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The characteristics of open-tubular capillary electrochromatography columns with series/mixed stationary phases constructed with magnetic nanoparticle coating

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ABSTRACT

Novel open-tubular capillary electrochromatography (OT-CEC) systems with core/shell magnetic nanoparticles modified by amino or C_{18} groups as stationary phase were constructed by immobilizing nanoparticles in the capillary with permanent magnets. Influence of preparation method of OT-CEC column with series stationary phases (continuous two-dimension) on column performance and effect of dispersant on capability of OT-CEC column prepared by stationary phases with mixed functionalities (mixed stationary phases) were investigated in details to achieve stable preparation. Organic acids were used to evaluate the OT-CEC systems, and the relative column efficiency of salicylic acid was 420,000 plates/m for series stationary phases, while that of benzoic acid reached 480,000 plates/m for mixed stationary phases. The excellent within-column and between-column repeatability ($n=5$) testified with the RSDs of retention time were less than 0.44% and 10.20% for series stationary phases and 1.65% and 4.29% for mixed stationary phases. The two OT-CEC systems were further applied to separation of the aqueous extract of *Rhizoma gastrodiae*. Comparing with normal OT capillary column, the new systems show extra high column efficiency due to large surface areas of nanoparticles and multiple separation mechanisms, and they have great potential in the method development for the analysis of complicated samples.

1. Introduction

Monodisperse silica particles were usually adopted in capillary electrochromatography (CEC) as stationary phase because of easy packing, high column efficiency, good stability and low back pressure [1–3], and have been widely used in different areas [4–6]. According to the Van Deemter equation, the column efficiency increases with the decrease of particle size, which gave birth to ultra-high pressure liquid chromatography (UHPLC) [7–9]. The system using 1.7 μm particles at elevated pressure up to 100 MPa was proposed by Waters (Manchester, UK). The column efficiency was enhanced three times than 5 μm particles under the same flow rate, and the resolution was increased more than 70%. However, high back pressure involved in packing procedure and difficulty of achieving homogeneous packing when using small particles, especially nanoparticles as stationary phase, limit the requirement of higher column efficiency and faster separation speed. The distribution of particle size and the structure of chromatography column, etc. should also be considered. When particle size reduces further to submicron scale (even nano scale), the solvent transport unit which can provide higher pressure will be needed for the homogenous packing of column. Zou and coworkers [10] synthesized 400 nm silica particles and packed them into capillary under 3000 psi for CEC application. The column efficiency of 210,000 plates/m for thiourea was obtained.

Comparing with packed column, OT-CEC has the advantage of none eddy diffusion, which helps to achieve higher column efficiency. In addition, OT-CEC column is suitable for rapid analysis [11–13] because of large EOF. One of the trends in the development of OT-CEC is using nanoparticles as a stationary phase. Bare gold nanoparticles were immobilized in the sol-gel-pretreated fused-silica capillary as stationary phase by Sykora and coworkers [14] for the first time, and it was applied for the separation of hydrophobic polyaromatic hydrocarbons as well as hydrophilic cationic antimicrobial peptides. Yang and coworkers [15] developed enantioselective OT-CEC with thiolated β-CD modified gold nanoparticles as stationary phase. Efficient enantioseparation by this method was demonstrated by the analysis of three drug enantiomers with good run-to-run repeatability. However, the major defect of OT-CEC with

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Abbreviations: Id, inside diameter; Od, outside diameter; OT-CEC, open-tubular capillary electrochromatography; OTMS, octadeclytrimethoxysilane; PEDA, N-(3-(trimethoxysilyl)-propyl)-ethylenediamine

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nanoparticles as stationary phase is the tedious coating procedure and unstable coating.

Since 1970s, core/shell magnetic silica coated nanoparticles have been applied as enrichment materials and protein transport carrier due to its good dispersion, fine biocompatibility and easy modified silica surface. Slovakova et al. [16] used strong magnets to immobilize trypsin grafted magnetic beads in a microchip for protein digestion. The device represented an inexpensive way of fabricating an OT-like-column, and high performance and good reproducibility were confirmed by capillary electrophoresis (CE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). In our previous work [17], a novel one-dimensional (1D) OT-CEC system using amino modified magnetic nanoparticles coating as stationary phase was successfully constructed by using a series of external magnets to fix magnetic nanoparticles. High relative column efficiency (220,000 plates/m for anthranilic acid) was obtained, and the excellent within-column and between-column repeatability (less than 1.51% and 5.29%, respectively) have been testified.

