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Electrochromatographic characterization of methacrylate ester-based monolith and capillary electrochromatography separation of flavonoids

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ABSTRACT

A porous polymethacrylate ester-based monolithic column for capillary electrochromatography (CEC) was designed by mean of *in situ* co-polymerizing lauryl methacrylate (LMA), ethylene dimethacrylate (EDMA) and 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) in a ternary porogenic solvent including cyclohexanol, 1,4-butanediol and water. After investigating the influence factors of the CEC monolithic columns, four flavonoids (i.e., Rutin, Quercetin, Kaempferol, and Quercitrin) were separated and assayed to evaluate this monolithic column with CEC method. Under optimum conditions, the CEC method exhibited high separation efficiency, with rapid separation time of 3–4 min, for the four flavonoid samples using 10 mM phosphate buffer containing 70% acetonitrile (pH 9.0). Importantly, the proposed method could provide a promising approach for rapid separation and detection in biomedicine.

1. Introduction

Flavonoids which exist abundantly in medicinal plants, are a widely distributed group of polyphenolic compounds with health-related properties [1]. These properties are mainly correlated to their antioxidant activity, such as anticancer, antiviral, anti-inflammatory, effects on capillary fragility, and their ability to inhibit human platelet aggregation [2,3]. Various methods have been developed for the separation and detection of flavonoids in medicinal plants, such as polyamide chromatography, gas chromatography (GC) [4,5], high performance liquid chromatography–mass spectrometry (HPLC–MS) [6–8], capillary electrophoresis (CE) [9–12], micellar electrokinetic capillary chromatography (MECC) [13–16] and microemulsion electrokinetic chromatography (MEEKC) [17]. To the best of our knowledge, there are few reports about the separation and determination of flavonoids by capillary electrochromatography (CEC) using methacrylate ester-based monolithic columns [3,18].

CEC has attracted much attention as a new micro-separation technique, because it combines high selectivity of HPLC and high efficiency of capillary electrophoresis (CE) [19–24]. Column preparation plays a key role for the successful development of the CEC chromatographic technique. Recently, various separation columns have been developed, such as open tubular column CEC (OT-CEC), packed column CEC (P-CEC) and monolithic column CEC (M-CEC). Moreover, the advantages and disadvantages of these columns have been discussed and evaluated in the literature [25–28]. Monolithic columns have been extensively applied in CEC due to simple preparation without frit formation and wide selection of monomers with differently functional groups including acrylamide-, styrene-, and methacrylate ester-functionalized silica columns [29–31]. Methacrylate ester-based monolithic columns represent rich advantages such as adjustable polarity, superior control against pore characteristics, and high stability under extreme pH conditions. Furthermore, methacrylate ester-based monolithic columns prepared with desired monomers and cross-linking agent together with suitable porogenic solvents, UV light or γ-ray for the initiation have been developed for an array of new applications [32,33].

The aim of this work was to exploit a porous monolithic column which was based on hydrophobic methacrylate ester for the application in CEC. The monolithic column was fabricated by *in situ* co-polymerization of lauryl methacrylate (LMA), ethylene dimethacrylate (EDMA) and 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) in a ternary porogenic solvent including cyclohexanol, 1,4-butanediol and water. The prepared monolithic column was used for the separation and determination of four multi-phenolic flavonoids. The separation performance and mechanism was deduced on the basis of the separation conditions.

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2. Materials and methods

2.1. Reagents and materials

LMA, EDMA, AMPS, 1,4-butanediol, 3-(methacryloyloxy)propyltrimethoxysilane (γ-MAPS), and cyclohexanol were purchased from Alfa Aesar (Shanghai, Alfa Aesar China). A fused-silica capillary of 100 μm i.d. was obtained from Yongnian Optic Fiber Plant (Yongnian, HeBei, China). Azobisiso-butyronitrile (AIBN), acetonitrile (ACN), NaH_2PO_4 and methanol (HPLC grade) were obtained from Sinopharm Chemical Reagents (Shanghai, China). Rutin, Quercetin, Kaempferol, and Quercitrin were supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Water was purified with a Milli-Q purification system (Millipore, Bedford, MA).

