithic column, respectively. It can be seen that the capillary is fully filled with the homogenous monolithic matrix, which is well attached to inner wall of the capillary. Uniform pore structure was obtained in both segment A and segment B, and pore structure of segment A was smaller than segment B due to the use of SPMA monomer in the former. The pore size of the monolithic matrix is about 1–2 μm. Hence, the mass-transfer resistance would be decreased.

3.2. Generation of electroosmotic flow (EOF) of the biphasic hybrid monolithic capillary column

An important aspect of CEC is magnitude of the EOF velocity, which influences the analysis speed and band spreading. Since the EOF is generated by the charged sites on the surface of stationary phase which is affected greatly by pH value, it is important to study the effect of the pH value on the magnitude of the EOF velocity. Fig. 4 shows the effect of pH value on the EOF of VTMS monolithic column, SPMA–VTMS hybrid monolithic column, SPMA–VTMS/VTMS biphasic hybrid monolithic column and SPMA–VTMS/HO-C_6-S-VTMS biphasic hybrid monolithic column. At low pH values, the EOF of VTMS monolithic column was hardly to determinate, this is related to non-dissociation of silanols on VTMS monolith surface under acidic condition. The EOF increased when pH was higher than 4, which is due to the dissociation of silanols of monolith surface under higher pH conditions. Nevertheless, higher EOF velocity was measured on the two biphasic hybrid monolithic column and SPMA–VTMS hybrid monolithic column in all pH ranges used, which corresponds to dissociation of sulfonic acid groups on the monolith surface. This result also indicated that segment A, in which sulfonic acid groups are integrated, acts as the important source for the generation of EOF. The EOF of SPMA–VTMS/VTMS biphasic hybrid monolithic column is greater than that after modification with 6-mercapto-1-hexanol in all pH range tested. It implies that the surface of SPMA–VTMS/VTMS biphasic hybrid monolithic column is well covered by 6-mercapto-1-hexanol, and thus resulting in the non-dissociation of surface silanol groups. This result also indicated that the modification via thiol-ene reaction on alkenyl functionalized monolith was successful. It is worth noting that the EOF of HO-C_6-S-VTMS hybrid monolithic column was hardly to determine in all pH range tested (date not show), so in the proposed method, HO-C_6-S-VTMS hybrid monolithic column cannot be used alone without SPMA–VTMS segment being integrated.

3.3. Effect of functional group bonded to segment B on the separation

It is found that thiol compounds can be derivatived to the surface of alkenyl-functionalized monolith via thiol-ene reaction. In this work, 6-mercapto-1-hexanol and octadecanethiol was selected as derivative ligands for the derivatization of segment B to which alkenyl groups were introduced. Fig. S3 shows the separation of p-phenylenediamine, aniline, p-toluidine, N-methyl aniline, N,N′-dimethylaniline and diphenylamine on SPMA–VTMS/C_{18}-S-VTMS biphasic hybrid monolithic column, SPMA–VTMS/VTMS biphasic hybrid monolithic column and SPMA–VTMS/HO-C_6-S-VTMS biphasic hybrid monolithic column respectively. As shown in Fig. S3a, six amines were washed out in 2 min and their separation was unsatisfactory without any derivatization done to the biphasic monolithic column, so further modification was required. Separation was greatly improved when biphasic hybrid monolithic column was modified with octadecanethiol. Baseline separation of p-toluidine, N-methyl aniline, N,N′-dimethylaniline, diphenylamine was achieved and higher column efficiency was obtained (Fig. S3b), however, baseline separation of p-phenylenediamine and aniline couldn't be achieved. Fig. S3c shows the separation of all six amines on SPMA–VTMS/HO-C_6-S-VTMS biphasic hybrid monolithic column, complete baseline separation was achieved and sharp peaks were obtained. Compared to SPMA–VTMS/C_{18}-S-VTMS biphasic hybrid monolithic column, higher column efficiency with narrow and symmetrical peak shapes were obtained on SPMA–VTMS/HO-C_6-S-VTMS biphasic hybrid monolithic column. This might be related to the alcohol hydroxyl on 6-mercapto-1-hexanol, which can provide additional hydrogen bonding interaction between analytes and monolith surface.

