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ORIGINAL PAPER

Determination of sulfamethoxazole in milk using molecularly imprinted polymer monolith microextraction coupled to HPLC

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Abstract We report on a new method for the selective extraction of the antibiotic sulfamethoxazole (SMO) in milk that is making use of a molecularly imprinted polymer (MIP) monolith as the sorbent. The monolith was synthesized in the tip of a micropipette using SMO as the template and a combination of acrylamide and 4-vinylpyridine as the co-functional monomers. The monolith was connected to syringes in different sizes and used for microextraction without any other treatment and showed high selectivity and enrichment ability for SMO. It was applied to the selective extraction and sensitive determination of SMO in milk. The linear range is from 5–600 μg L^{-1}, the correlation coefficient (r^2) is 0.9984, and the detection limit (at S/N=3) is 1 μg L^{-1}. Recoveries range from 93.6 to 101.7 %, with relative standard deviations of <6.1 %.

Keywords Sulfamethoxazole · Molecularly imprinted polymer · Polymer monolith microextraction · High-performance liquid chromatography

Introduction

Sulfamethoxazole (SMO, Fig. 1) is a sulfonamide antibiotic which is largely applied in human and veterinary medicine to fight infectious diseases and in animal feeds to promote livestock growth [1, 2]. Widespread use of sulfonamide drugs in veterinary without proper withdrawal period led to accumulation of sulfonamides in meat, eggs, milk and fish [3–5]. Because of continual overuse of sulfonamides, much attention has been paid to their potential carcinogenesis and the worry of the development of antibacterial resistance. For these health concerns, the European Commission (EC), America, China and other countries have adopted a maximum acceptable limit of residual sulfonamide in foods of animal origin, for examples in meat (100 μg kg^{-1}), milk and eggs (10 μg L^{-1}) [6]. Therefore, sensitive and selective methods are required for monitoring sulfonamide residues to guarantee the safety of food. Up to now, the main approaches for the detection of SMO in food samples included high performance liquid chromatography (HPLC) [7–9], gas chromatography (GC) [10], liquid chromatography–mass spectrometry (LC–MS) [11], and capillary electrophoresis (CE) [12].

The complexity of food matrices and contaminants presented in food at low concentration levels require performance analysis only after some clean-up and preconcentration steps. Solid-phase extraction (SPE) is a routine sample preparation technique for extracting analytes from food samples. Compared with liquid-liquid extraction (LLE), SPE has the advantages of simplicity, rapidity and less consumption of organic solvents. However, the generic nonselective sorbents used in SPE usually result in the coextraction of many matrix components. Although immunoaffinity chromatography (IAC) is capable of differentially adsorbing target analytes, it still has some disadvantages such as lack of stability and high costs of antibody preparation. Recent research has been oriented towards the development of efficient, economical, and miniaturized sample preparation methods. As a result, solid-phase microextraction (SPME) [13, 14] and liquid-phase microextraction (LPME) [15] have been developed. Compared with LLE, SPME is a solvent free process that includes

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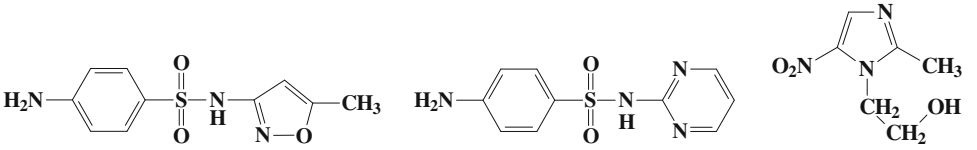

Sulfamethoxazole (SMO) Sulfadiazine (SD) Metronidazole (MNZ)

Fig. 1 The molecular structures of SMO, SD and MNZ

simultaneous extraction and preconcentration of analytes from aqueous samples or the headspace of the samples. However, SPME is expensive, its fiber is fragile and has limited lifetime, and the sorbents usually lack selectivity. LPME was developed as a solvent-minimized sample pretreatment procedure that is inexpensive, and since very little solvent is used, there is minimal exposure to toxic organic solvents. However, this method suffers from some disadvantages as follows: fast stirring would tend to format air bubble, extraction is time-consuming and equilibrium could not be attained after a long time in most cases [16].