2.2. Apparatus

The CEC experiments were performed on an Agilent ^{3D}CE (Agilent Technologies, Waldbronn, Germany) system equipped with a diode array detector system (DAD) and an external nitrogen pressure source. The cassette holding the capillary column was kept at 25 °C. Agilent CE ChemStation was used for the instrument control, data acquisition and data analysis. Prior to use, all mobile phases for CEC were degassed with a KQ3200E ultrasonic bath (Kuisan, China). SEM photographs of monolithic materials were taken with an environmental scanning electron microscope XL30 (Philips, Eindhoven, Netherlands).

2.3. Preparation of polymeric monolithic columns

Prior to experiment, the inner wall of the fused-silica capillary was functionalized with 3-(trimethoxysily)propyl methacrylate as described in the literature [34]. The polymeric monolithic columns were prepared as follows: (i) 300 μL LMA, 200 μL EDMA, 2.5 mg AMPS (0.5% with respect to the monomers), and 750 μL of a ternary pore-forming solvent (cyclohexanol: 1,4-butanediol: water = 6:3:1, w/w) were mixed in a centrifuge tube; (ii) 5.0 mg AIBN (1% with respect to the monomers, as an initiator) was added into the centrifuge tube, and the mixture was sonicated for 10 min to obtain clear solution; (iii) purging with nitrogen for 10 min, the mixture was injected into the pretreated capillary column (25 cm; total length: 35 cm); (iv) the capillary column was sealed at 60 °C with both ends in water bath for 12 h. Finally, the resulting monolithic capillary columns were flushed with methanol for about 2 h using HPLC pump. A detection window was burned with a flame torch adjacent to the monolithic material. The morphology of polymeric monolithic column was characterized by SEM (Philips, Eindhoven, Netherlands).

2.4. Electrochromatography procedures

Prior to experiment, the four flavonoid standards were dissolved in methanol, and then were diluted to 0.1 mg/mL. Phosphate buffer with various pHs were prepared by adding phosphoric acid to disodium hydrogen phosphate. All buffers were filtered with a 0.22-μm membrane filter. Standard solutions and running buffer were degassed by ultrasonication for 10 min. Each monolithic column was kept at 25 °C in the instrument and equilibrated with the mobile phase by applying a stepwise increase in voltage up to 25 kV under 8 bar pressure at both ends of the column until the baseline signal was stabilized. The separations were performed at 20 kV. The sample standard solutions were injected electrokinetically with 10 kV for 6 s in CEC. Separation of flavonids in the CEC experiment was performed using a prepared capillary of 35 cm (10 cm inlet to detector) × 100 μm i.d. × 365 μm o.d. Detection wavelength was performed at 254 nm.

3. Results and discussion

3.1. Monolithic morphology

To achieve a permanent porosity in polymer-based monoliths, the polymerization is usually performed in the presence of porogens, which produce phase separation of the solid polymer from the unreacted porogens [35]. In this paper, a ternary porogenic solvent containing cyclohexanol, 1,4-butanediol and water was used as porogen for the preparation of the porous monolithic column.