3.4. Effect of working buffer on separation

As known, the buffer pH strongly affects the net charge of analytes and the monolith surface, and the buffer ionic strength and organic solvent concentration can also affect the thickness of electrical double layer, thereby, all of such factors can affect the analytes separation. In this section, the effects of buffer pH, ionic strength and organic solvent concentration on the separation of amines were investigated. Taking into account the effect of sample matrix on the separation, a low concentration sample was used and the injection time was set to 30 s, thus the column will be filled with sample matrix before separation was carried out.

Fig. S4a shows the effect of buffer pH on separation, while the buffer ionic strength and organic solvent concentration are kept unchanged. At pH 6.64, the separation of the six tested amines is unsatisfied, probably due to the dissociation of amines under acidic condition. With pH increasing to 7.17, as their degree of dissociation is depressed, and the mobilities increased. Satisfactory separation of amines was obtained at this pH. When the pH is further increased to 8.10, the retention time of p-toluidine, N-methyl aniline, N,N′-dimethylaniline and diphenylamine significantly increase and satisfactory separation is obtained. However, the separation of p-phenylenediamine, aniline at pH 8.10 became

worse compared to pH 7.17. Given this situation, pH 7.17 was used as the working buffer pH in following separations.

The effect of buffer concentration on separation was investigated in 13.3–26.6 mM KH_2PO_4–Na_2HPO_4 buffer (pH 7.17). The result is shown in Fig. S4b, it is found that the increasing of the buffer concentration can prolong the migration time of amines, which is probably caused by the compression of the electrical double layer. At low concentration of phosphate, e.g., 13.3 mM, p-phenylenediamine and aniline cannot be effectively separated. Whereas, higher phosphate concentration can increase the separation efficiencies of amines. With the buffer concentration of 20 mM, baseline separation of six amines is obtained. Therefore, taking into account of the separation performance, 20 mM phosphate was used as a proper buffer concentration.

Fig. S4c shows the effect of organic solvent (with acetonitrile as example) concentration on separation, migration time of six amines show a characteristic convergence when the organic solvent concentration increases from 15% to 50%. This result indicated that the retention of SPMA–VTMS/HO-C_6-S-VTMS biphasic hybrid monolithic column exhibits reversed phase mechanism. It was noteworthy that the migration time of p-phenylenediamine, aniline slightly increased with increasing of organic solvent concentration, it is probably caused by the compression of electric double layer at high concentration of organic solvent, leading to the decrease of EOF. Good separation is obtained at low concentration of organic solvent, hence, 15% organic solvent was selected as an optimal concentration in next experiments, in which both satisfactory separation and sharp peaks were obtained.

3.5. Effect of segment B length on separation

Column length is one of the major influencing factors in separation. Herein, the effect of segment B length on separation of six amines was investigated. As shown in Fig. S5, no satisfactory separation of the tested amines was obtained at column length 18 cm, while baseline separation was obtained when length of segment B increased to 23 cm. Further increase of column length can improve the separation. Taking into account the analysis time, 23 cm of the column length of segment B was used.

3.6. Enrichment effect of the system

In order to prove the effect of enrichment in the two-stage monolithic column system, HO-C_6-S-VTMS hybrid monolithic column and SPMA–VTMS/HO-C_6-S-VTMS biphasic hybrid monolithic column were compared, and the result is shown in Fig. 5, the chromatogram f and chromatogram c, respectively, show the enrichment effect of the six amines on HO-C_6-S-VTMS hybrid monolithic column and SPMA–VTMS/HO-C_6-S-VTMS biphasic hybrid monolithic column when the injection time is 30 s. Obviously, almost without any effect of enrichment could be achieved without anion enrichment segment being integrated, on the contrary, good enrichment effect is obtained using the two-stage monolithic column system. As we know, the injection time and sample matrix compositions are the most important factors that affect the enrichment process, therefore, following investigations were focused on these factors mentioned above.

