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Preparation and evaluation of a porous monolithic capillary column for microextraction of estrogens from urine and milk samples online coupled to high-performance liquid chromatography

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ABSTRACT

A porous monolithic capillary column based on poly (methacrylic acid-co-ethylene glycol dimethacrylate) (poly (MAA-co-EGDMA)) was prepared using methanol and polyethylene glycol 6000 as mixed porogens. The monolith has the characteristics of good permeability, high extraction efficiency and long lifetime. Improved permeability of the monolith could realize sample loading with high flow rate. A simple and convenient construction that employed valve-switch technique was designed for online coupling of the monolithic capillary column to high performance liquid chromatography. In order to obtain optimum extraction efficiency, the extraction conditions including sample pH, sample volume, extraction and desorption flow rate were investigated. Under the optimum conditions, the enrichment factors were 180–362 for five estrogens, indicating remarkable preconcentration ability of the monolithic capillary column. The dynamic binding capacity (DBC) was estimated to be 3.73 mg mL^{-1} via frontal analysis. Finally the monolithic capillary column was successfully applied to online enrichment of estrogens from urine and milk samples followed by high performance chromatography. Low detection limits (S/N = 3) of the proposed method were achieved in the range of 0.04–0.35 μg L^{-1}. The recoveries were 95.6–106.1% and 76.5–116.8% for the spiked urine and milk samples respectively, with the RSDs of 1.7–9.9%.

1. Introduction

Miniaturization is important trend in the field of sample preparation. Solid-phase microextraction (SPME), now considered to be a fairly mature sample preparation technique, has advantages of simplicity, low cost, ease of use and rapid pre-concentration [1,2]. The original SPME technique uses a glass fiber with polymer coatings which has the merit of being easily connected to GC injection. In-tube solid phase microextraction (in-tube SPME) is an evolution from fiber-based SPME technique that uses a capillary column as an extraction device [3]. The main advantage of this technique is its convenience to couple on-line with high-performance liquid chromatography (HPLC). In addition, it was developed to overcome some problems related to the use of conventional fiber-based SPME, such as fragility and low sorption capacity.

The sorbing phase for in-tube SPME have been classified in three formats, packed particles, open-tubular coatings and monoliths. Compared with the other two forms of sorbents, monolithic column, which has satisfactory phase ratios, ensures high sample loading [4]. Monolithic column is generally formed in situ from reactant solution without the need for frits, and is easy to prepare owing to the availability of various precursors. Most importantly, monolithic materials have binary porous structure, mesopores and macropores. The presence of micron-size macropores ensures fast dynamic transport and low backpressure in application, thus allows a high flow-rate to achieve high analytical speed [5–7].

Basically, monolithic columns are divided into two groups: rigid organic polymer-based monoliths and silica-based monoliths. Both of the two formats have been adopted as the sorbents of in-tube SPME. The drawback of the silica-based monoliths is that they are apt to hydrolysis of the Si–O–C linkage, especially under moderately acidic or slightly alkaline conditions. Organic polymer monoliths, which show stability within the entire range of pH and exhibit excellent biocompatibility, are very suitable to serve as in-tube SPME media. Feng and co-workers [8–11] have prepared a series of organic polymer monoliths, including poly (methacrylic acid-co-ethylene glycol dimethacrylate) (poly (MAA-co-EGDMA)), poly (glycidyl methacrylate-co-ethylene glycol dimethacrylate) (poly (GMA-co-EGDMA)), poly (acrylamide-co-vinylpyridine-co-N,N′-methylene bisacrylamide) (poly (AA-VP-Bis) monolith. They termed this in-tube SPME technique as polymer monolith microextraction (PMME) technique. The PMME technique combined with HPLC was applied to determination of basic drugs, angiotensin II

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receptor antagonists, and sulfonamides in several kinds of edible animal-based products such as milk, egg, fish and chicken. The organic–inorganic hybrid silica monoliths, which combine the advantages of silica with organic polymer monoliths, have also been reported as extraction sorbent for in-tube SPME in dealing with water, milk and urine samples [12–14].

The development and evolution of new monolithic columns have offered significant improvement in performance of extraction and separation. For the preparation of monolith with homogeneous and narrow-distributed pores, polymerization protocols in the presence of templates (e.g. surfactants, silica spheres and gels) have been proposed in recent years [15]. For instance, a metal–organic coordination gel template method developed by Yang et al. has improved the porosity of the monolith significantly [16]. They also prepared a temperature-responsive poly (N-isopropylacrylamide-co-N,N′-methylenebisacrylamide) [poly(NIPAAm-co-BIS)] monolith via a free-radical polymerization technique [17]. To improve the selectivity of the PMME technique, the molecularly imprinted polymer (MIP)-based monolith was prepared, and allowed selective enrichment of target analytes from complicated samples [18]. Monolithic capillary column containing hydroxyapatite nanoparticles was prepared for separation of proteins and enrichment of phosphopeptides [19]. Moreover, porous monoliths were reported to combine with micro-scale analytical technique, such as microchip, capillary electrophoresis to improve sensitivity and separation ability [20,21].

