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Hybrid organic–inorganic monolithic stationary phase for acidic compounds separation by capillary electrochromatography

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Abstract

A novel type of organic–inorganic hybrid porous silica-based monolithic stationary phase for capillary electrochromatography (CEC) has been developed by sol–gel chemistry combined with supramolecular template-based approach in a simple and rapid manner. Both chromatographic interaction and electrophoretic migration contribute to the separation of acidic compounds by the monolithic column. Eight organic acids were separated rapidly with column efficiency up to 267 000 theoretical plates/m. The influences of buffer concentration and organic modifier content on the separation have been investigated. In addition, the hybrid monolithic column was used to separate triterpenoids from *Ganoderma lucidum*.

Keywords: Stationary phases, electrochromatography; Sol–gel; Monolithic columns; *Ganoderma lucidum*; Organic acids

1. Introduction

Capillary electrochromatography (CEC) has been regarded as a very promising electrokinetic separation technique that combines the high efficiency of capillary electrophoresis (CE) and the high selectivity of high-performance liquid chromatography (HPLC) [1,2]. The combination of chromatographic partition and electromigration mechanisms provides unique selectivity over either of the individual technique. Both charged and neutral analytes can be separated simultaneously in CEC. The hampered development of CEC may be due to technical problems associated with column technology. The column, as the "heart" of CEC system, not only serves as the separation media but also supplies the force to drive the mobile phase through the column [3]. In general, CEC columns can be classified into three types: open-tubular [4–6], packed [7–9] and monolithic columns [10–32]. While open-tubular columns can be easily fabricated and display high efficiency, they suffer from low sample capacity and low detection sensitivity. At present, conventional packed CEC columns with commercially available HPLC silica beads have been most widely used. However, they possess some inherent limitations such as tedious packing procedure, frequent bubble formation, frit fabrication and lack of reproducibility. As an alternative to the packed columns, monolithic columns have attracted increasing attention in recent years due to their simple preparation procedure, no need for retaining frits and improved separation performance [10,11].

Monolithic stationary phases are prepared by polymerization of monomers in the presence of a porogen and characterized by a bimodal pore structure consisting of large through pores for flow and small diffusion pores for adsorption. Depending on the nature of materials, there are two major classes of monolithic columns: organic polymer-based monolithic columns [12–19] and silica-based monolithic columns [20–32]. The former ones have excellent pH stability, and their porous properties can be easily tailored by tuning the composition of the porogenic solvent, monomers and cross-linking agent in the monomer solution. However,

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they undergo swelling in organic solvents, which leads to the change of pore structure and lack of mechanical stability. Moreover, the existence of micropores negatively affects the efficiency and peak symmetry for small molecules. The silica-based monolithic columns can be prepared with independent control of the size of silica skeletons and through pores via the sol–gel process. The sol–gel network offers high permeability, high mechanical strength, high efficiency and good solvent resistance. Nevertheless, the preparation of conventional silica-based monolithic columns involving individual silica matrix forming and subsequent chemical bonding is not only time-consuming but also difficult to control the entire process, which leads to the problems in reproducibility [21].

As an alternative, initial incorporation of functional monomers instead of further derivatization of the silica rod can overcome the shortcomings as mentioned above. Sol–gel chemistry [33] provides a versatile approach to the synthesis of organic–inorganic hybrid materials under mild reaction conditions. Hayes and Malik [32] have prepared silica monolith with surface-bonded C_{18} ligands in a single step by sol–gel process for CEC. Co-condensation of siloxane and organosiloxane precursors by the sol–gel technique in the present of different surfactant templates to produce functionalized amorphous xerogels silica has also been extensively investigated [34–37]. In these materials, an organic moiety is covalently linked via a nonhydrolyzable Si–C bond to a siloxane species, which hydrolyzes to form a silica network. In our study, this type of organically functionalized silica materials was applied to the preparation of monolithic stationary phase for CEC in one step.