Multi-dimensional chromatography has become one of the hotspot in analysis of complicated samples, and it shows strong power in investigation of proteomics, environmental science and herbal substance genomics [18,19]. Comparing with conventional 1D separation, multi-dimensional separation can strongly enhance the peak capacity and conveniently adjust separation selectivity. Two-dimensional (2D) system combines two separation modes with different selectivity directly (without interface) or by using interface [20–22]. Continuous 2D system [23–26] (series stationary phases, a separation mechanism followed another separation mechanism) adopts integration mode with no interface and takes the advantages of simple preparation, good repeatability and the property of total analysis. Yates and coworkers [27] described an automated continuous 2D system for multi-dimensional protein identification technology (MudPIT) by packing strong cation-exchange (SCX) resin and reversed-phase (RP) resin into one column, and a dynamic range from 10,000 to 1 between the most abundant and least abundant proteins/peptides in a complicated peptide mixture had been demonstrated. Later, they constructed another triphasic microcapillary column packed with RP material, SCX material and another RP material [28]. The column was useful in discovering co- and post-translational modifications of proteins. According to this strategy, they then assessed the effectiveness for the enrichment of phosphopeptides by using protein-based immobilized metal affinity chromatography (IMAC) as a pre-enrichment step prior to peptide-based IMAC, and 4470 unique phosphopeptides were identified in mammalian cells [29].

Mixed stationary phase, which involves multiple separation mechanisms simultaneously at the same cross section of capillary, has attracted wide interest in the separation of proteins and peptides [30–32]. Lei et al. [33] incorporated $Fe_3O_4@SiO_2–NH_2$ or SBA-15 nanoparticles into polymethacrylate monolithic column to develop novel stationary phase with mixed mechanism. The relative column efficiency of organic acids reached 290,000 plates/m and the results indicated that the incorporation of nanoparticles enhanced selectivity and column efficiency due to high specific surface area and mixed separation mechanism.

OT-CEC columns prepared with series/mixed magnetic nanoparticles stationary phases will have great potential in a wider research and application area. In this paper, silica surface of core/shell magnetic nanoparticles was modified by N-(3-(trimethoxysilyl)-propyl)-ethylenediamine and octadecyl-trimethoxysilane to obtain ion-exchange (IE) and RP functional groups. Magnetic nanoparticles and external magnetic field were used to construct series/mixed stationary phases OT-CEC columns with nanoparticle coating based on our previous work [17]. Organic acids and the aqueous extract of *Rhizoma gastrodiae* were used to evaluate the systems.

2. Materials and methods

2.1. Reagents and instrumentation

N-(3-(trimethoxysilyl)-propyl)-ethylenediamine (PEDA, 97%), octadecyl-trimethoxysilane (OTMS, 99%), isophtalic acid, salicylic acid, phthalic acid, benzoic acid and anthranilic acid were purchased from ACROS organic (New Jersey, USA); toluene was purchased from Suzhou Qiangsheng Chemical Industry Co., Ltd. (Suzhou, China). All above chemicals are analytical grade. Methanol was purchased from Shandong Yuwang Industrial Co. Ltd. (Shandong, China), and it is chromatographical grade.

Concentric cylindrical permanent magnets of 2.0 mm id × 5.0 mm od × 2.5 mm h (the flux density of each magnet is 0.2 T) were purchased from local store of Dalian, China. LSP01-1A syring pump (Baoding Longer Precision Pump Co. Ltd., China) was used for the preparation of OT-CEC column. The CEC experiments were performed on an EASYSEP GHV001 CEC system (Unimicro Shanghai Technologies Co. Ltd., China). Data collection and instrument control were realized with a Unimicro Trisep-2003 Station. Fused silica capillaries of 75 μm id – 375 μm od were purchased from Xinnuo Optical Chromatography Co. Ltd. (Handan, China). Water used in all experiments was purified by a Sartorius Arium 611 system (SARTORIUS, Germany). JSM-6360LV scanning electron microscope (JEOL, Japan), Magna 550 Fourier infrared spectrometer (NICOLET, USA), EV7 vibrating sample magnetometer (ADE, USA) and NOVA 4200e surface area and pore size analyzer (Quantachrome Instruments, USA) were used for materials characterization.

2.2. Functionalization of core-shell magnetic nanoparticles

Preparation and amination of core-shell magnetic nanoparticles were performed according to reference (see S1 in supplementary materials) [17]. After amination, the surface of $Fe_3O_4@SiO_2$ particles was covered by NH_2 groups.

Alkylation of core-shell magnetic nanoparticles was similar to Ref. [10], and the preparation method was performed as follows: 0.68 g of $Fe_3O_4@SiO_2$ was heated at 100 °C in a water bath for 3 h. After washing by methanol, nanoparticles were dried in oven at 60 °C. Subsequently, 1.36 g of octadeclytrimethoxysilane and 34.11 mL of anhydrous toluene were added into the activated nanoparticles, refluxed for 17 h at 120 °C. After the treatment, the nanoparticles were washed, respectively, by acetone and ethanol for three times, and then filtrated through a 0.45 μm filter membrane by vacuum pump. $Fe_3O_4@SiO_2–C_{18}$ particles were finally produced after drying.