Due to their high selectivity, reusable, inexpensive to prepare, physiochemical stability and applicability in harsh chemical media, molecularly imprinted polymers (MIPs) have been used as sorbents in SPE and SPME to selectively extract analytes from complex matrices [17–20]. Traditionally, MIPs were synthesized in bulk polymerization followed by a grinding and sieving process to acquire the desired particles in shape and size, which limited the extraction efficiency. To overcome these disadvantages, the MIP monolithic columns were prepared by in situ polymerization directly inside appropriate columns (for SPE) or capillaries (for SPME). This strategy could avoid the tedious grinding and sieving procedures as well as the problems of costly particle loss, particle in homogeneity and molecularly imprinted spots loss and obtained a MIP monolith with good resolution and low backpressure at high flow rate. Polymer monolith microextraction (PMME) is one type of SPME in which a polymer monolith is used as the sorbent. The combination of MIP technology with PMME could exhibit excellent extraction selectivity in dealing with biological samples [21]. But, the MIP monolith synthesized in a capillary was fragile, and tedious post-processing was needed.

To date, several MIP sorbents using SMO as the template have been reported [22–24]. However, little attention has been paid to make use of MIP monolith and PMME for high selective extraction of SMO from complex matrices.

In this work, a SMO-MIP monolith was synthesized in a micropipette tip for the first time using the combination of acrylamide (AM) and 4-vinylpyridine (4-VP) as co-functional monomers, ethylene dimethacrylate (EGDMA) as the cross-linker, acetonitrile as the porogenic solvent. The robust micro-monolith could be connected with syringes in different sizes simply to perform PMME process without any post-processing. The derivated MIP monolith showed high selectivity and enrichment ability for SMO. Further, an MIP-PMME-HPLC procedure has been employed for the determination of SMO using the MIP monolith for the clean-up and preconcentration of SMO. The results indicated that the method can be applied for the rapid, selective and sensitive analysis of SMO in milk samples.

Experimental

Instruments

The chromatographic analysis was carried out on a Dionex Summit U3000 HPLC system equipped with a manual injector and a Photodiode Array Detector (PAD) (Dionex Technologies, USA, http://www.dionex.com). A personal computer equipped with a Chromeleon ChemStation program for HPLC was used to process chromatographic data. A amethyst-C18 column (4.6 mm×250 mm, 5 μm) from Sepax Technologies Inc. (Newark, USA, http://www.sepax-tech.com) was connected with a guard column (cartridge 2.1 mm×12.5 mm, 5 μm, Agilent Technologies, PaloAlto, CA, USA, http://www.home.agilent.com) filled with the same packing material. The mobile phase was a mixture of methanol–water (36:64, *v/v*) and the flow rate was 1.0 mL min^{-1}. The column temperature was set at 25 °C by a temperature controller for column oven (Nuohai Technologies, China, http://www.cznhdz.com). The UV detector was set at a wavelength of 270 nm for SMO and SD, 320 nm for MNZ, respectively. All injections were performed manually with a 20.0 μL sample loop. A DZF-6021 vacuum drying oven (Yiheng Instrument Factory Co. Ltd., Shanghai, China, http://www.yihengyiqi.com) was used for polymerization. An LSP01-1A longer pump (Baoding Longer Precision Pump Co. Ltd., China, http://www.longerpump.com) was used for pumping. Filter membranes of nominal pore size 0.45 μm were obtained from Xingya Scavenging Material Company (Shanghai, China, http://shxingya.cn.alibaba.com). The microscopic morphology of the monolith was examined by a Model X-650 scanning electron microscope (Hitachi, Tokyo, Japan, http://www.hitachi.com). The infrared spectroscopy of SMO, 4-VP, AM, MIP and NIP monolith was examined by a Fourier transform infrared spectrometer (Perkin Elmer, USA, http://www.perkinelmer.com).