In this paper, we used two sol–gel precursors—tetraethoxysilane (TEOS) and 3-aminopropyltriethoxysilane (APTES), which were used for the formation of silica matrix and the introduction of amino moiety to generate the chromatographic surface and reversed EOF, respectively. Because the resultant hybrid silica matrix was fabricated without drying procedure, no shrinkage or crack of gel within the capillary was observed. Through experiments, such monolithic CEC columns showed excellent properties in the separation of organic acids and triterpenoids from *Ganoderma lucidum*.

2. Experimental

2.1. Instrumentation and materials

All CEC experiments were performed on a Beckman P/ACE 5010 capillary electrophoresis system equipped with a UV-absorbance detector (Beckman, Fullerton, CA, USA). Data acquisition and processing were done using Gold software (version 8.10). No pressure was applied to the ends of capillaries. An HPLC pump (Elite Analytical Instruments, Dalian, China) was used to flush monolithic columns with ethanol to extract the surfactant. A manual syringe pump (Unimicro Technologies, Pleasanton, CA, USA) was used to condition monolithic columns with mobile phase and chase bubbles out of capillaries. A model XW-80A Vortex mixer (Jingke Industrial, Shanghai, China) was used for thorough mixing of sol solutions. Fused-silica capillaries of 75 μm i.d. and 375 μm o.d. were purchased from Yongnian Optic Fiber Plant (Hebei, China).

2.2. Chemicals and buffers

The chemicals for column preparation such as tetraethoxysilane (98%), 3-aminopropyltriethoxysilane (99%) were purchased from Acros organics (Geel, Belgium), which were used directly without further purification. HPLC-grade acetonitrile (ACN) was obtained from Scharlau Chemie (Barcelona, Spain). Cetyltrimethyl ammonium bromide (CTAB), citrate acid, sodium citrate, acetone, methanol, organic acids and other chemicals were purchased from Beijing Chemical Reagents (Beijing, China). All solutions were prepared with water purified by a CLEAR SG system (Barsbüttel, Germany). Mobile phase was prepared by adjusting the buffer to desired pH value, then mixing with the appropriate amount of organic modifier. The mobile phase was degassed in an ultrasonic bath for 15 min prior to use.

2.3. Extraction and preparation of Ganoderma lucidum

The spores of *G. lucidum* (Leyss. ex Fr.) Karst. were provided by China–America Joint Venture Shenyang Wanxiang Biology Feeding Co. Ltd. (Liaoning, China) and authenticated by Professor Yushu Huo (Health Science Center at San Antonio, Texas University, USA).

The triterpenoid constituents were extracted from *G. lucidum* using a previously described procedure [38]. Briefly, the sample was dried and ground into fine power, then 2 g of powdered sample was mixed with 100 mL alcohol and then placed on a rotating shaker for 24 h. Filtrate was collected, and alcohol was evaporated under reduced pressure. A final volume of 1.5 mL sample was prepared with the addition of methanol.

2.4. Column preparation

2.4.1. Pretreatment of the inner wall of capillary

In order to clean and activate the inner surface of capillary for effective attachment of silica skeletons, the capillary was rinsed first with 0.2 M HCl solution for 30 min and then with water until the pH value of the outlet solution was 7.0. Subsequently, it was flushed with 1 M NaOH for 2 h, then water and methanol for 30 min, respectively. Finally, the capillary was purged with nitrogen at 160 °C for 3 h prior to use.

2.4.2. Preparation of monolithic columns

The monolithic hybrid matrix was prepared as follows: 112 μL TEOS, 118 μL APTES, 215 μL ethanol, 32 μL water

and 8 mg CTAB were mixed in a 1.5 mL eppendorf vial. The solution was thoroughly vortexed at room temperature for 30 s. The homogeneous mixtures were then introduced into the pretreated capillary of appropriated length by a syringe, and an empty segment was kept at the end of the capillary column. The ends of capillary were connected with a piece of Teflon tubing to form a circle, and reacted at room temperature for 24 h. The hybrid gel formed within the capillary was then rinsed with ethanol to extract CTAB, and then washed with water. For comparison, the column without CTAB was prepared under the same condition.

A detection window was then created at the end of the continuous bed by removing the polyimide coating using a razor blade. The capillary column was then cut to desirable length, and carefully installed in a CE cartridge.