2.3. Preparation of OT-CEC column

2.3.1. Pretreatment of capillary

Briefly, a capillary was orderly washed with HCl (0.1 mol/L), water, NaOH (0.1 mol/L) and water for 30 min, and then purged with nitrogen for 2 h.

2.3.2. Preparation of OT-CEC column with series stationary phases

Thirty pairs of concentric cylindrical permanent magnets were oppositely nipped to the capillary of 30 cm (Fig. 1(a)), and the length of capillary covered by magnets was 13 cm. The first pair of magnets was placed at a distance of 3.5 cm from the inlet of capillary, and the last pair of magnets was placed at a distance of 5.5 cm from the detection window. 2.5 mg/mL of $Fe_3O_4@SiO_2–NH_2$ suspension (dispersed in H_2O) was pushed into the capillary from the entrance of capillary at a flow rate of 12.50 μL/min

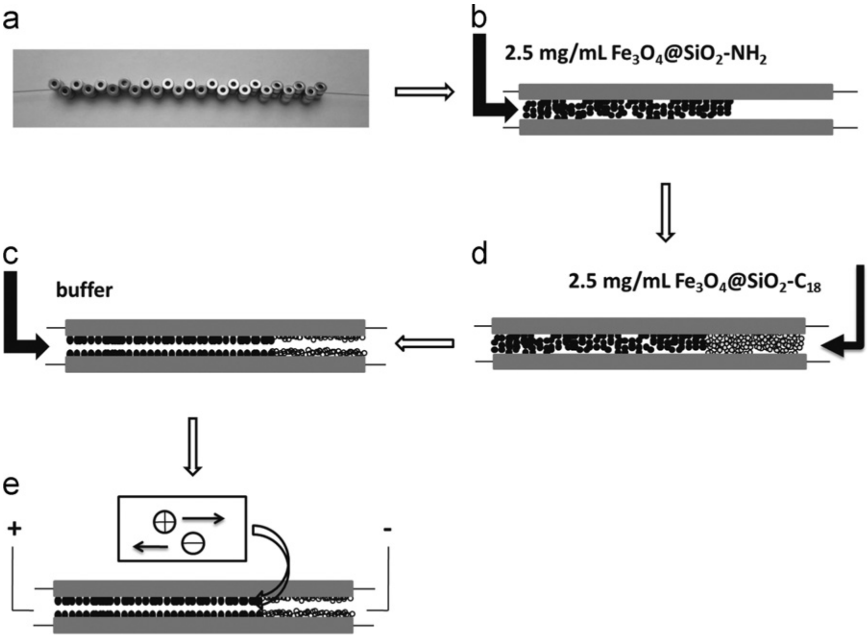

Fig. 1. Preparation of OT-CEC column with series stationary phases (I): (a) Placement of magnets, (b) loading of $Fe_3O_4@SiO_2–NH_2$, (c) loading of $Fe_3O_4@SiO_2–C_{18}$, (d) column cleaning, (e) electric balance. Gray rectangles represent magnets. Solid ball represents $Fe_3O_4@SiO_2–NH_2$, and hollow ball represents $Fe_3O_4@SiO_2–C_{18}$.

(Fig. 1(b)) till the front of nanoparticles suspension, which could be observed through the gap of magnets, reached certain place of magnetic field, which is decided by the lengths of two dimensions. Whereafter, the same concentration of $Fe_3O_4@SiO_2–C_{18}$ suspension (dispersed in acetone) was flushed from the exit of capillary at the same flow rate (Fig. 1(c)) until it reached the boundary of $Fe_3O_4@SiO_2–NH_2$ bed. When approaching the magnet zone, the nanoparticles were immobilized on the inner wall of the capillary to form a bed. 20 mmol/L of Na_2HPO_4 solution (pH 7.0) with 50% ACN (mobile phase) was used to wash away un-captured nanoparticles at the same flow rate and keep the consistency of mobile phase (Fig. 1(d)). The effective length of nanoparticle coating was 13 cm, and the operation length of capillary was 22 cm with a total length of 30 cm.

2.3.3. Preparation of OT-CEC column with mixed stationary phases

Mixtures of $Fe_3O_4@SiO_2–NH_2$ and $Fe_3O_4@SiO_2–C_{18}$ nanoparticles with different mass ratios were weighed into a centrifuge tube, and then 20 mM phosphate buffer was added into it. The total concentration of nanoparticle suspension was approximately 5.0 mg/mL. The preparation method of OT-CEC column with mixed stationary phases was the same as that in Ref. [17]. 5.0 mg/mL mixed nanoparticles suspension was flushed through the capillary at the flow rate of 12.50 μL/min, and the magnetic nanoparticles were gradually fixed onto the inner wall of the capillary by external magnetic field. Then 20 mmol/L of Na_2HPO_4 solution (pH 7.0) with 50% ACN was used to wash away un-captured nanoparticles at the same flow rate. After preparation, the effective length of nanoparticle coating was 13 cm, and the operation length of capillary was 22 cm with a total length of 30 cm. The first pair of magnets was placed at a distance of 3.5 cm from the inlet of the capillary, and the last pair of magnets was placed at a distance of 5.5 cm from the detection window with total magnets of 30 pairs.