Chemicals

Ethylene dimethacrylate (EGDMA) purchased from Acros (New Jersey, USA, http://www.gchems.com) was extracted with 5 % aqueous sodium hydroxide and water, then dried over using anhydrous magnesium sulfate. 2, 2′-azobisisobutyronitrile (AIBN) was obtained from Shanghai No.4 Chemical Reagent Corp. (Shanghai, China, http://www.tjx0079.eb80.com) and recrystallized in anhydrous ethanol before use. 4-Vinylpyridine (4-VP) was obtained from Acros (New Jersey, USA, http://www.gchems.com). Acrylamide (AM) purchased from Fuchen Chemical Reagent Company (Tianjin, China, http://www.tjfch.com) was distilled under vacuum prior to use. Methanol and acetonitrile (HPLC grade) were obtained from Tedia Company Inc. (Ohio, USA, http://www.tedia.com). Sodium chloride, phosphoric acid and other reagents used were all of analytical grade. Double-distilled deionized water was filtered through a 0.22-μm fiber membrane before using..

Sulfamethoxazole (SMO), sulfadiazine (SD) and metronidazole (MNZ) were purchased from Sigma (St Louis, MO, USA, http://www.sigma-aldrich.com). The stock standard solutions of SMO, SD and MNZ were prepared in methanol at a concentration of 1 mg mL^{-1} and stored at 4 °C in refrigerator. Working standard solutions of analytes were prepared by appropriate dilution of the stock solution using purified water.

Preparation of molecularly imprinted monolith

For the preparation of the sulfamethoxazole imprinted polymer monolith, the template sulfamethoxazole (0.05 mmol) was dissolved in 400 μL of acetonitrile in a clean PE tube and mixed with AM (0.1 mmol) and 4-VP (0.1 mmol) as the co-functional monomer. The mixture was surged ultrasonically for 4 h. Then, 1 mmol of cross-linker EGDMA and 9.7 mg of initiator AIBN were added and deoxygenated by nitrogen purging for about 10 min. Next, 50 μL of the homogeneous solution was filled into a micropipette tip which had been sealed at one end. Subsequently, the other end of the pipette tip was sealed with silicon rubber. After polymerization at 60 °C for 24 h, the silicon rubber was removed. The resultant MIP monolith was washed with methanol to remove the template molecules. A reference, non-imprinted polymer monolith (NIP), was prepared simultaneously as the same procedure including washing, but in the absence of the template.

Preparation of the extraction device

As shown in Fig. 2, the SMO imprinted polymer monolith could be connected with syringes in different sizes simply without any other treatment. A syringe infusion pump (Baoding Longer Precision Pump Co. Ltd., China) was employed for the delivery of sample solution, washing solution and desorption solvent.

MIP-PMME procedure

The MIP monolith was washing with 2.0 mL of acetonitrile and 1.0 mL of water, respectively. Then, an aliquot of 5.0 mL sample solution (adjusted pH=2.0 with H_3PO_4) was loaded at a flow rate of 0.05 mL min^{-1} with the aid of an infusion pump. The MIP monolith was washed with 0.5 mL water at a flow rate of 0.2 mL min^{-1} to remove the matrix interferences. Then, the analytes were eluted with 0.1 mL of acetonitrile at a flow rate of 0.05 mL min^{-1}. The eluent solution in the PE tube was removed using a 100 μL HPLC microsyringe and injected into the HPLC system for analysis directly. All experiments were performed repeatedly and means of results were used in plotting of curves or in tables.