2.5. Separation conditions

The total length and effective length of the capillaries were 27 and 20 cm, respectively. Before CEC experiments, the monolithic column was preconditioned with running buffer for 30 min with a manual syringe pump. Then it was further conditioned on CE instrument by electrokinetically driving the mobile phase for another 30 min under low applied voltage. Acetone was chosen as the EOF marker. The separation temperature was kept at 20 °C. During the experiments, the columns were equilibrated for about 30 min after the mobile phase was changed.

2.6. Characterization of the monolithic columns

A short length of the monolithic column was cut off and sputtered with gold for observing of the cross-section of the column by a JEOL JSM-5600LV scanning electron microscopy (SEM) (Tokyo, Japan). Pore size distribution of the porous monolithic material was measured by nitrogen adsorption method (Nowa 4000, Quantachrome, USA).

3. Results and discussion

3.1. Column Preparation

In general, the sol–gel reaction involves the following steps [33]: (1) the hydrolysis of alkoxysilane; (2) the condensation of hydrated silica to form siloxane bonding (Si–O–Si); and (3) the polycondensation of linkage of additional silanol group to form the cyclic oligomers and eventually cast a silicate network. The properties of sol–gel matrix including pore size and chromatographic ability can be tailored by a number of factors, such as the amount and type of the monomers, amount of water, pH, solvent nature, additives and reaction temperature.

By the co-condensation of TEOS and APTES in the present of CTAB, we obtained the silica-based hybrid material with chromatographically active amino groups bonded to the silicon atoms. Therefore, there was no need to conduct a separate step for surface derivatization of the created gel. This is a striking advantage over other methods. The incorporation of sol–gel precursor, APTES, possesses an amino moiety, allowing for weak anion-exchange (WAX) interactions of acidic analytes with the monolithic stationary phase. This reagent also serves to yield a positively charged surface, thereby providing the EOF from the cathode to the anode. Furthermore, three ethoxy groups of APTES allow for sol–gel reaction with the silica matrix. In addition, the silanol groups at the surface of the silica capillary can be incorporated into the polycondensation reactions, anchoring the material to the capillary walls. In our experiments, no shrinkage or crack of the resultant hybrid organic–inorganic monolith appeared since the matrix was prepared without drying.

There are some common criteria in the choice of the co-condensation reaction system, including the need to avoid phase separation of the precursors to obtain uniform distributions of functional groups and to avoid Si–C bond cleavage during the sol–gel reaction and surfactant removal. From the kinetic point of view, the formation of an organic–inorganic hybrid mesostructure is the result of the delicate balance of two competitive processes—organization of the template and polymerization [39]. The synthesis routes rely on cationic surfactant, where the main interaction between the template and the organic–inorganic hybrid species is weak electrostatic interaction under this experimental condition. The surfactant CTAB acts as supramolecular template in the formation of the sol–gel monolith. And it can be easily removed by a simple solvent extraction. In our experiment, we find that the column permeability increases with the amount of CTAB. However, increasing the column permeability leads to a substantial deterioration of separation efficiency. So, appropriate amount of CTAB should be taken into consideration.

It is noted that alkoxysilane-based sol–gel precursors usually show poor solubility in water. Therefore, we choose ethanol as one of the sol solution components to achieve a homogeneous system with all the sol–gel ingredients effectively dissolved in it. As we know, the functionalities of TEOS and APTES are four and three after hydrolysis, respectively. An increase in the ratio of TEOS to APTES will induce an increase in the condensation degree. Taking into account the mechanical strength and permeability, the solution was prepared by a mixture of TEOS and APTES in a stoichiometric molar ratio of 1:1. Water is also critical for the sol–gel process. At the beginning water is indispensable for hydrolysis, and it is then produced as a byproduct of the condensation reaction. In our experiment, the amount of water added corresponded to the stoichiometric quantity required for complete hydrolysis of the alkoxy groups of the monomers. In the reaction solution, no catalyst was added, because the amino groups of APTES serve as an “internal catalyst”. When monomers were added, gels were formed within a few minutes due to the basic properties of the func-

tional groups. Hydrogen bonds between the amino groups of APTES and silanol groups of hydrolyzed species possibly play a major role in the build-up of the gel network [40].