2.4. Electrophoresis conditions

Separation voltage was -8 kV with total capillary length of 30 cm, and injection voltage was -2 kV, 5 s. Room temperature was used for all separation. UV–vis detection wavelength was set at 214 nm. Data collection frequency was 16 Hz.

2.5. Preparation of mobile phase

Totally, 150 mM phosphate buffers with different pH values were prepared as mother solution first, and the mobile phase was prepared by mixing different volumes of mother solution, ACN and water.

2.6. Preparation of the aqueous extract of *Rhizoma gastrodiae*

The aqueous extract of *Rhizoma gastrodiae* was prepared according to Ref. [17]. 8 g of *Rhizoma gastrodiae* with 80 mL of water were heated to 100 °C for 5 h. After cooling down to room temperature, the solution was filtered through a 0.45 μm filter membrane via Büchner funnel filtration.

3. Results and discussion

3.1. Characteristic of nanoparticles

In order to characterize the properties of modified magnetic nanoparticles, scanning electron microscope (SEM), infrared spectrogram (IR), vibrating sample magnetometer (VSM) and surface area and pore size analyzer were used to investigate the size, characteristic IR absorption after modification, saturation magnetization (Ms) and surface area of nanoparticles. The results are shown in Table 1. (Figures are shown in S3 in supplementary materials.)

It is shown in Table 1 that $Fe_3O_4@SiO_2–NH_2$ and $Fe_3O_4@SiO_2–C_{18}$ particles are in nanoscale and provide large specific surface area to enhance column efficiency and retention. After modification, the characteristic absorption of 3301 cm^{-1} corresponds to the stretching vibration of NH_2, and the characteristic absorption of C_{18} is 2955 and 2978 cm^{-1}. They demonstrate that NH_2 and C_{18} groups have been grafted to the surface of SiO_2. The saturation magnetizations of $Fe_3O_4@SiO_2–NH_2$ and $Fe_3O_4@SiO_2–C_{18}$ achieve

Table 1
Properties of modified magnetic nanoparticles.

	$Fe_3O_4@SiO_2–NH_2$	$Fe_3O_4@SiO_2–C_{18}$
Average diameter of microspheres	100 nm	
Characteristic IR absorption after modification	3301 cm^{-1} for NH_2	2955 cm^{-1} and 2978 cm^{-1} for C_{18}
Ms	55.5 emu/g	51.6 emu/g
Surface area	53.3 m^2/g	78.5 m^2/g

55.5 and 51.6 emu/g, respectively, which are essential to form a stable bed under the magnetic field. The surface areas of particles, which can improve the column efficiency, are 53.3 m^2/g for $Fe_3O_4@SiO_2–NH_2$ and 78.5 m^2/g for $Fe_3O_4@SiO_2–C_{18}$.

3.2. *Optimization of preparation methods of OT-CEC column with series stationary phases*

In our previous work [17], magnetic field intensity, concentration and flow rate of nanoparticles suspension were investigated, respectively, to optimize the preparation of OT-CEC columns, and a relatively stable column was obtained using $Fe_3O_4@SiO_2–NH_2$ nanoparticles. The nanoparticles bed can be observed from the gap between every pair of oppositely nipped magnets in the preparation process. It was found that the injected magnetic particles gradually gathered in the capillary covered by magnetic field, which can control the amount of particles loaded into the capillary column. Optimization of preparation method of OT-CEC column with series stationary phases was discussed below.

Firstly, thirty pairs of magnets were oppositely nipped to the capillary, then 2.5 mg/mL of $Fe_3O_4@SiO_2–C_{18}$ suspension (dispersed in acetone) was injected from the entrance of capillary into the capillary until it reached the tenth pair of concentric magnets (the length from the first pair of magnets to the tenth pair of magnets is 4.3 cm). Then 2.5 mg/mL of $Fe_3O_4@SiO_2–NH_2$ suspension (dispersed in H_2O) was injected from the same direction into the capillary until we saw particles immobilized by the 30th pair of magnets. After washing away un-captured nanoparticles with running buffer, an OT-CEC column with series stationary phases ($Fe_3O_4@SiO_2–NH_2$:$Fe_3O_4@SiO_2–C_{18}$ =2:1) was ready for further experiments (Fig. S2-1 in supplementary materials).