The estrogens were found to have relationship with the development of breast cancers. Increased risk for breast cancer has been reported in women with high circulating and urinary estrogen levels, as well as in those exposed to increased estrogen levels over time. However, free estrogens in biological samples usually occur at extremely low levels and are difficult to detect. Current methods for measuring estrogens have involved immunoassay [22], gas chromatography–mass spectrometry (GC–MS) [23], HPLC with ultraviolet detection (UV) [24,25], fluorescence detection (FD) and HPLC–MS [26]. Immunoassays are sensitive methods, but suffer from cross-reactions due to the similar structures of the estrogens. Chromatographic method could determine individual estrogen. However, their sensitivity and selectivity limit their direct use for determination of estrogens at trace concentration. Usually, several sample pretreatment procedures before instrumental analysis are necessary, including solid-phase extractions, ion-exchange column separations, liquid–liquid extractions, or derivatization procedures. These procedures are usually performed manually and separately, leading to long analytical time and laborious operation. Recently, newly developed sample pretreatment method, including cloud point extraction [27], stir bar sorptive extraction (SBSE) [28,29], membrane extraction [30] and solid-phase microextraction (SPME) [31–33] have been reported for monitoring estrogens either in pharmaceutical formulation, in environment matrices or in biologic samples. In our previous work, the MIP coated SPME fiber using 17β-estradiol as template was prepared for determination of four estrogens in fishery samples [34]. However, the method was not sensitive enough owing to the low sample capacity of the SPME fiber. A microextraction medium with improved loading capacity would facilitate the increase of enrichment factor. In addition, the possibility to analyze estrogens using an on-line approach in complicated matrices is interesting and inspiring.

The aim of our work was to develop a new online sample preparation method with a well-designed monolithic capillary column and a simple online extraction device for determination of estrogens in complicated samples. For this purpose, a poly (MAA-co-EDGMA) monolithic capillary column with improved porosity was prepared and coupled online to HPLC for analysis of estrogens from urine and milk samples. The structure of the polymer monolith consists of polar groups and carboxyl groups in the hydrophobic bone structure. These specific characteristics provide hydrogen bond interaction, hydrophobic interaction between the monolith framework and estrogens. The analytical method could quantify very low quantities of five estrogens simultaneously within short time.

2. Experimental

2.1. Chemicals and materials

Estradiol (E2), estrone (E1) and ethinyl estradiol (EE) were purchased from Zizhu Tiangong Technology Co., Ltd. (Beijing, China). Diethylstilbestrol (DES) and hexestrol (99%, HXS) were obtained from Yuancheng Gongchuang Technology Co., Ltd. (Wuhan, China). The chemical structures of the five estrogens were shown in Fig. S1, Supplementary material. Methacrylic acid (MAA), azo(bis)-isobutyronitrile (AIBN) and polyethylene glycol 6000 (PEG6000) were from Damao Chemical Regent Company (Tianjin, China). Trimethylolpropane trimethacrylate (TRIM) and ethylene glycol dimethacrylate (EGDMA) were purchased from Corel Chemical Plant (Shanghai, China). 3-(Methacryloxy)propyltrimethoxysilane (γ-MPS) was obtained from Shengda Fine Chemical Industry Corporation. Methanol, acetonitrile (HPLC grade) was purchased from Sigama. Water used for HPLC was doubly distilled and filtered through a 0.45 μm nylon filter. Other chemicals were of analytical pure.

2.2. Preparation of capillary monolithic column

Fused-silica capillaries (O.D. 375 μm and I.D. 320 μm), purchased from Yongnian Optic Fiber Plant (Hebei, China), were activated with 1 mol L^{-1} NaOH and then 1 mol L^{-1} HCl. After rinsed with purified water, they were dried at 100 °C in oven for 1 h. Furthermore, the capillaries were pre-treated by γ-MPS and cut into 10 cm long. Afterwards, 47 μL of MAA, 400 μL of EGDMA and 1100 μL of methanol were mixed together in a test tube, and then PEG 6000 (160 mg) and initiator AIBN (4.5 mg) were added. After sonicated for 20 min, the solution was filled into the treated capillary. The capillary was sealed by silicone rubber at each end and kept at 60 °C for 16 h. The unreacted reagents and porogens were washed by methanol with HPLC pump.

2.3. Measurement of the dynamic binding capacity

The dynamic binding capacity was measured by using dihydrodiethylstilbestrol as the model analyte. Dihydrodiethylstilbestrol solution in distilled water at the concentration of 1.0 mg L^{-1} was purged through the monolithic capillary column at the flow rate of 0.15 mL min^{-1}. Dynamic binding capacity was calculated according to Eq. (1).

$$DBC = \frac{C_0(V - V_0)}{V_C} \quad (1)$$

where DBC is the dynamic binding capacity (mg mL^{-1}), C_0 is the feed concentration of dihydrodiethylstilbestrol (mg L^{-1}), V is the volume of dihydrodiethylstilbestrol solution pumped into the column at 50% breakthrough (mL), V_0 is the dead volume of the HPLC system (mL), and V_C is the total column volume of the monolith (mL).