Preparation of milk samples

Preliminary analyses showed that the milk samples purchased from the local retail market were analyte-free. 5 mL of milk sample were spiked with known variable amounts of SMO and mixed with 6 mL of acetonitrile using a vortex mixer (WH-3, Luxi Analysis Instrument Factory Co. Ltd., Shanghai, China). The sample solution was centrifuged at 7 °C for 10 min at 10000 rpm (Xiangzhi Centrifuge Instrument Co. Ltd., Changsha, China). Then, the supernatant was completely transferred to another 50 mL volumetric flask. After evaporation of the solvent under a gentle nitrogen flow, the residue was redissolved in 30 mL of purified water (adjusted pH=2.0 with H_3PO_4). Finally, the reconstituted solution was stored at 4 °C and filtered through a 0.45 μm membrane filter prior to use. Blank sample was prepared in the same way as above but without the compound-spiking step.

Results and discussions

In order to obtain the optimized extraction conditions, enrichment factor (EF) and extraction recovery (ER) were used to evaluate the extraction efficiency of MIP monolith under different conditions.

$$EF = \frac{C_{elu}}{C_0},\ ER = \frac{n_{elu}}{n_0} \times 100 = \left[\frac{C_{elu} \times V_{elu}}{C_0 \times V_{aq}}\right] \times 100$$

$$= EF \times \left(\frac{V_{elu}}{V_{aq}}\right) \times 100$$

where C_{elu}, n_{elu} and V_{elu} are SMO concentration and its

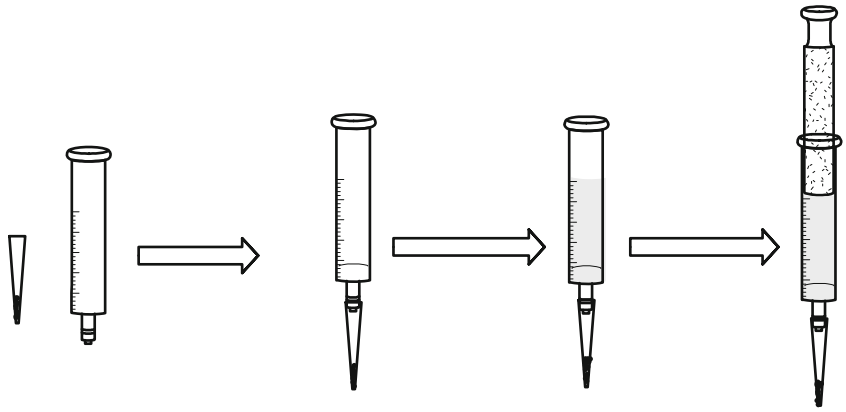

Fig. 2 Scheme of the novel PMME device

number of moles in eluent, the volume of eluent, respectively. C_0, n_0 and V_{aq} are SMO concentration and its number of moles in sample solution, the volume of sample solution, respectively.

The imprinting factor (IF) and selective factor (SF) were used to evaluate the recognition abilities of the SMO-MIP monolith.

$$IF = \frac{EF_{MIP}}{EF_{NIP}},\ SF_{SMO/SD} = \frac{Q_{SMO}}{Q_{SD}},\ SF_{SMO/MNZ} = \frac{Q_{SMO}}{Q_{MNZ}}$$

Where EF_{MIP} is the enrichment factor of SMO extracted in MIP monolith and EF_{NIP} is the enrichment factor of SMO extracted in NIP monolith under the same conditions. Q_{SMO}, Q_{SD} and Q_{MNZ} are the adsorption capacities of SMO, SD and MNZ in MIP monolith, respectively.

Optimization of synthesis conditions

For non-covalent molecular imprinted polymers, acrylamide (AM) and 4-vinylpyridine (4-VP) are commonly used functional monomers, MIPs prepared using these two kinds of monomers, either separately or in combination, have shown various recognition properties. MIPs made from mixed functional monomers could exhibit more efficient recognition properties than those from single monomer (Zheng, Li, Chang, Wang, & Li, 2002). In this study, SMO-MIP monolith was prepared using the combination of AM and 4-VP as co-functional monomers, ethylene dimethacrylate (EGDMA) as the cross-linker, acetonitrile as the porogenic solvent according to the reported method (Liu, Ouyang, Zhao, Shangguan, Chen, & Liu, 2006). The volume of acetonitrile was optimized in this study. The results showed that the mean pore size decreased while increasing the volume of acetonitrile. The pores in the monolith had too small pore diameter to allow the sample solution to flow through when 0.6 mL of acetonitrile was used. However, if the volume of acetonitrile decreased down to below 0.3 mL, the monolith would become flexible and provide poor recognition ability for SMO. The MIP monolith made from 0.4 mL of acetonitrile indicated felicitous flow-though pore size and it was selected as the appropriate porogenic solvent volume.