3.7. Effect of injection time on analyte enrichment

The effect of injection time on analyte enrichment was studied in detail, ranging from 2 s to 90 s. The result is shown in Fig. 5. Obviously, the peak area of all six amines increases with the increasing of injection time. Baseline separation of the amines is obtained when

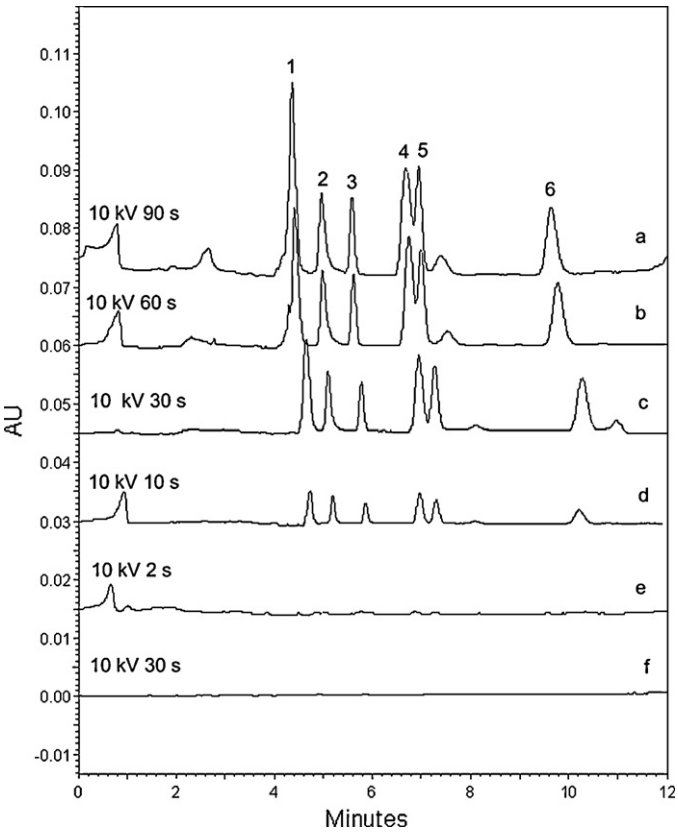

Fig. 5. Effect of injection time on the enrichment. Peak 1 p-phenylenediamine, peak 2 aniline, peak 3 p-toluidine, peak 4 N-methyl aniline, peak 5 N,N′-dimethylaniline, and peak 6 diphenylamine. Conditions: injection, 10 kV; separation solution, KH_2PO_4–Na_2HPO_4 buffer (pH 7.17) in 15% acetonitrile; sample matrix, water; sample concentration, 10 μM each; applied voltage, 15 kV; and detection wavelength 203 nm.

the injection time is less than 30 s (10–30 s). With 30 s injection time, the enrichment factor for p-phenylenediamine, aniline, p-toluidine, N-methyl aniline, N,N′-dimethylaniline, diphenylamine are124, 170, 44, 104, 97 and 108, respectively (Table S1). However, when the injection time increases to 60 s, peak of N-methyl aniline and that of N,N′-dimethylaniline overlap severely, the total enrichment factor is 180, and the enrichment factor for p-phenylenediamine, aniline, p-toluidine, diphenylamine are 179, 270, 68 and 134, respectively (Table S1). The enrichment factors at 90 s injection time are similar to 60 s injection time, The total enrichment factor for N-methyl aniline and N,N′-dimethylaniline is 202, and the enrichment factor for p-phenylenediamine, aniline, p-toluidine and diphenylamine are 234, 282, 70 and 145, respectively (Table S1). The enrichment factor increased slowly from 60 s to 90 s except for p-phenylenediamine. This result indicated that saturated adsorption is achieved at injection time 90 s. When the injection time was 30 s, the limits of detection and limits of quantitation for the six tested amines were about 1 μM (n = 3, RSD < 3.5%) and 0.2 μM (n = 3, RSD < 4.7%), respectively.

3.8. Effect of sample matrix on analyte enrichment

Fig. 6 illustrates the effect of acetonitrile concentration on enrichment. At acetonitrile concentration less than 20%, the peak area of aniline, p-toluidine, N-methyl aniline, N,N′-dimethylaniline, diphenylamine keep relatively stable, while with the increase of acetonitrile concentration, the peak area of p-phenylenediamine increase significantly. The peak area of all six tested amines decrease sharply as acetonitrile concentration increases from