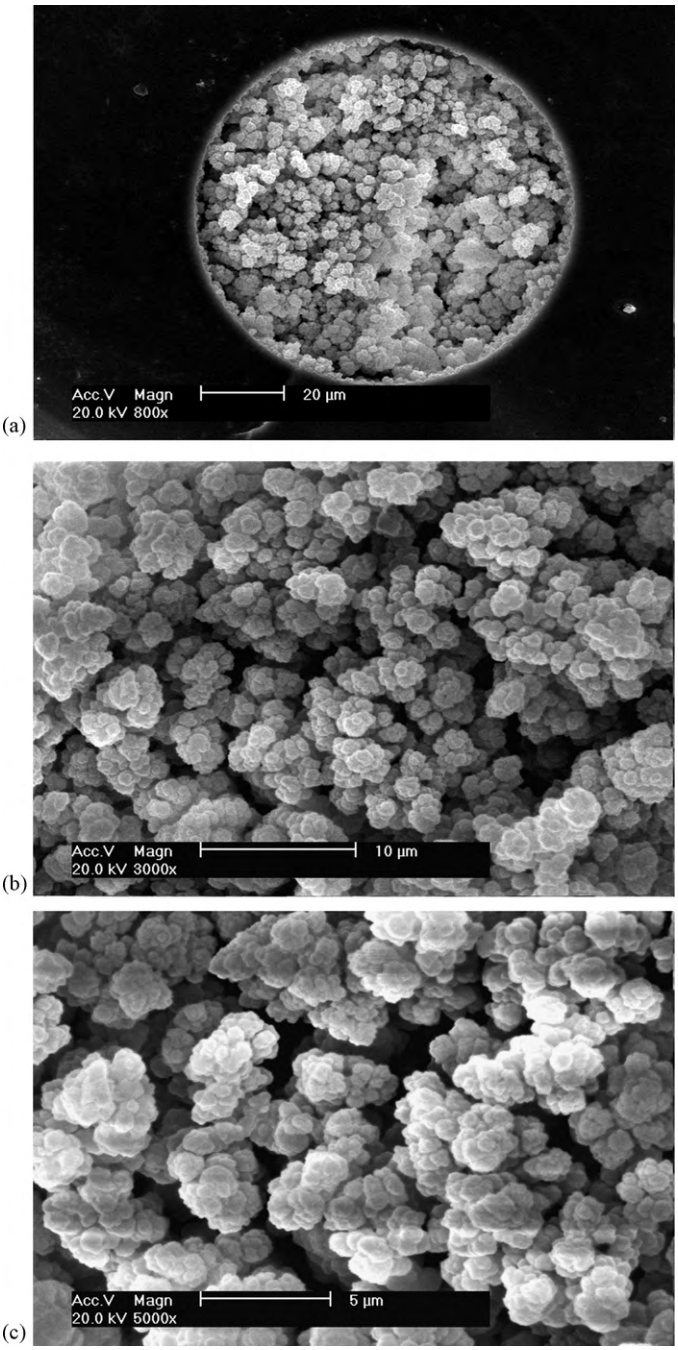

Fig. 1. Scanning electron microphotographs of monolithic columns: (a) 800×; (b) 3000×; (c) 5000×.

Table 1
Reproducibility of EOF, retention factor, and efficiency on column.

	EOF (%RSD)	Retention factors (%RSD)			Capillary efficiency (%RSD)		
		Thiourea	Phenol	Benzene	Thiourea	Phenol	Benzene
Column-to-column (n = 9)	2.0	3.6	3.8	4.0	3.5	3.5	3.7
Run-to-run (n = 5)	1.7	1.6	1.7	1.7	2.7	2.8	3.0
Day-to-day (n = 3)	2.6	2.4	2.3	2.5	4.5	4.7	4.8

Fig. 1 shows the monolithic bed, which is linked to the pretreated capillary wall for the stability of the column. This monolithic bed exhibits a uniform structure, continuous microglobules and large through-pores. The uniform structure could not only provide the possibility of fast analysis, but also offer high separation efficiency.

3.2. Performance of monolithic column

To evaluate the advantage of the homemade monolithic column, thiourea, phenol and benzene mixture were used as a model system. The EOF was measured with thiourea as unretained marker, and calculated in the experiment. The column performance was evaluated using a mixture which included phenol, benzene, toluene, phenylethane and naphthalene. According to hydrophobic interaction mechanism, it was possible well separated at an applied voltage of 20 kV with 10 mM phosphate buffer (pH 7.0). The theoretical plates of the five compounds were 1.11×10^5, 0.85×10^5, 0.91×10^5, $0.82 \times 10^5$and 1.05×10^5 plates/m on the monolithic column, respectively.

3.3. Reproducibility and stability

The reproducibility of the monolithic columns was assessed by the relative standard deviation (RSD) with different columns using thiourea, phenol and benzene as the model system. The measurement was performed using thiourea as the unretained marker. The results are listed in Table 1. The RSD of the EOF velocity, separation efficiency and retention factors k^* of column-to-column (n = 9) were <2.0%, <4.0% and <3.7% for the model system, respectively. Run-to-run (n = 5) and day-to-day (n = 3) repeatability were very satisfactory with low relative standard deviation (RSD < 2.6%) for EOF, (RSD < 2.5%) for k^* and column efficiency (RSD < 4.8%) in the CEC mode. Therefore, the reproducibility and stability of the prepared monolithic columns were acceptable.