The characterization and specificity evaluation of the MIP monolith

The MIP monolith morphological structure was investigated by scanning electron microscope. The results showed that there were many macropores and flow-through channels inlaid in the network skeleton of SMO imprinted monolith, which provided flow paths through the column. Due to the size and density of the macropore network, the monolith had a high internal porosity and, consequently, a large permeability and low column hydraulic resistance. The pores allowed the mobile phase to flow through with low flow resistance.

Figure 3 showed that the infrared spectrum of SMO-MIP monolith was different from that of SMO, AM and 4-VP. Compared with the infrared spectrum of AM, the N−H stretching vibration wide peak at the band 3000–3500 cm^{-1} became weak and the C=O stretch vibration peaks at 1614 and 1678 cm^{-1} shifted to that of 1733 cm^{-1} in the infrared spectrum of the associated complexes. On the other hand, as compared with the infrared spectrum of 4-VP, the stretching vibration wide peak at 3000–3500 cm^{-1} became weak and the C=C stretch vibration peak at 1633 cm^{-1} disappeared. While compared with the infrared spectrogram of SMO, the N−H stretch vibration peak at 3100–3500 cm^{-1} disappeared. The NIP and MIP monoliths showed similar locations and appearances of the major bands. These results showed that the polymers have been successfully synthesized.

In order to evaluate the selectivity of the MIP monolith, SD with similar structure to SMO as the analogue and MNZ as the non-analogue (Fig. 1) were tested. For sampling, 5.0 mL of mixed standard solution including 30 ng mL^{-1} SMO, SD and MNZ respectively was loaded on the MIP and NIP monoliths at a flow rate of 0.2 mL min^{-1}, and 100 μL of methanol was used to elute analytes. The eluents were analyzed by HPLC directly. The results indicated that the