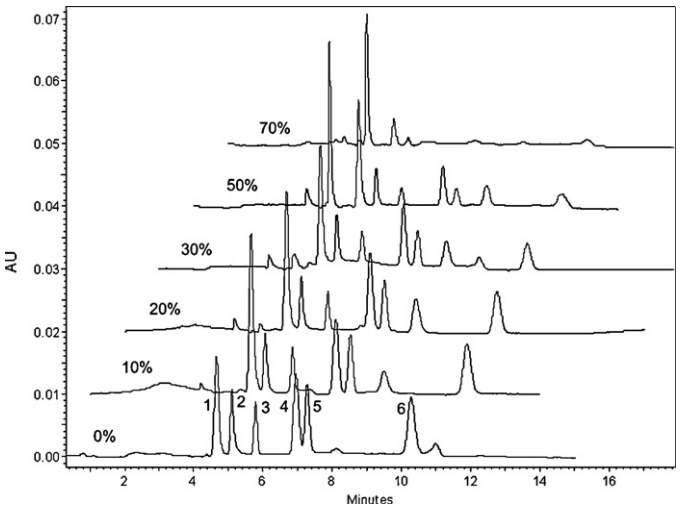

Fig. 6. Effect of acetonitrile concentration in sample matrix on the analyte enrichment. Conditions: injection, 10 kV for 30 s; separation solution, KH_2PO_4–Na_2HPO_4 buffer (pH 7.17) in 15% acetonitrile; sample matrix, solution of varied acetonitrile percentage; sample concentration, 10 μM each; applied voltage, 15 kV; and detection wavelength 203 nm. (Analyte peak designations are the same as in Fig. 5.)

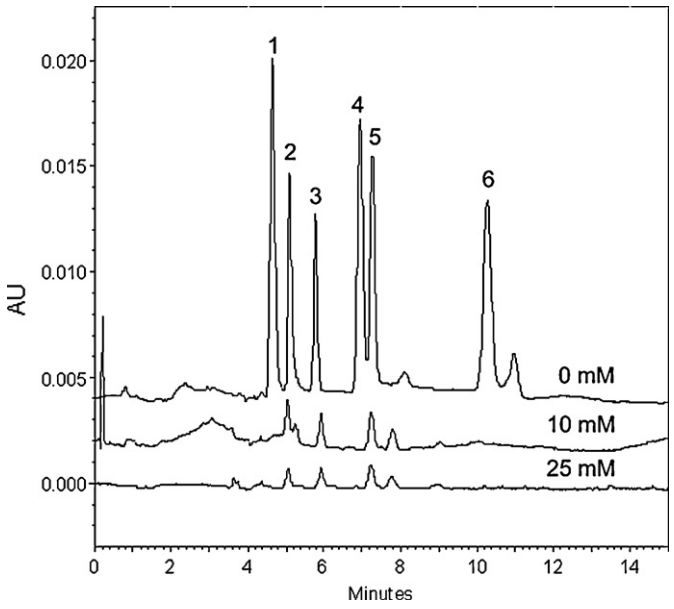

Fig. 7. Effect of electrolyte salt concentration in sample matrix on the analyte enrichment. Peak designations are the same as in Fig. 5. Conditions: injection, 10 kV for 30 s; separation solution, KH_2PO_4–Na_2HPO_4 buffer (pH 7.17) in 15% acetonitrile; sample matrix, varied concentration of sodium chloride in 10% acetonitrile; sample concentration 10 μM each; applied voltage, 15 kV; and detection wavelength 203 nm.

30 to 70%. Especially for p-toluidine, N-methyl aniline, N,N′-dimethylaniline, and diphenylamine, the peak of these amines almost completely disappear when 70% acetonitrile is used. Therefore, a low content of the organic solvent was necessary for improving the enrichment efficiency, while high content of the organic solvent would reduce the enrichment efficiency due to volume shrinkage of charged polymer occurring in the organic phase, which lead to a decrease in surface area of monolithic column. In following experiment, 10% acetonitrile was selected as the optimized organic phase content for the sample matrix.

The enrichment of analytes on ion exchange column is strongly affected by the electrolyte salt concentration of matrix due to the competition of matrix ions with analytes for adsorption on ion exchange sorbent. The effect of electrolyte salt concentration in sample matrix on enrichment of six amines on SPMA–VTMS/HO-C_6-S-VTMS biphasic hybrid monolithic column was studied. As shown in Fig. 7, as the concentration of sodium chloride increases, the peak area of amines decreases rapidly, especially for diphenylamine, the peak can scarcely be recognized when the concentration of sodium chloride increased to 10 mM. Obviously, the decline of enrichment performance is related to the competition of sodium ions with amines for adsorption on SPMA ion exchange monolithic column. It is worth noting that significant decrease of peak area isn't observed when the concentration of sodium chloride increases from 10 mM to 25 mM. Hence, in order to avoid the intolerance to high ionic strength of ion exchange sorbent, the ionic strength of sample matrix should be kept as low as possible to allow all components to bind to the ion exchange sorbent.