3.2. Structural properties of the monolithic stationary phase

The SEM photographs in Fig. 1 show that the morphology of the monolithic columns prepared without CTAB and with CTAB, respectively. As we all know, sol–gel reaction can be catalyzed by either acid or base. In the former process, the rate of the hydrolysis reaction is significantly faster than the condensation reaction, providing favorable conditions for the formation of linear polymers. In the latter one, the condensation rate is faster than the hydrolysis rate, it is easy to form highly branched polymeric structures and uniform particles formation. In our experiment, the reaction was self-catalyzed by the amino groups of APTES, which was similar to the base-catalyzed condition. From Fig. 1, it can be observed that the hybrid monolithic column prepared with CTAB was more uniform and regular than the column prepared without CTAB. Also it had the appearance of amorphous silica aggregates with more through pores in the silica matrix, which greatly improve the permeability of the monolithic column. The through pores might be introduced by the processes of sol–gel transition and organization of the template.

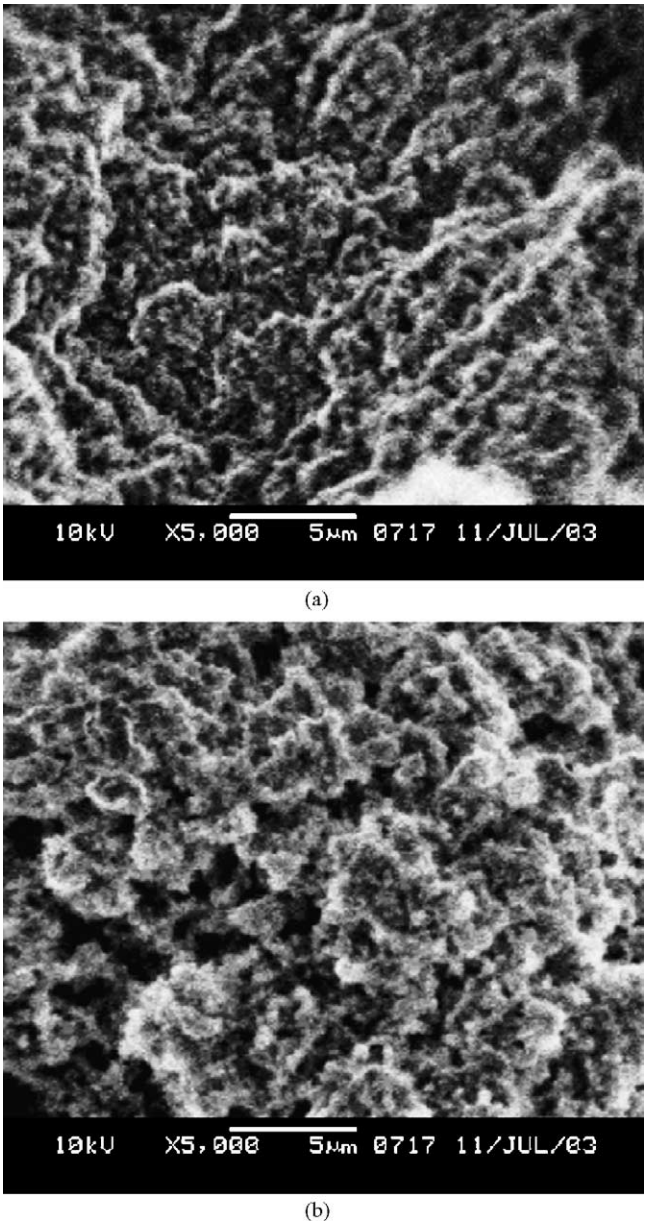

Fig. 1. SEM photographs of the cross-section of the monolithic columns. 10 kV, magnification 5000×. (a) prepared without CTAB; (b) prepared with CTAB.

The pore size distribution of the material prepared with CTAB was measured by nitrogen adsorption method in the dry state. The average pore diameter of the monolithic material is 14.2 nm with a BET surface area of 105.3 m^2/g, which is less than the conventional silica monolithic material. This result can not reflect the actual pore structure of the hybrid organic–inorganic monolithic column because the measurement was performed on the material that dried absolutely, whereas the column was prepared in the wet state and without drying.