In this method, $Fe_3O_4@SiO_2–C_{18}$ nanoparticles were first loaded into the column. However, in the moving process of $Fe_3O_4@SiO_2–NH_2$, only small amount of $Fe_3O_4@SiO_2–C_{18}$ nanoparticles were immobilized by the last tenth pair of magnets observing by microscope. It is caused by distribution broadens of nanoparticles. This filling method led to the mixing of $Fe_3O_4@SiO_2–NH_2$ and $Fe_3O_4@SiO_2–C_{18}$ and loss of $Fe_3O_4@SiO_2–C_{18}$, which would cause uneven coating and relatively smaller surface areas, resulting in bad repeatability and poorer resolution. Even so, high column efficiency of 400,000 plates/m had been achieved and the resolutions of five organic acids separated by column prepared by this method were 1.51, 3.22, 1.98 and 1.32 in sequence (elution order was: isophtalic acid, salicylic acid, phthalic acid, benzoic acid and anthranilic acid).

Secondly, twenty pairs of magnets were oppositely nipped to the capillary, and 2.5 mg/mL of $Fe_3O_4@SiO_2–NH_2$ suspension was flushed into the capillary until fully filling the capillary covered by magnets. Then, another ten pairs of magnets were oppositely nipped to the capillary next to (after) the previous magnets. 2.5 mg/mL of $Fe_3O_4@SiO_2–C_{18}$ suspension was injected from the exit of capillary into the capillary until it reached the $Fe_3O_4@SiO_2–NH_2$ bed (the 21st pair of magnets). At last, running buffer was used to wash away un-captured nanoparticles (see Fig. S2-2 in supplementary materials).

In this method, when the rest ten pairs of magnets were installed, the former $Fe_3O_4@SiO_2–NH_2$ was easily "flowed" to the latter magnets driven by magnetic force. It caused the uneven distribution of stationary phase especially in the border of two stationary phases, which also led to poor resolution and repeatability. The resolutions of five organic acids were 1.22, 3.48, 1.49 and 1.18. Moreover, frequently interruption of CEC separations suggested bad stability of column.

The OT-CEC column prepared according to Fig. 1 possessed homogeneous dispersion of nanoparticles, and obtained high relative column efficiency and large resolution. The relative column efficiency of salicylic acid is 420,000 plates/m, and the resolutions of five acids are 2.19, 2.27, 2.55 and 1.44.

3.3. *Effect of dispersant on preparation of OT-CEC column with mixed stationary phases*

Anion exchange and RP stationary phases have different surface properties. $Fe_3O_4@SiO_2–NH_2$ particles are hydrophilic and can be easily dispersed in aqueous solution. However, $Fe_3O_4@SiO_2–C_{18}$ particles are strong hydrophobic, and it is easy to disperse them in weak polar solvents. In order to obtain good dispersion for both $Fe_3O_4@SiO_2–NH_2$ and $Fe_3O_4@SiO_2–C_{18}$ particles, medium polarity solvents including acetone, ethyl acetate, isopropanol, pyridine and tetrahydrofuran, and mixed solvents including acetone/H_2O, n-propanol/H_2O, ethanol/H_2O and running buffer (phosphate solution) were used to investigate the dispersion of $Fe_3O_4@SiO_2–NH_2$/$Fe_3O_4@SiO_2–C_{18}$. The dispersion property and sediment time of nanoparticles in these solvents are shown in Table 2.

According to Table 2, mixture of n-propanol and H_2O (1:1) was the best solvent to disperse $Fe_3O_4@SiO_2–NH_2$/$Fe_3O_4@SiO_2–C_{18}$. Homogenous dispersion was achieved by using n-propanol/H_2O, which provided enough time for preparing of capillary column by magnetic field. However, it is difficult to generate stable EOF when using this solvent as the mobile phase. Necessary step of solvent switching may lead to the formation of bubble when performing CEC separation. To avoid this, 20 mM phosphate buffer was used as a dispersion solvent and stable suspension was observed within 10 min. The nanoparticles sediment can be observed after 15 min. Therefore, ultrasonic dispersion every 10 min is necessary to guarantee the repeatability of column preparation. Eventually, relative column efficiency of 480,000 plates/m for benzoic acid was obtained, and the resolutions of five acids are 2.48, 2.17, 3.02 and 1.32 ($Fe_3O_4@SiO_2–NH_2$:$Fe_3O_4@SiO_2–C_{18}$=2:1).