2.4. In-tube SPME–HPLC procedures

The in-tube SPME coupled online to HPLC system was established and illustrated in Fig. 1. The whole system consists of a six-port injection valve (Valve 1), two six-port valves (Valve 2 and 3), a sample loop and a 10 cm-length capillary. The monolithic capillary column was mounted on the injection loop of valve 2. The

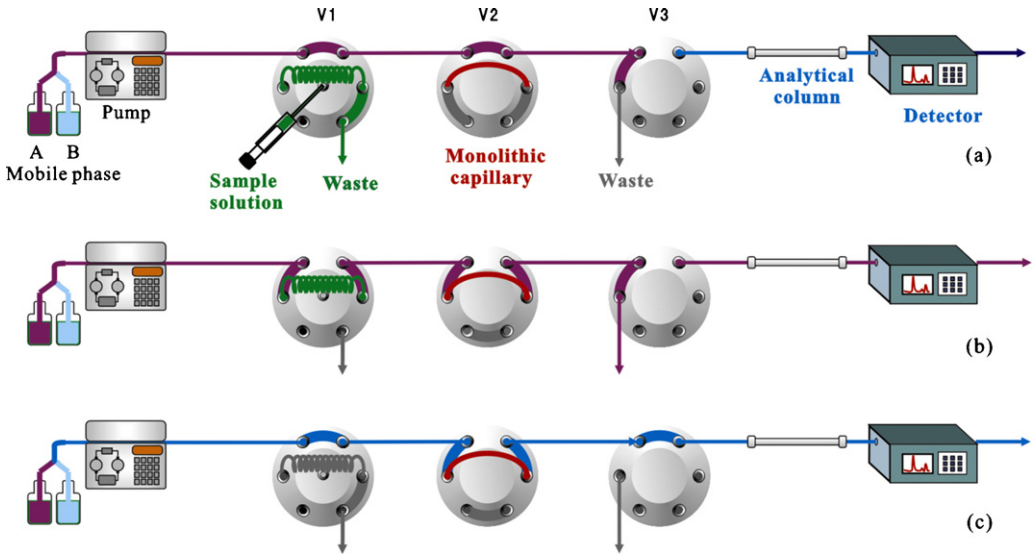

Fig. 1. Schematic diagram of the online microextraction system coupled to HPLC. (a) Sample loading and precondition; (b) Extraction and cleaning-up; (c) Desorption procedure.

in-tube SPME–HPLC procedures could be divided in three steps as described in Table 1. (a) Sample loading and precondition. Valve 1 and 2 were initially set to LOAD position, and valve 3 was set to INJECT position. Before extraction, the carrier solution was driven by the HPLC pump to flow through the monolith for precondition of the extraction system. At the same time, the sample loop was filled with 9 mL of the sample solution using a syringe. (b) Extraction and cleaning-up. The three valves were switched to the INJECT position. The sample solution was driven by the carrier solution, usually the water or buffer solution of the mobile phase, to flow through the monolithic capillary column at the flow rate of 0.20 mL min^{-1}. Afterwards, the monolithic capillary was cleaned up in order to eliminate the residual sample solution in the capillary after the sample solution loading. (c) Desorption procedure. Valve 1 and 3 was switched back to LOAD position. The extracted analytes were desorbed from the monolithic capillary column to the analytical column with the mobile phase at the flow rate of 0.20 mL min^{-1}, followed by adjusting the flow rate of mobile phase to 1.0 mL min^{-1} for chromatographic separation after Valve 2 was switched to LOAD position.

The schematic diagram was some different from that reported before [8], in which a high pressure pump was required to drive the carrier solution besides the HPLC pump serving as the driven force of the mobile phase. In this study, construction of the online microextraction system coupled to HPLC required no additional pump and less changes of the HPLC system, thus was simple, convenient and easy for popularization. The precision of the method could be guaranteed by accurate control of the valve-switching time interval.

2.5. Chromatographic measurements

The HPLC system assembled from Shimadzu LC-20A (Shimadzu, Japan) consists of a model LC 20AB pump and a model SPD-20A UV detector. A LC-solution workstation (Shimadzu, Japan) was utilized to control the system and also for data analysis. A Diamonsil C18 (250 mm × 4.6 mm, 5 μm particle size, Dikma) column was used. The mobile phase consisted of 45% acetonitrile and 55% distilled water. The mobile phase flow-rate through the column was 1.0 mL min^{-1} at room temperature. UV wavelength was set at 225 nm.

Urine samples were obtained from a female volunteer. Milk samples were purchased from local retail markets. These samples were stored at −20 °C before use. A portion of 9 mL sample was mixed and homogenized with 100 μg L^{-1} estrogen standard solutions to obtain the spiked samples at the concentration of 1.0 μg L^{-1} and 5.0 μg L^{-1}. Then 300 μL of perchloric acid was added to the milk samples for protein precipitation. The sample was homogenized and centrifuged for 5.0 min at 3000 rpm and the supernatant was collected for analysis. All sample solution was filtered through a 0.45 μm pore cellulose filter prior to in-tube SPME–HPLC analysis.