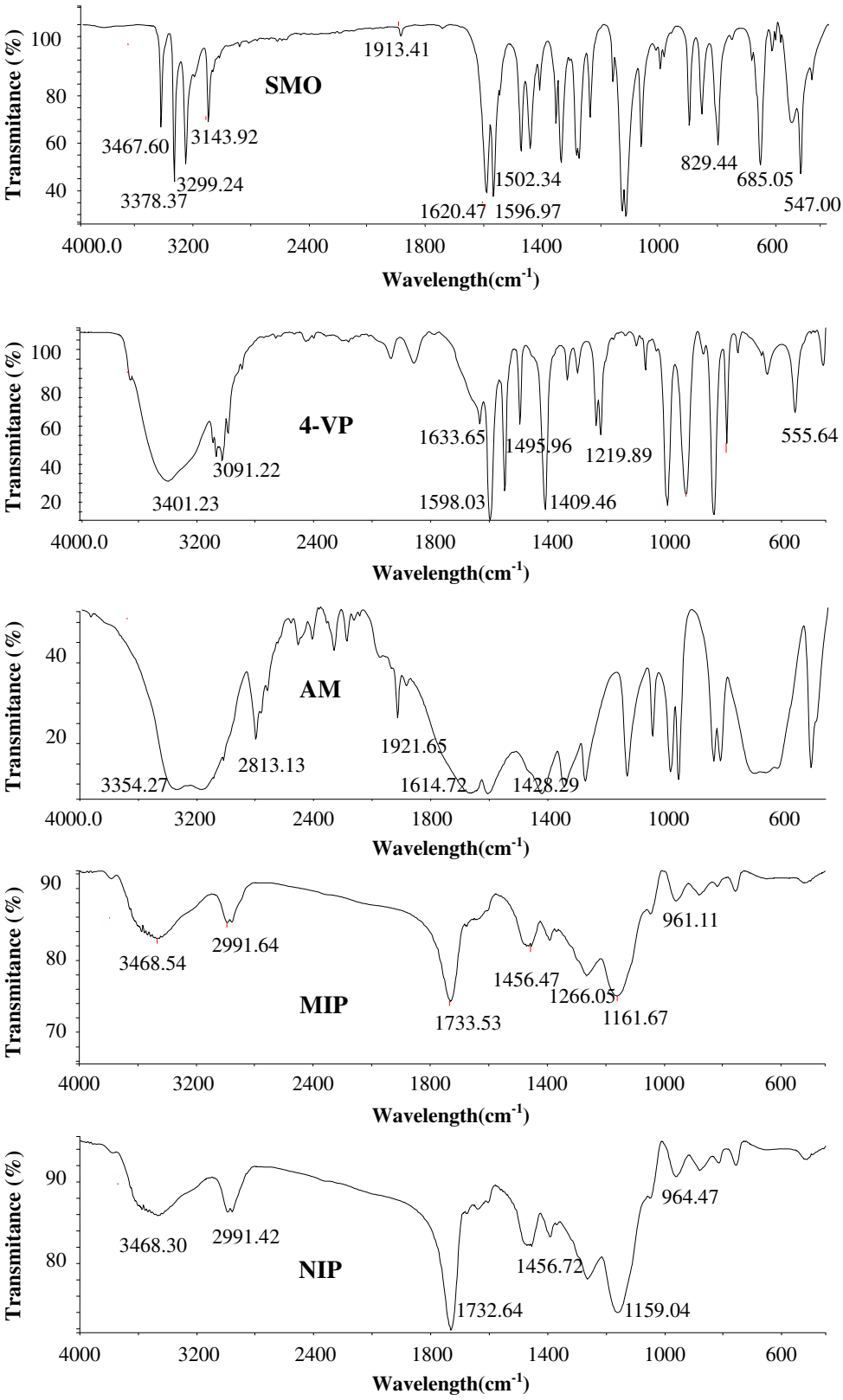

Fig. 3 FT-IR spectra of SMO, AM, 4-VP, MIP and NIP

MIP had a higher affinity for SMO than NIP, where IF was 2.53. The MIP-PMME possessed a little higher extraction efficiency for SD than the NIP-PMME, possibly due to the similar structure between SMO and SD. The data also showed that the retention of MNZ on MIP monolith was weaker than that on NIP monolith. These results indicated that SMO-MIP

monolith possessed special recognition sites and complementary spatial structure for SMO binding.

To estimate the adsorption capacity of SMO, SD and MNZ on the MIP monolith, an adsorption experiment was carried out under optimized conditions. 5 μg mL^{-1} SMO standard solutions were continuously passed through the MIP monolith at 0.05 mL min^{-1} until the peak area of SMO in effluent was equal to that in standard solution. Then the adsorption capacity of SMO on the MIP monolith was calculated on the basis of the SMO concentrations in standard solution and effluent, and the volumes of standard solution and effluent. The adsorption capacities of SD and MNZ on the MIP monolith were determined using the same method. The experimental results showed that $SF_{SMO/SD}$, $SF_{SMO/MNZ}$ were 3.5 and 34.3, respectively. These results demonstrated the acceptable selectivity of the synthesized MIP monolith for SMO analysis, and the SMO-MIP monolith could be used for clean-up and enrichment of SMO effectively.

Optimization of MIP-PMME conditions

Several parameters associated with the MIP-PMME efficiency, such as the flow rate, volume, pH and salt concentration of sample, the type and volume of washing solution, the type, volume and flow rate of eluent were optimized in this study. A sample solution of 5 mL spiked with SMO at 30 ng mL^{-1} was used for performing the experiments.