3.4. CEC separation

Four flavonoids were separated with the developed monolithic column (Fig. 2). To investigate the separation performance and the mechanism of this poly (LMA-co-EDMA) monolith, various experimental conditions including the pH of mobile phase, ACN content, buffer concentration and applied voltage were further studied.

3.4.1. Effect of ACN content

To describe the elution of charged solutes in CEC, the retention factor (k^*) is defined by the following equation:

$$k^* = \frac{(t_R - t_0)}{t_0} \quad (1)$$

where k^* is the retention factor, t_R was the migration time of the analyte and t_0 is the migration time of the EOF marker in CEC.

The k^* value reflects the concurrence of both chromatographic and electrophoretic processes. The influence of ACN concentration on the retention factor (k^*) of four flavonoids is shown in Table 2. Seen from Table 2, k^* decreases dramatically with the ACN concentration increasing from 25% to 80%, and the elution order is Rutin, Quercitrin, Quercetin and Kaempferol. To explain this behavior, it is possible to presume that flavonoids are presented as either neutral molecules or partial ionization flavonoids at the pH condition (pH 9.0). Hence, the different separation behaviors were attributed to their differences in their different hydrophobicity.

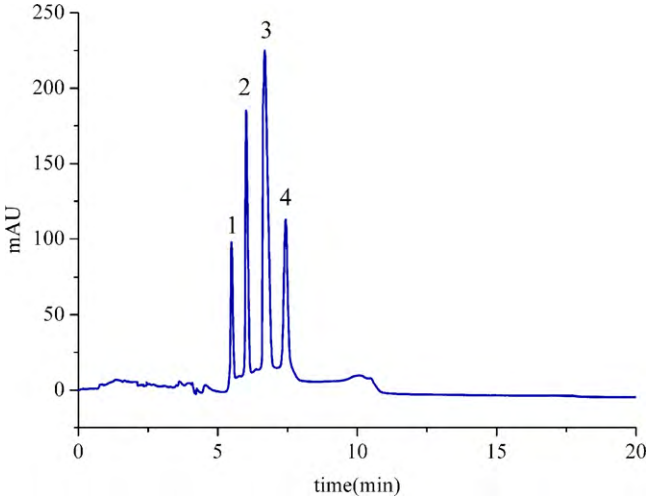

Fig. 2. Electrochromatogram of four flavonoids in LMA-based monolithic column, Separation conditions: mobile phase: 10 mmol/L phosphate buffer and ACN (30/70, v/v), pH 9.0; applied voltage 20 kV; column temperature 25 °C; injection 10 kV for 6 s; 1: Rutin; 2: Quercitrin; 3: Quercetin; 4: Kaempferol.

3.4.2. Effect of the pH

It is well known, the pH of the mobile phase often affects the solute ionization and polarity of the monolithic stationary phase. Thus, it plays an important role on the retention time of the analytes. The effect of mobile phase pH value on the retention time of four flavonoids was investigated by changing the pH of running buffer solutions. The pH of the stock 0.1 M phosphate buffer was initially diluted to 10 mM, and then adjusted with phosphoric acid to the desired pH. The effect of pH value on the retention factors was shown in Table 3. It could be seen that, the retention factors decreased with the increment of the pH from 2.5 to 10.0. The retention factors were obviously changed between various molecules from 4.0 to 8.0; and it was also observed that these changes were weak with the increasing the pH of mobile phase from 2.5 to 4.5 and

Table 2
Effect of ACN content on the retention factors (k^*).

ACN (%)	k^*			
	k^*_{rutin}	$k^*_{quercitrin}$	$k^*_{quercetin}$	$k^*_{kaempferol}$
25	1.31	1.49	1.70	1.90
45	1.01	1.20	1.33	1.41
60	0.84	0.97	1.14	1.21
70	0.54	0.80	0.96	1.06
80	0.43	0.63	0.66	0.87

Experimental conditions: capillary column, 25 cm effective length, 35 cm total length × 100 μm i.d.; mobile phase, 10 mmol/L phosphate buffer pH 9.0; supplement pressure: 8 bar; applied voltage: 20 kV; detection wavelength: 254 nm; other conditions as in Fig. 2.