3.3. Separation of organic acids

The analysis of acidic compounds in the reversed-phase CEC is relatively difficult because the opposite migration direction of analytes and EOF leads to a very long analysis time or samples even cannot be eluted out. Therefore, the column with positively charged surface is suitable for the separation of negatively charged analytes. Strong anion-exchange CEC was successfully used to separate acidic compounds [41]. Here, we used the organic–inorganic hybrid stationary phase for the separation of acidic compounds in the WAX-CEC mode.

The surface of the WAX monolithic column is positively charged, which results in the direction of EOF from cathode to anode. Therefore, acidic compounds will migrate with the direction of EOF, and fast separation of acidic solutes can be achieved. The separation mechanism of charged compounds on the monolithic column is the combination of electrophoretic mobility and anion exchange interaction. However, the relative contributions of electrophoretic mobility differences and differential chromatographic interactions to the retention behavior of the acidic compounds are opposite. The higher charge-to-mass ratio of the solute, on the one hand, the higher the electrophoretic mobility of acidic compounds, the faster the migration of the solute, the shorter the retention time; on the other hand, the stronger the chromatographic interactions with the stationary phase, the longer the retention time. As shown in Fig. 2, eight organic acids were separated within 7 min with 40% acetontrile in 10 mM citrate buffer (pH 3.8) as the mobile phase. The charged solutes were all eluted before void time, showing

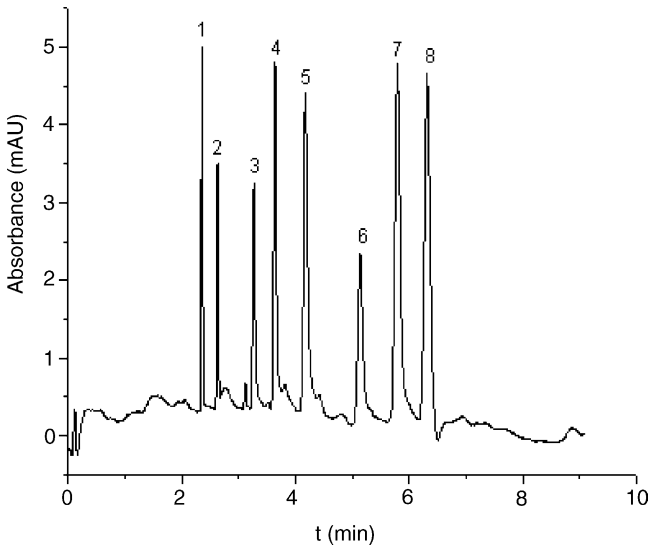

Fig. 2. Chromatogram for separation of organic acids. Experimental conditions: mobile phase, 10 mM citrate buffer containing 40% acetonitrile (pH 3.8); applied voltage, 10 kV; detection wavelength, 214 nm; sample concentration, 0.01–0.06 mg/mL; electrokinetic injection, 2 kV × 3 s. Peaks: (1) *p*-toluenesulfonic acid; (2) *p*-aminobenzosulfonic acid; (3) *p*-nitrobenzoic acid; (4) *o*-chlorobenzoic acid; (5) *m*-bromobenzoic acid; (6) benzoic acid; (7) α-naphthyl acetic acid; (8) *p*-hydroxybenzoic acid.

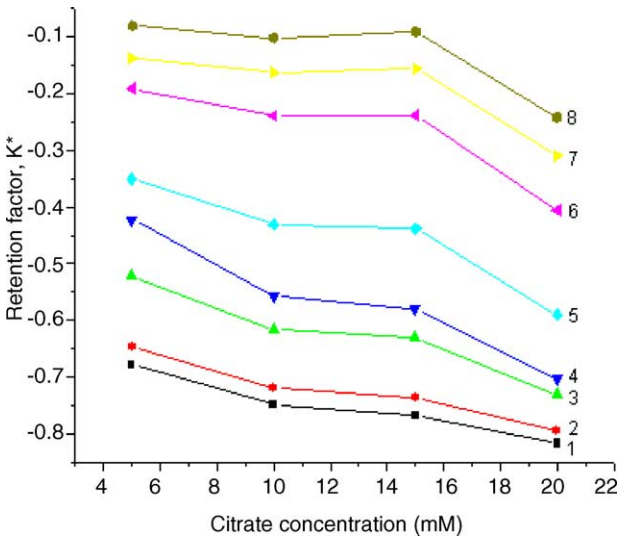

Fig. 3. Effect of the citrate concentration on retention factors (k^*). Experimental conditions: mobile phase, citrate buffer containing 50% acetonitrile (pH 3.8) with buffer concentrations ranging from 5 to 20 mM. Other conditions are the same as in Fig. 2.