Effect of sample flow rate

The flow rate of the sample solution was optimized in the range of 0.05 ~ 0.40 mL min^{-1}. The results showed that EF and ER of SMO decreased with increasing the flow rate of the sample solution. This may be due to the plenitudinous mass transfer of the analyte from sample solution to MIP monolith at lower flow rate. Thus, 0.05 mL min^{-1} was chosen as the optimized flow rate of sample solution in the following experiments.

Effect of washing solution

The washing solution was adjusted by optimizing the proportion of methanol in water. The experimental results indicated that EF and ER of SMO decreased obviously by increasing methanol content in the washing solution. This may be due to that the loss of analyte increased with increasing the methanol content in washing solution. There was no observed difference in EF and ER of SMO after washing with 0.5 mL and 1 mL of purified water. Therefore, 0.5 mL of purified water was selected as the optimized washing solution.

Effect of the type, volume and flow rate of eluent

The selection of an appropriate eluent is of high important for the PMME process. Considering the consistency to the mobile phase used in liquid chromatography, the eluent is limited to solvents such as methanol, acetonitrile and purified water. Different proportions of methanol with water, acetonitrile with water as eluent were tested. The experimental results indicated that the addition of water in eluent was not advantageous for eluting SMO. However, better result was achieved when acetonitrile was used as the eluent. The experimental results also showed that EF and ER decreased when the amount of acetic acid in eluent increased. So, acetonitrile was selected as the eluent in the following experiments.

In order to study the effect of eluent volume on the extraction efficiency, different volumes of eluent (acetonitrile) were tested. The results showed that 0.1 mL eluent was sufficient to elute analyte from the monolith. Further increasing the volume of the eluent was not preferred because EF decreased with the increasing of eluent volume. Thus, 0.1 mL of acetonitrile was selected for subsequent works.

The flow rate of the eluent was optimized in the range of 0.01 ~ 0.3 mL min^{-1}. The results showed that no significant change in the extraction efficiency was found when the flow rate of eluent changed in the range of 0.01 ~ 0.05 mL min^{-1}. Then the extraction efficiency decreased with the flow rate increasing. Therefore 0.05 mL min^{-1} was selected as the optimized flow rate of eluent in the following experiments.

Effect of sample volume

The effect of sample volume was monitored by loading sample solution (containing 30 ng mL^{-1} of the analyte) from 2.0 to 10.0 mL at a constant flow rate. The experimental results showed that EF of SMO increased with the increasing of sample volume from 2.0 to 10.0 mL. This indicated that the extraction capacity was not reached even when 10.0 mL of sample solution was loaded. However, ER began to decrease when the sample volume increased. For obtaining higher EF and ER for SMO, 5.0 mL of sample solution was selected in the MIP-PMME procedure.

Effect of sample pH

The sample pH is a significant factor, which may affect the molecule form of the analyte and closely relate to the interaction between analytes and the MIP monolith. The effect of the sample pH on the extraction efficiency for SMO was investigated using several buffer solutions with pH 2 ~ 8. The experimental results showed that EF and ER decreased when sample pH increased from 2 to 8. This could be explained by the fact that SMO itself share both hydrophilic

and hydrophobic characters. For MIP preparation, the main interaction was hydrogen bonding. During the recognition step in aqueous solution the hydrogen bonding should be highly suppressed due to strong hydrogen bonding properties of water. However, the synergetic effect of cavity restriction and hydrophobic interaction might contribute to the selective and even stronger binding of SMO on the MIP. Although the SMO molecule has one primary amine group and one secondary amine group, the *pK*a of the SMO molecule is 5.6 due to the strong acidity of sulfonylamino group. SMO was supposed to be neutral at low pH which was propitious to interaction with MIP. Finally, the sample solution was adjusted to pH 2 in the subsequent experiments.

Effect of salt concentration

The effect of salt concentration of the sample on the extraction efficiency was also investigated. The results indicated that EF and ER of SMO increased slightly as the concentration of NaCl increased from 0 to 30 % (*w/v*). Actually, good extraction efficiency had been achieved with no addition of NaCl in sample solution, and high concentration of salt would increase the pressure of the monolith in the MIP-PMME process.