3. Results and discussion

3.1. Investigation of porogen on the column permeability

Organic polymer monolithic materials are obtained by in situ polymerization in a “mold”. Therefore, the selection of porogen is crucial. A porogen is used to obtain appropriate permeability of the monolithic capillary column so as to pump the sample solution through the column with low flow resistant. For monolith preparation, solvent with smaller molecular weight is usually used to create mesopores, while compounds with higher molecular weight such as dodecanol and PEG are used to create macropores. In this study, PEG 6000 combined with different solvents including methanol, acetonitrile and DMF were used as the mixed porogens for in situ preparation of monolithic framework within a silica capillary. In order to examine the permeability of the resultant monolithic

Table 1
Program for in-tube SPME/HPLC process of Fig. 1.

Step	Event	Valve 1	Valve 2	Valve 3	Mobile phase
a	Sample loading and precondition	Load	Load	Inject	100% water
b	Extraction and cleaning-up	Inject	Inject	Inject	100% water
c	Desorption	Load	Inject	Load	45% acetonitrile and 55% water

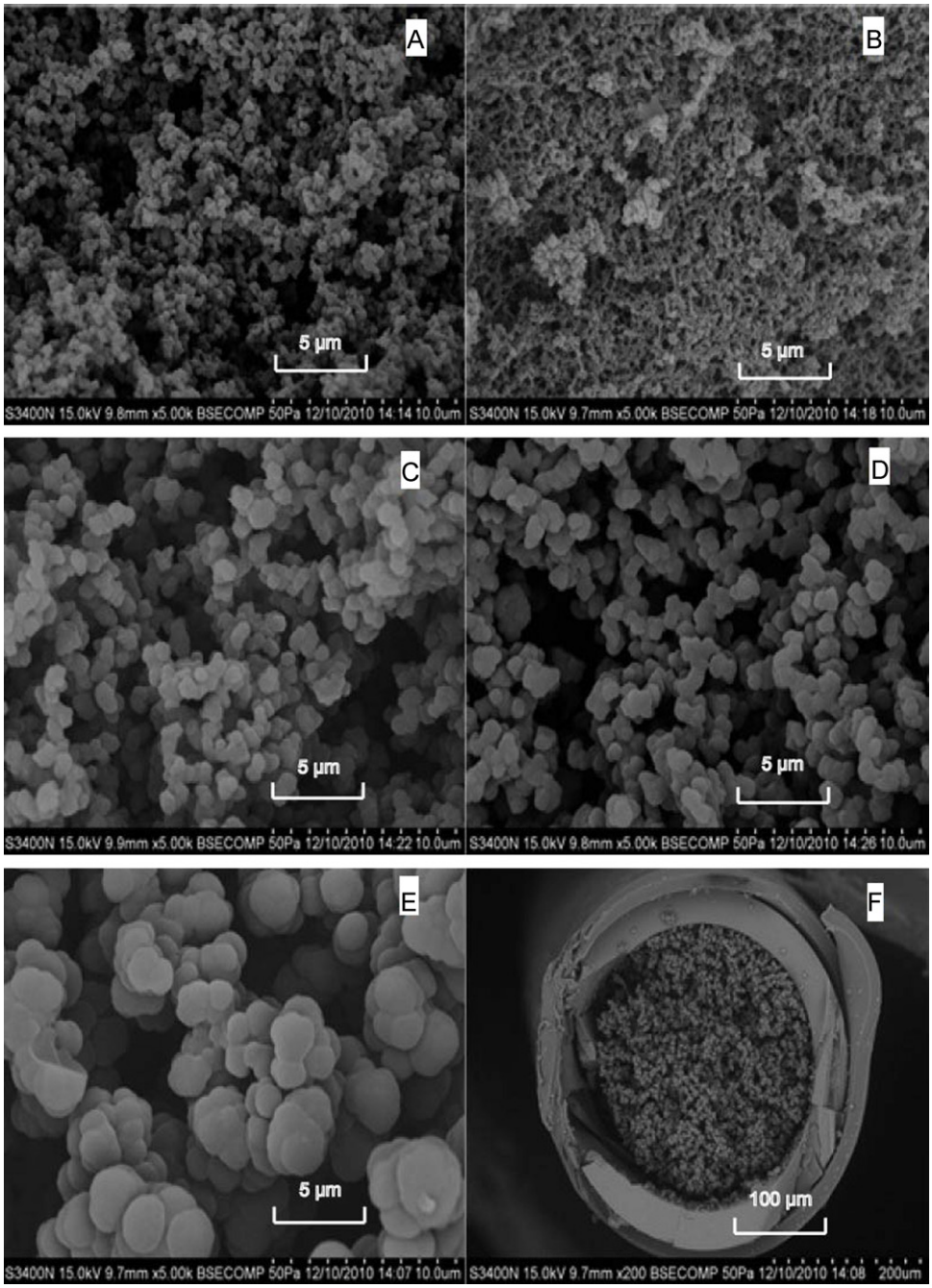

Fig. 2. SEM images of the poly (MAA-co-EGDMA) monolith prepared with different porogens. (A) Column II; (B) Column IV; (C) Column I; (D) Column III; (E, F) Column V; The magnification is 5000 folds for A–E and 250 folds for F.