that their retention on the monolithic stationary phase was relatively weak. The highest separation efficiency of up to 267 000 theoretical plates/m was achieved for the first eluted solute. The relative standard deviation (R.S.D.) values for all retention times were less than 1.85% for five consecutive runs, which showed the good stability of the monolithic column. The column-to-column reproducibility ($n = 5$) was evaluated in terms of R.S.D. values for the retention times of *p*-toluenesulfonic acid, *p*-aminobenzosulfonic acid and *p*-nitrobenzoic acid, and were found to be 7.92, 8.89 and 8.67%, respectively.

3.4. Effect of the buffer concentration on the separation of organic acids

To describe the elution of charged solutes in CEC, here we defined a nominal retention factor (k^*) based on the chromatographic formalist [42] as followings:

$$k^* = \frac{t_m - t_0}{t_0}$$

where t_m and t_0 denote the migration time of the analyte and that of an inert neutral tracer, respectively. Obviously, the k^* value reflects the concurrence of both chromatographic and electrophoretic processes.

Since all the eight solutes were eluted before void time, the k^* values were negative. The influence of ionic strength on the separation was investigated at various phosphate buffer concentrations from 5 to 20 mM in the mobile phase containing 50% (v/v) acetonitrile while keeping pH at 3.8. It was observed that the electroosmotic mobility decreased about 44% when citrate buffer concentration increased from 5 to 20 mM. In general, elution power of mobile phase in ion-exchange chromatography depends on the ionic strength of mobile phase. As the ionic strength of mobile phase increased, on the one hand, the electrostatic interactions were reduced and the retention of all analytes decreased; on the other hand, the electrophoretic mobility of acidic compounds decreased, resulting in an increase of k^* value. As shown in Fig. 3, it can be observed that the retention factors tend to decrease slightly with the increase of ionic strength, which indicates the separation of the acidic analytes is the result of a combination of electrokinetic migration and chromatographic interaction, and the latter one plays predominant role in comparison with the role of electrophoretic migration in the separation under these conditions.

3.5. Effect of acetonitrile content on the separation of organic acids

The effect of the acetonitrile content in the range from 20 to 60% (v/v) on the separation while keeping the ionic strength constant in the mobile phase was also studied. It was observed that the electroosmotic mobility decreased about 56% when the acetonitrile content increased from 20 to 60%. Because both the ratio of dielectric constant to viscosity (ε/η) and the ζ-potential of the surface of the WAX material decreased with the increase of acetonitrile content in this scale, resulting in a decrease of the electroosmotic mobility. The increase of acetonitrile content will decrease the electrophoretic mobility and lead to the longer migration time. According to Fig. 4, the electrochromatographic retention factors showed slightly decreasing tendency with the increase of acetonitrile content in the mobile phase, which

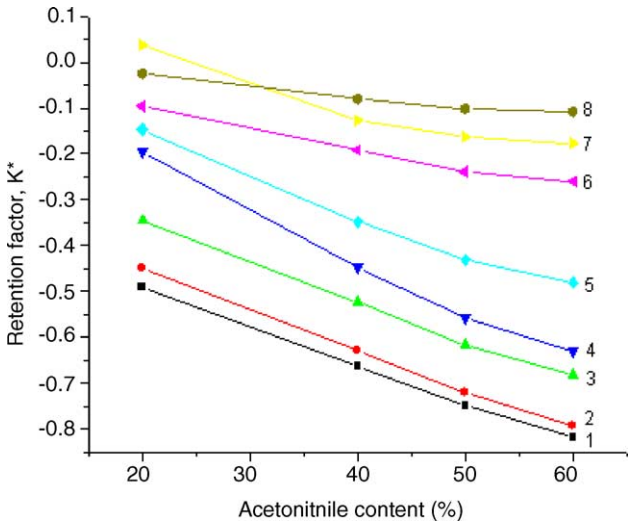

Fig. 4. Effect of acetonitrile content on retention factors (k^*). Experimental conditions: mobile phase, 10 mM citrate buffer (pH 3.8) containing 20, 40, 50 and 60% acetonitrile, respectively. Other conditions are the same as in Fig. 2.