Table 2
The dispersion and sediment time of nanoparticles in solvents.[a]

Solvent	Dispersion property	Initial sediment time (min)
Acetone	Poor Suspension	<1
Ethyl acetate	General suspension, colloid	3
Isopropanol	General suspension	4
Pyridine	Poor suspension	<1
Tetrahydrofuran	General suspension, colloid	5
50% acetone/H_2O	General suspension	3
50% n-propanol/H_2O	Good suspension	15
50% ethanol/H_2O	Poor suspension	<1
20 mM phosphate buffer	Good suspension	10

[a] The volume of testing vessel is 1.5 cm dia. – 4.5 cm h. The sediment distance is 3.0 cm.

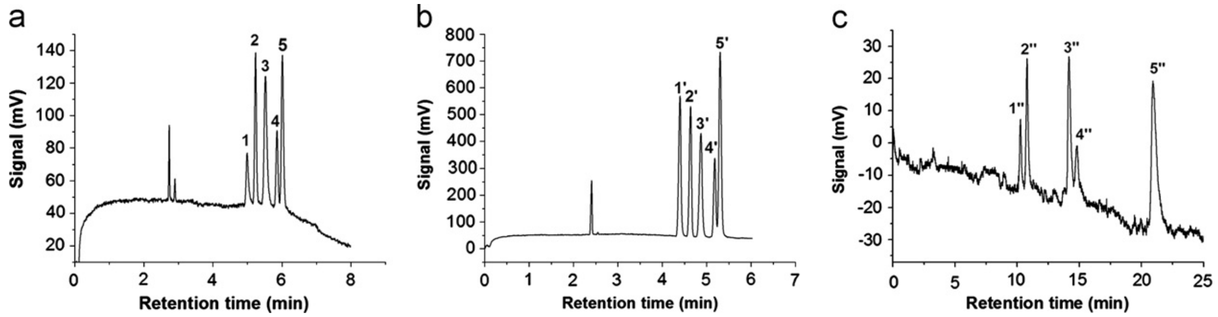

Fig. 2. Electrochromatograms of organic acids in OT-CEC columns with series/mixed stationary phases. (a) OT-CEC column with series stationary phases, $Fe_3O_4@SiO_2{-}NH_2/Fe_3O_4@SiO_2{-}C_{18}=2/1$; (b) OT-CEC column with mixed stationary phases, $Fe_3O_4@SiO_2{-}NH_2/Fe_3O_4@SiO_2{-}C_{18}=2/1$; (c) bare capillary. Experimental conditions: buffer, 20 mM Na_2HPO_4 (pH 7.0) with 50% ACN; samples: 1, 1′, 1″-isophtalic acid, 2,2′,2″-salicylic acid, 3,3′,3″-phthalic acid, 4,4′,4″-benzoic acid, 5,5′,5″- anthranilic acid.

Table 3
Retention factors of solutes with different ratios of $Fe_3O_4@SiO_2{-}NH_2$ to $Fe_3O_4@SiO_2{-}C_{18}$ in OT-CEC with series stationary phases. [a]

NH_2/ C_{18}	t_0 (min)	k'				
		Isophalic acid	Salicylic acid	Phthalic acid	Benzoic acid	Anthranilic acid
1/1	3.973	0.29	0.52	0.52	0.75	0.83
2/1	2.737	0.82	0.91	1.02	1.14	1.20
3/1	2.402	0.96	1.14	1.40	1.67	1.79
4/1	2.265	1.40	1.65	1.75	2.16	2.28

[a] Experimental conditions: electrode setting was from (−) to (+); buffer, 20 mM Na_2HPO_4 (pH 7.0) with 50% ACN. Others see Section 2.4.

Table 4
Retention factors of solutes with different pH in OT-CEC with series stationary phases.

pH	k'				
	Isophalic acid	Salicylic acid	Phthalic acid	Benzoic acid	Anthranilic acid
7.0[a]	0.82	0.91	1.02	1.14	1.20
4.2[b]	0.84	0.89	0.94	1.30	1.82

[a] Experimental conditions: electrode setting was from (−) to (+); mobile phase, 20 mM phosphate buffer with 50% ACN. Others see Section 2.4.

[b] To keep the consistency of buffer system, 20 mM phosphate solution at pH 4.2 with 50% ACN was used as a mobile phase.

3.4. Evaluation of OT-CEC column

Fig. 2(a) (b) shows the separation of five organic acids by series/mixed stationary phases OT-CEC columns with magnetic nanoparticle coating.