column with different porogens, we investigated the backpressure at several volumetric flow rates. The plot of volumetric flow rate vs. back pressure was shown in Fig. S2, Supplementary material. From this plot, the permeability K_F was summarized in Table 2. The values of K_F were estimated by the following equation.

$$K_F = \frac{F\eta L}{\pi r^2 \Delta P}$$

where K_F is the permeability, F is the flow rate of the pump, η is the solvent viscosity, L is the column length, πr^2 is the cross sectional area of the column, ΔP is the back pressure. As shown in Table 2, significantly higher permeability was observed on monolithic capillary column with methanol/PEG6000 as porogen than that with acetonitrile/PEG 6000 or DMF/PEG 6000 as porogen. The permeability K_F for the former was about 10 folds of that for the latter. Poly(ethylene glycol) (PEG) is a hydrophilic polymer possessing flexible long chain, which probably has a complete stretch in methanol, and thus provides the best porogenic effect.

MAA-co-EGDMA monolith has been prepared and reported before in which toluene and dodecanol were used as the porogen [8]. This porogenic composition has also been investigated in our study. It was found that several hours were required to eliminate dodecanol from the polymer framework. However, only a few minutes were enough to wash porogen when methanol/PEG 6000 were used. In addition, the monolith capillary column with methanol/PEG 6000 as porogen has very high permeability that a flow rate of 0.2 mL min^{-1} can be adopted, other than 0.04 mL min^{-1} used in the reference [8]. The high flow rate for extraction and

Table 2
Effect of porogens on the permeability of the monolith.

Column	Porogen	Flow rate (mL min^{-1})	ΔP ($\times 10^6$ Pa)	K_F (Darcy)
Column I	PEG6000 160 mg DMF 1100 μL	0.01	2.1	0.098
Column II	PEG6000 500 mg Acetonitrile 700 μL	0.01	1.7	0.12
Column III	PEG6000 300 mg DMF 800 μL	0.01	1.7	0.12
Column IV	PEG6000 300 mg Acetonitrile 900 μL	0.01	3.1	0.067
Column V	PEG6000 300 mg Methanol 1100 μL	0.10	2.0	1.04

desorption was benefit to accelerating extraction speed, thus shorten the whole analytical time.

3.2. Characteristics of the monolithic column

Fig. 2 shows the scanning electron microscope (SEM) images of the MAA-co-EGDMA monolith prepared with different porogens. PEG 6000 was used as the template to create macropores and dissolved in different good solvent including acetonitrile, DMF and methanol. It was obvious from Fig. 2 that the monolith prepared with methanol/PEG 6000 as porogen revealed the largest pore size and most loose structure. The loose morphology is essential to ensure fast dynamic transport and low backpressure in applications. The SEM results corresponded well with the permeability study. It can be observed from Fig. 2 that using methanol/PEG 6000 as porogen provided more through-pores, and thus resulted in the highest permeability (Table 2). Additionally, the monolithic materials showed good attachment to the inner side of the capillary and homogeneous structure. The morphology of monolith was not notably influenced by the percentage of PEG 6000 in good solvent from Fig. 2.

The infrared spectrum of the monolith was investigated (Fig. S3, Supplementary material). The broad absorption band at 3449 cm^{-1} corresponding to the stretching vibration of O–H bonds was attributed to the hydroxyl groups of MAA molecules (monomer). The band observed at 2956 cm^{-1} is indicative of C–H stretching while that at 1728 cm^{-1} can be attributed to C=O stretching. The absorption peak around 1636 cm^{-1} was attributed to the stretching vibration of residual vinylic C=C bonds.

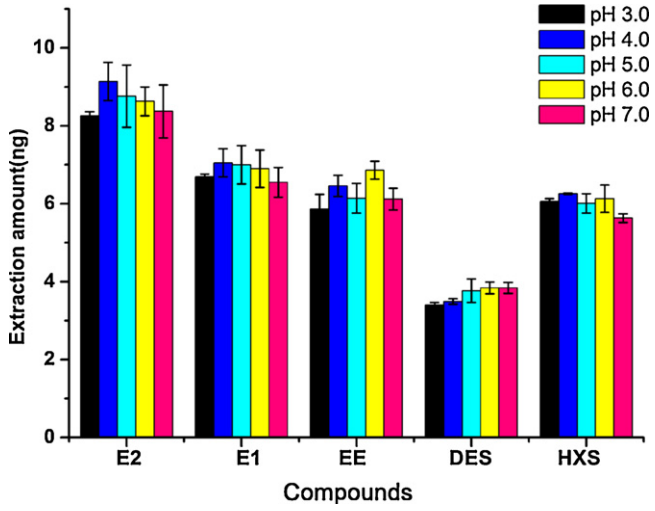

Fig. 3. Effect of the sample pH value on the extraction amounts of estrogens. Sample volume: 1.0 mL; Flow rate: 0.2 mL min^{-1}; Concentration of sample: 10.0 μg L^{-1}; Desorption with 200 μL mobile phase at the flow rate of 0.2 mL min^{-1}.