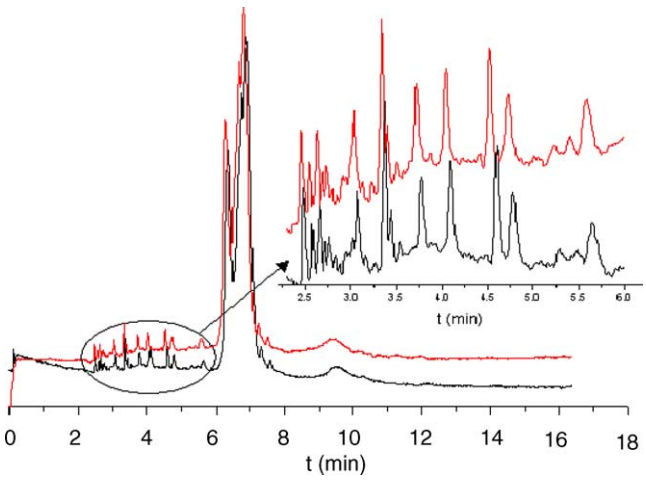

Fig. 5. Chromatograms for crude triterpenoids of *G. lucidum* in two consecutive runs. Experimental conditions: mobile phase, 10 mM citrate buffer containing 40% acetonitrile (pH 3.8); applied voltage, 10 kV; detection wavelength, 254 nm; electrokinetic injection, 3 kV × 8 s.

suggested that the interaction between the analytes and the stationary phase weakened with the increase of acetonitrile content.

3.6. Separation of crude triterpenoids of Ganoderma lucidum

Natural products and their active components as sources for new drug discovery have attracted attention in recent years. *G. lucidum*, a famous tradition Chinese medicine, has been used to treat various human diseases for hundreds of years. Modern research has revealed that *G. lucidum* contains a variety of chemical ingredients, including simple and complex carbohydrates, organic germanium, triterpenoids, adenosine, alkaloids, numerous mineral elements and amino acids [43]. Among them, triterpenoids and polysaccharides have been the focus of the international scientific community. Triterpenoids show the abilities of lowering blood pressure as well as blood lipids, and polysaccharides are found to possess potent anti-tumor effect, attributed to their immuno-modulation property.

More than 100 new triterpenoids including ganoderic acid derivatives have been reported. And the use of HPLC and thin-layer chromatography (TLC) for the fingerprint profiling of triterpenoids present in the fruiting bodies of *G. lucidum* has also been demonstrated [38,44]. However, to the best of our knowledge, there are no reports about the separation of triterpenoids with CEC. In this paper, we setup a rapid and high-resolution method with the hybrid monolithic column.

Fig. 5 showed the separation of crude triterpenoids of *G. lucidum* in two consecutive runs. The larger peaks were the peaks of solvent and uncharged samples under this condition. Most acidic triterpenoids can be well separated in 6 min and the reproducibility was satisfactory. Further optimization of separation conditions and quantitative analysis of the components were under studying. From the above-mentioned results, we can see that compared with traditional methods, such as HPLC and TLC, the CEC-based method has some advantages of low sample loading, fast speed and high separation efficiency.

4. Conclusions

A novel type of sol–gel derived hybrid organic–inorganic porous silica-based monolithic column for CEC has been developed in a single step process and successfully used for the separation of ionic compounds. It showed good mechanical strength and permeability. This monolithic column offered significant promise to the fast separation of acidic compounds. Good separation of crude triterpenoids in *G. lucidum* showed this monolithic column also had great potential in the analysis of complex system.

Acknowledgements

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