3.9. Repeatability and stability

The run-to-run reproducibility was evaluated using aniline as a standard analyte on a single biphasic capillary monolithic column. RSDs for retention time and peak area of the analytes were less than 2.3% for 5 runs in CEC. Both column-to-column and batch-to-batch reproducibilities for the prepared monolithic columns were also evaluated in term of the RSDs of retention time and peak area of analytes, which were less than 4.1 and 7.8% for 4 runs, respectively. These results indicate that the system we established here has acceptable repeatability and stability.

3.10. The adsorption capacity of SPMA–VTMS hybrid monolithic column

Breakthrough curves provide valuable information about the adsorption capacity and mass transfer efficiency of the preconcentration. Fig. 8 shows the results of frontal analysis using blank capillary column, VTMS monolithic column and SPMA–VTMS hybrid monolithic column. Dead volume was measured used a 10 cm blank capillary column (id, 50 μm) and the breakthrough time was found to be 2.56 min (a). For the VTMS monolithic

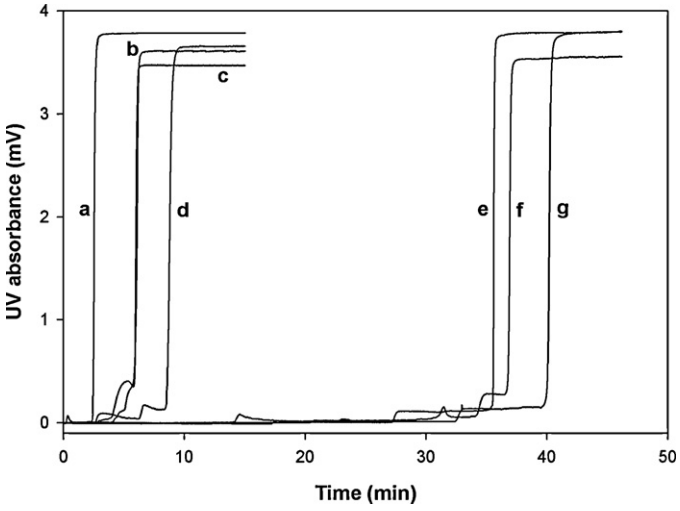

Fig. 8. Breakthrough curves obtained by fontal analysis. Curve (a), blank capillary column (length, 10 cm; id, 50 μm), curves (b)–(d) represent breakthrough curves of p-toluidine, single peptide (Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and p-phenylenediamine on VTMS hybrid column (length, 10 cm; id, 75 μm), curves (e)–(g) represent breakthrough curves of p-toluidine, single peptide and p-phenylenediamine on SPMA–VTMS hybrid column (length, 10 cm; id, 75 μm); sample, 2 mM anatyle dissolved in water; flow rate 0.5 μL/min; and UV detection at 203 nm.

column, the breakthrough curves for p-toluidine (b), single peptide (Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg) (c) and p-phenylenediamine (d) obtained at flow rate 0.5 μL/min are almost identical, and the fronts are very sharp. This indicates that mass transfer is very fast. Their breakthrough times of these curves are 6.10, 6.14, and 8.88 min, respectively. The adsorption capacities for p-toluidine, single peptide and p-phenylenediamine dissolved in water were calculated to be 3.54×10^{-4}, 3.58×10^{-4} and 6.32×10^{-4} μmol/cm. The breakthrough curves for the SPMA–VTMS hybrid monolithic column were as sharp as for the VTMS monolithic column, but longer breakthrough time was obtained, which might be resulted from the successful integration of SPMA monomer. Their breakthrough times from curves e, f, and g (Fig. 8) were determined to be 35.6, 36.9, and 40.26 min, respectively. The adsorption capacities for p-toluidine, single peptide and p-phenylenediamine dissolved in water was calculated to be 3.3×10^{-3}, 3.43×10^{-3} and 3.77×10^{-3} μmol/cm, respectively. The adsorption capacity values were calculated based on three measurements, and the RSD values were found to be less than 2.5%.