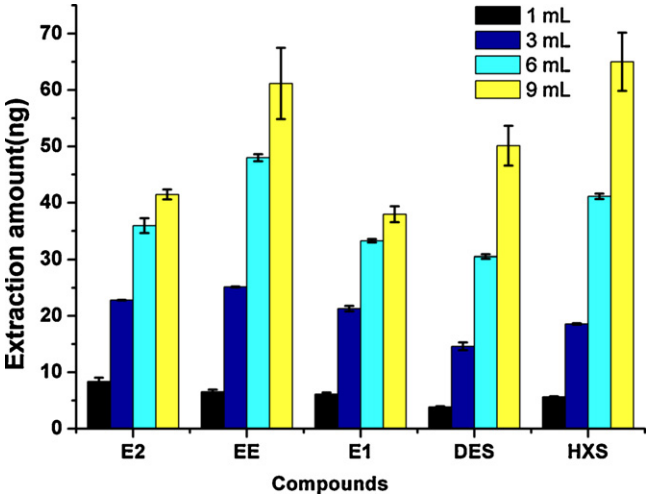

Fig. 4. Effect of the sample volume on the extraction amounts of estrogens at concentration of 10.0 μg L^{-1}. Other conditions see Fig. 3.

3.3. Effect of extraction conditions on extraction efficiency

Before the proposed monolithic capillary column was applied to sample analysis, several parameters, such as pH of the sample solution, sample flow rate, sample volume, wash and desorption steps, which related to the extraction efficiency, were investigated. The extraction was performed with 10.0 μg L^{-1} standard solution of five estrogens.

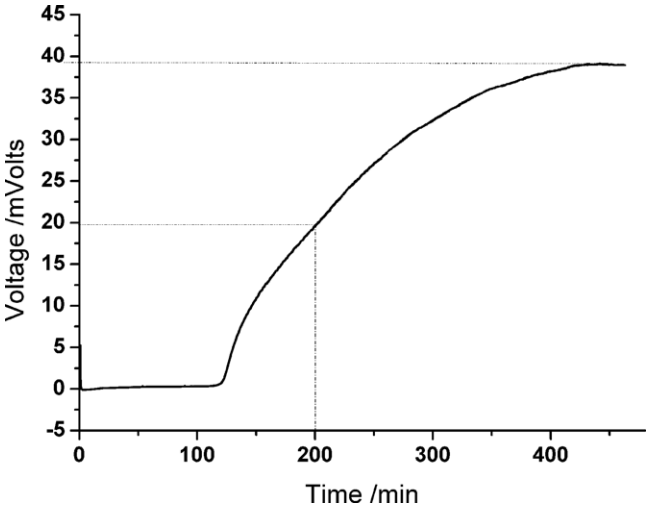

Fig. 5. The breakthrough curve of dihydrodiethylstilbestrol on the monolithic capillary column. Loading concentration: 1.0 mg L^{-1}; Sample flow rate: 0.15 mL min^{-1}.