Considering the relative column efficiency and the resolution of organic acids, especially the resolution of the most difficult separated substances (benzoic acid and anthranilic acid), the ratio of $Fe_3O_4@SiO_2{-}NH_2$ to $Fe_3O_4@SiO_2{-}C_{18}$ of 2:1 was chosen as the optimal condition, and organic acids were well separated by both series/mixed stationary phases OT-CEC columns (separation electrochromatograms with different ratio of $Fe_3O_4@SiO_2{-}NH_2$ to $Fe_3O_4@SiO_2{-}C_{18}$ are shown in S4 in supplementary materials). In these two systems prepared by magnetic nanoparticles, EOF from anode to cathode was provided by residual silanol groups on the surface of capillary and $Fe_3O_4@SiO_2$ particles, and negative EOF was provided by amino groups on the surface of $Fe_3O_4@SiO_2{-}NH_2$ particles. The synergy effect led to small EOF from anode to cathode, and the positive EOFs were 2.36×10^{-4} and 1.82×10^{-4} $cm^2/V\ s$ for OT-CEC columns with series/mixed stationary phases, respectively. Organic acids were eluted from the capillary by electrophoretic force. The separation mechanism included electrophoretic and chromatographic mechanisms, and the latter involved IE and RP interactions. Fig. 2(c) shows the separation of five organic acids by electrophoresis, and compared with Fig. 2(a) and Fig. 2(b), high selectivity and column efficiency in CEC illustrated the difference with CE and the combination of multiple mechanisms. After calculation, the relative column efficiency of salicylic acid was 420,000 plates/m by using series stationary phases, while that of benzoic acid reached 480,000 plates/m by using mixed stationary phases. At the same condition, the column efficiency of OT-CEC column with only $Fe_3O_4@SiO_2{-}NH_2$ coating as stationary phase was 290,000 plates/m for anthranilic acid and the resolutions of five acids were 0, 4.44, 0 and 1.21 in order. The RSDs of retention time were less than 0.44% and 1.65% for five consecutive runs and 10.20% and 4.29% for five columns using OT-CEC columns with series/mixed stationary phases, respectively, which indicated good repeatability and stability. (The RSDs of retention time for OT-CEC column are shown in S5 in supplementary materials.) No change of resolution was observed for at least 30 consecutive runs. All results suggest that the OT columns prepared by $Fe_3O_4@SiO_2{-}NH_2$ and $Fe_3O_4@SiO_2{-}C_{18}$ nanoparticles have an advantage of good separation efficiency resulting from large surface area of nanoparticles and combination of different separation mechanisms.

3.5. Mechanisms of OT-CEC column

To demonstrate the RP separation ability of $Fe_3O_4@SiO_2{-}C_{18}$ nanoparticles, mixture of aniline, o-xylene, 1,3,5-trimethylbenzene, phenanthrene or o-xylene, 1,3,5-trimethylbenzene, phenanthrene were used to evaluate the performance of OT-CEC columns with series/mixed stationary phases, and the neutral samples were eluted according to their hydrophobicity, which verified RP mechanism of the OT columns (results are shown in Fig. S6-1 and Fig. S6-2 in supplementary materials). After calculation, the relative column efficiency of o-xylene was 96,000 plates/m and 64,000 plates/m by using series stationary phases and mixed stationary phases, respectively. The shorter effective length of column of 8.67 cm ($NH_2/C_{18}=1/2$) led to the lower column efficiency.

Effect of different ratio of $Fe_3O_4@SiO_2{-}NH_2$ to $Fe_3O_4@SiO_2{-}C_{18}$ on column performance in OT-CEC column with series stationary phases was investigated. Positive EOF gradually reduced with the increase of $Fe_3O_4@SiO_2{-}NH_2$. The separation mechanism of organic acids changed with the proportion of amino and C_{18} groups. At pH 7.0, organic acids existed in charged species and interacted mainly with IE stationary phase instead of RP stationary phase. When keeping effective length of the capillary constant, the interaction between negative charged solutes and IE stationary phase enhanced with increasing the length of $Fe_3O_4@SiO_2{-}NH_2$, which led to the increase of retention factor,