Table 3
Effect of pH value on the retention factors (k^*).

pH	k^*			
	k^*_{rutin}	$k^*_{quercitrin}$	$k^*_{quercetin}$	$k^*_{kaempferol}$
2.5	1.22	1.25	1.28	1.30
4.0	1.17	1.21	1.24	1.28
6.0	0.88	0.94	0.96	0.98
7.5	0.63	0.68	0.71	0.75
9.0	0.59	0.64	0.67	0.70
10.0	0.55	0.57	0.62	0.65

Experimental conditions: mobile phase: 10 mmol/L phosphate buffer and ACN (30/70, v/v), pH 9.0; other conditions as in Table 2.

8.0 to 10.0. The reasons might be that flavonoids ($pK_a > 8.5$) were presented as neutral molecules in the acid condition, and the EOF was supplied by the sulfonic group, so the separation behavior was attributed to their difference in hydrophobicity. It is well known that flavonoids tend to be partially ionized (REF). The EOF was supplied by the sulfonic group. The separation behavior depended on their difference in hydrophobicity and electrostatic interactions. The result shows that at pH 10.0, the best separation of the four flavonoids was achieved, with the retention factors being weakened and the retention times shortened. However, the longevity of the column was shortened at this high pH, so pH 9.0 was selected in our experiments.

3.4.3. Effect of buffer concentration

The effect of the buffer concentration on the retention factors of four flavonoids was also studied by varying the concentration of phosphate buffer from 5 to 30 mM in the mobile phase of ACN/phosphate buffer (70/30, v/v). The results indicate that the retention factors of the four flavonoids increased slightly with the concentration increase from 5 to 30 mM. At pH 9.0, the four flavonoids would be negatively charged due to their partial ionization, thus both hydrophobic and electrostatic interactions would be responsible for their retention. The potential electrostatic repulsion between the phosphate residues of the flavonoids and negatively charged sulfonic groups decreased with increasing phosphate buffer concentration in the mobile phase. When phosphate buffer concentration increased, hydrophobic interactions also weakly decreased, with the results that there was a slight increase in the retention of the four flavonoids. However, the joule heating was also raised with increasing phosphate buffer concentration in the mobile phase, so to increase the lifetime of column (by lowering the joule heating) 10 mM was selected as the phosphate buffer concentration in these experiments.

3.4.4. Effect of applied voltage

The relationship between the retention behavior and applied voltage in CEC has been studied in the literature. The effect of electric field strength on the separation factor was investigated using applied voltage from 5 to 25 kV on the column with the mobile phase containing 70% ACN (v/v) and 10 mM phosphate buffers (pH 9.0). It was well known that the retention time decreased with the increase of applied voltage from 5 to 25 kV. The retention time of flavonoids was longest from 5 to 15 kV, and the resolution of flavonoids Quercetin and Quercitrin was poor when the applied voltage was 25 kV. In view of the retention time and resolution of flavonoids, the optimal separation of voltage was determined to be 20 kV.

4. Conclusion

A poly (LMA-co-EDMA) monolithic column has been prepared in fused-silica capillary using an *in situ* co-polymerization method. The sulfonic group on the surface of the stationary phase was dissociated to form the negative charge to generate the EOF. The monolithic column has been successfully applied to the separation of four flavonoids. The electrochromatography mechanism and electrophoretic migration of the separation of four flavonoids were studied systematically. The separation conditions such as pH, salt concentration and ACN content showed that the separation was a mixed mode of hydrophobic and electrostatic interaction. In these regards, capillary electrochromatography could play an important separation technique for the control of natural medicines.