Evaluation of the method

Under the optimized conditions, the method was applied for determination of SMO in milk samples. Blank milk samples were spiked at a range of 5–600 μg L^{-1} with SMO. Then, the spiked samples were analyzed by the MIP-PMME-HPLC method. The regression coefficient (r^2) was 0.9984 and the limit of detection (LOD), based on signal-to-noise ratios (S/N) of 3, was 1 μg L^{-1} for SMO in milk which was lower than the values reported in other works related to the determination of SMO [25–29].

The reproducibility of the method was determined by the within-day and between-day precisions at the concentration of 10, 100 and 500 μg L^{-1} for SMO in spiked milk samples. The results showed that the within-day precision (RSD, n= 5) was less than 1.7 %, while the between-day precision (RSD, n=5) was less than 4.7 %.

The developed MIP-PMME-HPLC technique was applied for the determination of SMO in milk to further elucidate the applicability and reliability of this method. Three batches of milk samples were collected from local supermarkets. The results showed that the three different batches of milk samples were free of SMO residue. To test the performance of this established method, the extraction recoveries were performed by spiking fresh milk samples with SMO standard solution. For each concentration level (10, 100 and 500 μg L^{-1}), three replicate experiments with the whole analysis process were made. The recoveries ranging between 93.6 ~ 101.7 % were obtained with RSDs less than 6.1 %(n= 3). Thus, the developed method is reliable for routine analysis of SMO in complex milk sample.

The chromatograms of spiking milk samples before and after treated by MIP-PMME and MIP-PMME were showed in Fig. 4. It can be seen that after treated by MIP-PMME, a majority of interfering substances in milk sample was eliminated, thus quantification of SMO can be successfully achieved. In comparison with the chromatogram of direct injection, a dramatic enrichment of the peak height was observed. The result indicated the remarkable preconcentration ability of the SMO-MIP monolith (EF=48.45).

The MIP monolith showed high stability since no significant changes in the back-pressure and extraction efficiency of the monolith were found in the experiment.

Conclusions

A novel, durable SMO-MIP monolith was synthesized in a micropipette tip for the first time. The monolith could be connected with syringes in different sizes simply without any other treatment to perform PMME process. The derivated MIP monolith showed high selectivity and enrichment ability for SMO. MIP-PMME followed by HPLC and PAD detection was developed as an analytical method for the selective and sensitive determination of SMO in milk. The optimum conditions of synthesis and extraction performance have been obtained. The experimental results revealed that this method provided high selectivity, lower solvent consumption, higher extraction efficiency and good linearity over the investigated concentration range. The

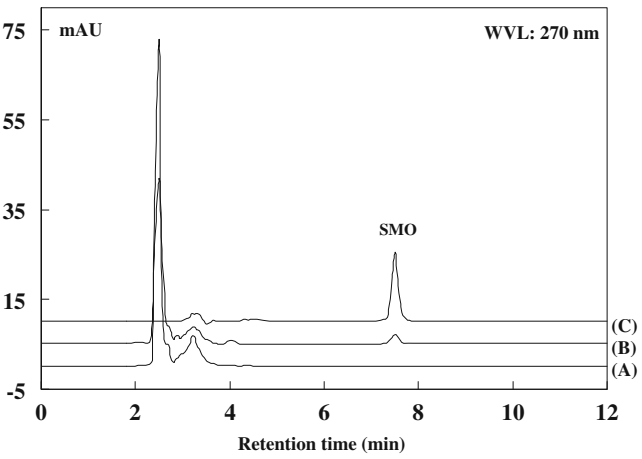

Fig. 4 HPLC chromatograms of spiked milk samples before (A) and after treated by NIP-PMME (B) and MIP-PMME (C). Sample solutions of SMO were spiked at 30 μg L^{-1}

performance of this procedure in the analysis of SMO in milk sample was satisfactory.

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