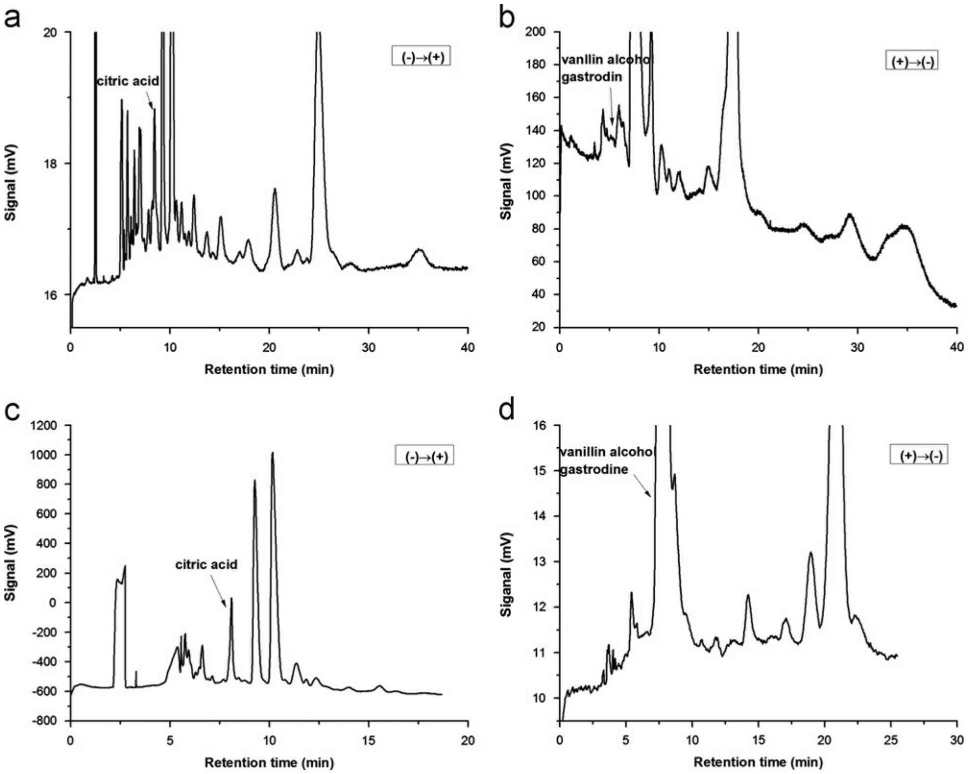

Fig. 3. The electrochromatograms of the aqueous extract of *Rhizoma gastrodiae* in OT-CEC columns with series/mixed stationary phases. Experimental conditions: buffer, 20 mM NaH_2PO_4 (pH 7.6) with 50% ACN; column, (a, b) series stationary phases, (c, d) mixed stationary phases; ratio of $Fe_3O_4@SiO_2–NH_2$ to $Fe_3O_4@SiO_2–C_{18}$, 2:1; injection voltage, (a) cathode, −4 kV, 4 s, (b)anode, −4 kV, 4 s, (c) cathode, −8 kV, 15 s, (d) anode, −6 kV, 6 s.

resulting in enhancement of retention (shown in Table 3). The best separation of five organic acids was achieved by using 2:1 ratio of $Fe_3O_4@SiO_2–NH_2$ to $Fe_3O_4@SiO_2–C_{18}$ (see Fig. 2(a)). Similar results were found in OT-CEC column with mixed stationary phases (see Table S7-1 in supplementary materials).

Five organic acids were separated at different pH in OT-CEC column with series stationary phases while keeping the same ratio of $Fe_3O_4@SiO_2–NH_2$ to $Fe_3O_4@SiO_2–C_{18}$ further investigate the mechanism, and the results are shown in Table 4. At pH 4.2, isophalic acid, salicylic acid and phthalic acid were negatively charged species, benzoic acid existed in both neutral and charged species, and anthranilic acid was electrically neutral. The migration of benzoic acid and anthranilic acid depended on EOF and interaction with RP stationary phase, the retention factors of benzoic acid and anthranilic acid increased compared to those of pH 7.0 (negatively charged), and the increasing tendency of anthranilic acid was more significant. The result suggests that RP stationary phase contributes more to the increase of retention when the hydrophobicity of solute was enhanced. Similar results were found in OT-CEC column with mixed stationary phases (see Table S7-2 in supplementary materials).

In conclusion, the separation mechanism of the OT-CEC column with series or mixed stationary phases is mixed mechanism of weak IE and RP processes.

3.6. Application

Rhizoma gastrodia is one of Chinese traditional medicine and commonly used for the treatment of neurasthenia, epilepsy, tetanus, nervous headache and other diseases in clinical. Gastrodin (4-hydroxyphthalic-β-D-glucopyranoside), vanilla alcohol (4-hydroxyl-3-hydroximethoxy) and citric acid are active ingredients in *rhizoma gastrodia*, which play a role of dilation of blood vessels, anti-inflammation and immunity and somnific action in human body. OT-CEC columns with series/mixed stationary phases were applied into the separation of the aqueous extract of *Rhizoma gastrodiae*. The electrochromatograms are shown in Fig. 3, and principal components of gastrodin, vanilla alcohol and citric acid were identified according to increasing the peak height by adding standards. Neutral pH was chosen to get acidic, neutral and basic components in one sample. Considering small EOF, multiple separations using different electrode setting were conducted to guarantee neutral, positive and negative charged components in *rhizoma gastrodia* can be eluted from OT-CEC columns. The results show great separation ability of prepared columns for complicated samples.

4. Conclusions

Continuous 2D and mixed stationary phases OT-CEC columns were prepared by fixing magnetic nanoparticles functionalized by amino and C_{18} groups separately with magnets. The separation selectivity can be adjusted conveniently by changing the ratio of two kinds of different nanoparticles. High specific area of nanoparticles and multiple separation mechanisms improved column efficiency and separation ability significantly. It has great potential in the method development of analysis of complex samples owning to simple preparation process.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.11.021.

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