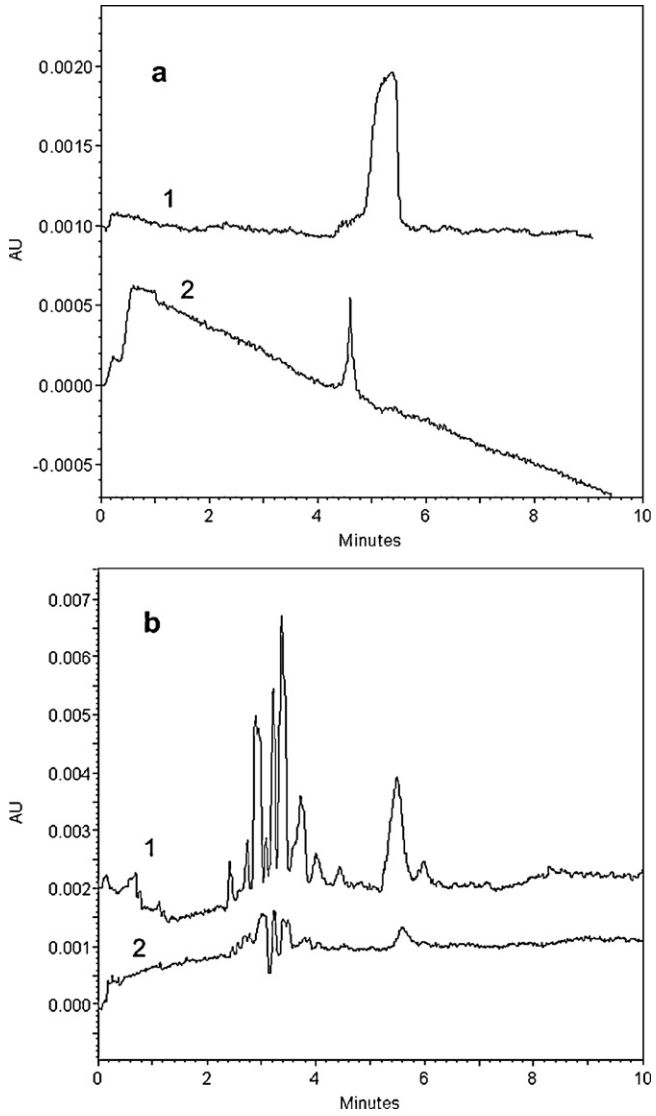

Fig. 9. Enrichment of peptide on SPMA–VTMS/C_6H_{12}OH-VTMS biphasic hybrid monolithic column. Conditions: (a1) sample, 0.01 mg/mL (Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg), injection, 10 kV for 3 min; (a2) sample, 1 mg/mL (Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg), injection, 10 kV for 3 s; (b1) sample, 0.001 mg/mL BSA hydrolysate, injection, 10 kV for 5 min; and (b2) sample, 1 mg/mL BSA hydrolysate, injection, 10 kV for 3 s. Separation buffer, 20 mM KH_2PO_4–Na_2HPO_4 buffer (pH 7.17) in 15% acetonitrile; sample matrix, 10% acetonitrile; applied voltage, 15 kV; and detection wavelength 203 nm.

3.11. Application to peptide enrichment

To demonstrate the applicability of the method to analysis of complex samples, we applied the proposed method to the analysis of peptide samples. Fig. 9a shows the enrichment of single peptide (Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg) with an enrichment factor of 783 folds compared to standard CE electrokinetic injection. Fig. 9b shows the enrichment of diluted BSA hydrolysate, peptide peaks can hardly be observed at the concentration of 1 mg/mL calculated according to BSA with 2 s of injection time, while more than ten peaks could be observed at concentration of 0.001 mg/mL with 3 min injection time. The result indicates that the proposed system is suitable for preconcentration of low abundance peptides in biological samples.

4. Conclusion

In this paper, we describe a novel biphasic silica hybrid monolithic column, the cation-exchange segment located at the inlet acts as preconcentration column, and the next ene-based monolithic segment serves as separation column, on which functional groups can be changed according to need. The applicability of this system was demonstrated using standard amines (p-toluidine, aniline, p-toluidine, N-methyl aniline, N,N′-dimethylaniline, and diphenylamine). Both good separation and enrichment effect were obtained by optimizing surface functional groups of stationary phase, separation buffer and sample matrix. The proposed system is also successfully applied to complex biological samples, such as single peptide, diluted BSA hydrolysate, and the result indicated that the system has a capacity for preconcentration of low abundance peptides.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.01.024.

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