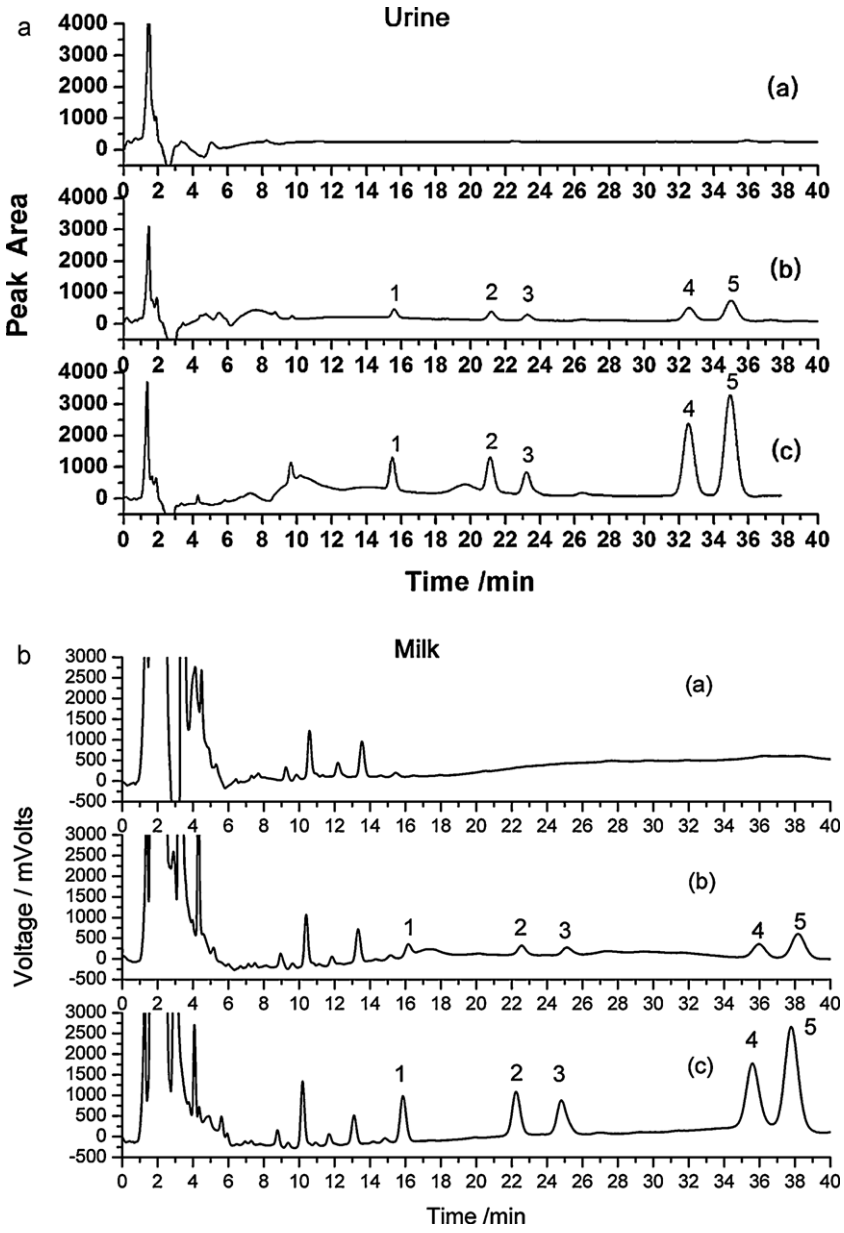

Fig. 6. Chromatograms of the urine and milk samples online extracted by the monolithic capillary column for determination of five estrogens. (a) unspiked; (b) spiked with 1.0 μg L^{-1} estrogens; (c) spiked with 5.0 μg L^{-1} estrogens. Peaks: 1 E2; 2 EE; 3 E1; 4: DES; 5 HXS.

The effect of pH value of the sample solution on the extraction amounts was investigated in the range from 3.0 to 7.0 (Fig. 3). No obvious change in extraction efficiency was observed. The microextraction process is therefore not pH-dependent, and requires no accurate control of the pH value in sample solution. The results may be explained that hydrophobic interaction and hydrogen bonding interaction play a dominant role to the extraction process.

The effect of sample flow rate in the range of 0.1–0.3 mL min^{-1} has been investigated (Fig. S4, Supplementary material). The experimental results showed that increase of flow rate had no obvious effect on the total extraction amounts. The results indicated that the mass transfer of the analyte from sample solution to monolith is a fast dynamic process. Sample flow rate of 0.2 mL min^{-1} was used considering the moderate back pressure created.

The effect of sample volume was monitored by loading estrogen standard solutions which contained 10.0 μg L^{-1} of the analytes from 1.0 mL to 9.0 mL at a constant flow rate of 0.2 mL min^{-1} (Fig. 4). The results showed that the extraction amounts of estrogens increased with the increase of sample volume. Therefore, it is easy to improve the analytical sensitivity by increasing the sample volume owning to the high loading speed.

To simplify the manipulation of microextraction, the analytes were desorbed directly by the mobile phase. The desorption flow rate was optimized in the range of 0.1–0.3 mL min^{-1} (Fig. S5, Supplementary material). No significant changes in the detected amounts of the analytes were observed, and 0.2 mL min^{-1} of desorption flow rate was selected. The desorption volume was also investigated from 100 μL to 400 μL. The results showed that the estrogens concentrated in the capillary can be completely transferred to the analytical column by 200 μL of desorption solvent. No carryover was found, which was confirmed by the blank analysis performed after desorption.

Table 3
Enrichment factor, linear regression data, detection limit and column-to-column reproducibility for five estrogens.

Analytes	Enrichment factor[a]	Extraction yields (%)[b]	Linear range ($\mu g\,L^{-1}$)	r	LOD ($\mu g\,L^{-1}$)	Column-to-column RSDs (%) ($n=5$)	
						Intra-batch	Batch-to-batch
Estradiol	180	40.0	0.50–15.00	0.9990	0.35	0.4	1.7
Ethinyl estradiol	267	59.3	0.50–15.00	0.9920	0.24	3.0	8.8
Estrone	190	42.2	0.50–15.00	0.9990	0.32	1.2	2.7
Diethylstilbestrol	362	80.4	0.05–100.00	0.9960	0.04	2.9	4.8
Hexestrol	312	69.3	0.05–100.00	1.0000	0.04	3.3	2.7

[a] Enrichment factor is calculated by comparing the peak areas obtained with monolithic microextraction and without preconcentration. The injection volume was 20 μL for direct injection. The sample solution was at the concentration of 25.0 $\mu g\,L^{-1}$ for estrogens.
[b] Percentage of enriched amounts of estrogens on the column over the total loaded

3.4. Loading capacity and enrichment factor

In order to examine the potential adsorption capacity of the monolith, the dynamic binding capacity (DBC) was examined via frontal analysis. A solution of 1.0 $mg\,L^{-1}$ dihydrodiethylstilbestrol was pumped through the column at a flow rate of 0.15 $mL\,min^{-1}$ at room temperature. The breakthrough curve on the monolithic capillary column of 10 cm length was indicated in Fig. 5. As a result of the breakthrough curve, a DBC of 3.73 $mg\,mL^{-1}$ for dihydrodiethylstilbestrol was measured, indicating a high loading capacity of the MAA-co-EGDMA monolith.