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References

[1] E.de. Rijke, P. Out, W.M. Niessen, F. Ariese, C. Gooijer, U.A.Th. Brinkman, J. Chromatogr. A 1112 (2006) 31.
[2] E. Bouskela, F.Z.G.A. Cyrino, L. Lerond, Br. J. Pharmacol. 122 (1997) 1611.
[3] C. Desiderio, A.D. Rossi, M. Sinibaldi, J. Chromatogr. A 1081 (2005) 99.
[4] M.P. Fernandez, P.A. Watson, C. Breuil, J. Chromatogr. A 922 (2001) 225.
[5] G.J. Soleas, J. Yan, D.M. Goldberg, J. Chromatogr. B 757 (2001) 161.
[6] W. Wu, C. Yan, L. Li, Z.Q. Liu, S.H. Liu, J. Chromatogr. A 1047 (2004) 213.
[7] M.J. Dubber, V. Sewram, N. Mshicileli, G.S. Shephard, I. Kanfer, J. Pharm. Biomed. Anal. 37 (2005) 723.
[8] A. Tolonen, J. Uusitalo, Rapid Commun. Mass Spectrom. 18 (2004) 3113.
[9] B.L. Lee, C.N. Ong, J. Chromatogr. A 881 (2000) 439.
[10] C.T. da Costa, D. Horton, S.A. Margolis, J. Chromatogr. A 881 (2000) 403.
[11] G. Chen, H. Zhang, J. Ye, Talanta 53 (2000) 471.
[12] X. Xu, H. Ye, W. Wang, L. Yu, G. Chen, Talanta 68 (2006) 759.
[13] P.J. Larger, A.D. Jones, C. Dacombe, J. Chromatogr. A 799 (1998) 309.
[14] M.A. Rodríguez-Delgado, M.L. Pérez, R. Corbella, G. González, F.J. García Montelongo, J. Chromatogr. A 871 (2000) 427.
[15] Q.C. Chu, L. Fu, Y.Q. Guan, J.N. Ye, J. Agric. Food Chem. 52 (2004) 7828.
[16] Y.Y. Peng, F.H. Liu, J.N. Ye, Chromatographia 60 (2004) 597.
[17] L.S. Yu, X.Q. Xu, G.N. Chen, Electrophoresis 29 (2008) 726.
[18] X.J. Chen, J. Zhao, Q. Meng, J. Chromatogr. A 1216 (2009) 7329.
[19] V. Pretorius, B.J. Hopkins, J.D. Schieke, J. Chromatogr. 99 (1974) 23.
[20] J.W. Jorgenson, K.D. Lukacs, J. Chromatogr. 218 (1981) 209.
[21] L.A. Colon, G. Burgos, T.D. Maloney, R.L. Rodriguez, Electrophoresis 21 (2000) 3965.
[22] F. Svec, E.C. Peters, D. Sykora, J.M.J. Fréchet, J. Chromatogr. A 887 (2000) 3.
[23] Y. Li, H.W. Liu, X.H. Jian, J.L. Li, Electrophoresis 21 (2000) 3109.
[24] J. Ding, B. Ning, G. Fu, Y. Lu, S. Dong, Chromatographia 52 (2000) 285.
[25] A. Rocco, S. Fanali, J. Chromatogr. A 1191 (2008) 263.
[26] X.C. Wang, H.X. Lu, X.C. Lin, Z.H. Xie, J. Chromatogr. A 1190 (2008) 365.
[27] P. Kubáň, V. Kubáň, J. Chromatogr. A 1190 (2008) 377.
[28] S. Oguri, C. Oga, H. Takeda, J. Chromatogr. A 1157 (2007) 304.
[29] F.M. Okanda, Z. El Rassi, Electrophoresis 26 (2005) 1988.
[30] J.J. Ou, J. Dong, X.L. Dong, Z.Y. Yu, M.L. Ye, H.F. Zou, Electrophoresis 28 (2007) 148.
[31] E.F. Hilder, F. Svec, J.M.J. Fréchet, J. Chromatogr. A 1044 (2004) 3.
[32] A.M. Adalid, J.M. Herrero-Martínez, S. Roselló, A. Maquieira, F. Nuez, Electrophoresis 28 (2007) 4120.
[33] M.J. Lerma-García, E.F. Simó-Alfonso, G. Ramis-Ramos, J.M. Herrero-Martí-nez, Electrophoresis 28 (2007) 4128.
[34] F.G. Ye, Z.H. Xie, X. Wu, X.C. Lin, Talanta 69 (2006) 97.
[35] M. Bedair, Z. El Rassi, J. Chromatogr. A 1013 (2003) 35.