The enrichment factors were calculated by comparing the peak areas obtained before and after online microextraction. In comparison with the chromatogram of direct injection, a significant enhancement of the peak height was observed after extraction by the MAA-co-EGDMA monolith, indicating the remarkable preconcentration ability of the column. The enrichment factors were measured from 180–362 for five estrogens. The extraction yields were based on the percentage of enriched amounts of estrogens on the column over the total loaded, and were estimated from 40.0% to 80.4% (Table 3).

3.5. Reproducibility and stability

The column-to-column reproducibility was assessed by calculating the relative standard deviation (RSD) for extraction of five estrogens (Table 3). Results revealed satisfactory reproducibility was obtained both for intra-batches (RSDs from 0.4% to 3.3%) and inter-batches (RSD from 1.7% to 8.8%). Moreover, the monolith showed high stability and could be used for extraction more than 100 times with no significant changes in column backpressure and extraction efficiency.

3.6. Application to analysis of estrogens in urine and milk samples

The developed in-tube-SPME–HPLC method was applied to the determination of estrogens in urine and milk samples. The analytical performance was tested regarding linearity, precision and sensitivity. To test the linearity of the calibration curves, various concentrations of estrogens mixed solution in the range of 0.02–100.00 $\mu g\,L^{-1}$ were analyzed. As shown in Table 3, the linear ranges were 0.50–15.00 $\mu g\,L^{-1}$ for E2, E1, EE and 0.05–100.00 $\mu g\,L^{-1}$ for DES, HXS. The sensitivity of this analytical procedure was evaluated in terms of the limit of detection (LOD), which was determined as the analytes concentration corresponding to signal-to-noise ratio (S/N) of 3. The LOD was found in the range of 0.04–0.35 $\mu g\,L^{-1}$. The sensitivity of the proposed method, though lower than the reported data by LC/MS [35,36], was higher than most of the methods using SPE, SPME and SBSE as sample preparation techniques followed by HPLC–UV detection [25,29,34,37]. Moreover, the online microextraction protocol in this method realized the sample preparation procedure to be environmentally friendly and easy to operate.

Table 4
Relative recoveries of five estrogens spiked in urine and milk samples.

Analytes	Recovery (%, RSD, %) ($n=5$)	
	1.0 $\mu g\,L^{-1}$	5.0 $\mu g\,L^{-1}$
Urine		
Estradiol	104.3 (8.8)	103.5 (5.7)
Ethinyl estradiol	95.6 (5.3)	97.1 (4.3)
Estrone	106.1 (6.3)	97.2 (1.7)
Diethylstilbestrol	105.9 (2.7)	94.6 (6.3)
Hexestrol	102.1 (4.6)	104.1 (5.8)
Milk		
Estradiol	106.0 (4.6)	116.8 (3.1)
Ethinyl estradiol	101.5 (3.7)	109.9 (3.8)
Estrone	110.1 (6.9)	113.7 (3.1)
Diethylstilbestrol	94.4 (2.5)	76.5 (9.9)
Hexestrol	97.0 (4.9)	86.7 (6.4)

Fig. 6 illustrated the chromatograms of urine and milk samples after treated by the monolithic capillary column. The results showed that estrogens were extracted effectively with significant increase of sensitivity. To test the performance of the established method, the recoveries were performed by spiking urine and milk samples with standard solution at concentration of 1.0 $\mu g\,L^{-1}$ and 5.0 $\mu g\,L^{-1}$ respectively. For each concentration level, five replicate experiments with the whole analysis process were made. The recoveries of estrogens in the spiked urine and milk samples were 95.6–106.1% and 76.5–116.8%, with the RSDs of 1.7–8.8% and 2.5–9.9%, respectively (Table 4).

4. Conclusions

In summary, we proposed an effective approach using a poly (MAA-co-EGDMA) monolithic capillary column as extraction medium for microextraction of estrogens from urine and milk samples online coupled to HPLC. The results indicated that the prepared monolith had good permeability and high extraction efficiency. A simple and convenient device was designed for online coupling the monolithic capillary column to the HPLC system. Effects of sample pH, flow rate, sample volume, and elution conditions were investigated to obtain the optimum experimental conditions. The established method was environmentally friendly, inexpensive and convenient, and expected to be useful in monitoring estrogens in biological, environmental and food samples.

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Appendix A. Supplementary data

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