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Determination of sulfonamides in bovine milk with column-switching high performance liquid chromatography using surface imprinted silica with hydrophilic external layer as restricted access and selective extraction material

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ABSTRACT

A novel restricted access-molecularly imprinted material (RA-MIP) with selectivity for sulfonamides was synthesized using initiator-transfer agent-terminator (iniferter) method, a "living"/controlled radical polymerization technique. The material was prepared by grafting two layers with different functions on the silica support. To perform a "grafting from" polymerization, iniferter was immobilized on the surface of silica. The internal sulfamethazine imprinted polymer and the external poly(glycidyl methacrylate) [poly(GMA)] were then grafted successively. The hydrophilic structures were formed on the external layer of the material by the hydrolysis of the linear poly(GMA) for protein removal. The result has shown that this restricted access-MIP grafted silica (RA-MIP-SG) not only has the selectivity for the template and its analog, but also has the ability of exclusion for bovine serum albumin (BSA). It indicated that the material possesses both properties of molecularly imprinted polymer (MIP) and restricted access material (RAM). Using RA-MIP-SG as pre-column, a column-switching HPLC method was established for the determination of sulfonamides in bovine milk. Direct sample injection was performed in the analysis, which provides a convenient analytical procedure. Good linearity in the range of 2–400 ng mL^{-1} ($R > 0.998$) has been obtained for seven sulfonamides in the study. The recoveries of all the analytes at three concentration levels are between 93% and 107% with the RSD < 8.0%. The limits of quantification and limits of detection are less than or equal to 2.7 ng mL^{-1} and 0.8 ng mL^{-1} respectively. It demonstrated this RA-MIP-SG can be applied in sample extraction and clean-up for the determination of sulfonamides in bovine milk by direct injection and column-switching HPLC with good efficiency and reliability.

1. Introduction

Pharmaceutical analysis in the biological samples is an important analytical area which has drawn increased attention in recent years in different respects such as pharmaceutical studies, disease diagnosis and food safety control. In the biological sample analysis, HPLC is the widely employed technique due to its excellent ability in separation and determination. In the HPLC process the off-line sample pretreatment for protein removal is often required to avoid the precipitation of the protein on the column, which is still the bottleneck for the analytical efficiency. In order to solve this problem, restricted access material (RAM) has been developed [1]. The RAM has interior phase for small molecule retention and external hydrophilic layer which is non-adsorptive for protein. On the RAM, the protein exclusion is generally performed by physical diffusion barrier based on the size of the pores, or by chemical diffusion barrier created by a hydrophilic network shielding. Direct injection can be performed using RAM as stationary phase, which eliminates the time consuming pretreatment procedure and facilitates the high throughput HPLC analysis.

On the other hand, selective extraction of the target analyte is advantageous in the biological sample analysis. Molecularly imprinted polymer (MIP), a synthetic material, has shown good potential in this application. With advantages such as predetermined enzyme-like selectivity and chemical stability, MIP has drawn considerable attention and has been applied in different fields such as separation [2], sensing [3], catalysis [4], drug delivery system [5] and solid-phase extraction [6]. In the biological sample analysis, the material combining the characteristics of RAM and MIP is ideal because the analytical efficiency will be greatly improved by simultaneous protein removal and selective extraction. Haginaka et al. developed a RAM-MIP with an MIP inner core and hydrophilic restricted access external layer [7]. They used multi-step swelling and thermal polymerization method for the polymer particle preparation, in which hydrophilic monomers were added after the imprinting polymerization to

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form external layer. The drawback of the MIP synthesized by the multiple swelling is the possibility of leakage of the template molecule. To solve this problem, structurally-related analog or isotopologue of the template has been used for the RAM-MIP synthesis [8,9]. The other kind of RAM-MIP was developed by Puoci et al. [10], in which glycidyl methacrylate (GMA) was copolymerized in the entire molecularly imprinted polymer. Then the epoxide rings on the polymer surface were opened by hydrolysis to form the hydrophilic structure. The applications of these restricted access-MIP material have shown advantages in the biological and environment sample analysis [11–13]. In order to prepare MIPs with aqueous compatibility and reduce the non-specific interaction, Sellergren and his colleagues [14,15] have synthesized "water compatible" MIP using hydrophilic co-monomer or by hydrolytically hydrophilizing the polymer matrix. De Lorenzi et al. have synthesized poly(2-hydroxyethyl methacrylate) capped MIP-silica via iniferter technique [16], which shows the water-compatibility and has exhibited a higher imprinting factor in highly aqueous conditions. The "water compatible" MIPs have better property in the bio-sample extraction.

We report a new research for the synthesis of restricted access-MIP material and its application in the direct-injection HPLC analysis of sulfonamides from milk in this presentation. In the study, surface-initiated iniferter (initiator-transfer agent-terminator) technique [17], a "living"/controlled polymerization method, was used in the material preparation. The iniferter technique has special advantages in making surface modified multifunctional material and has been introduced into the preparation of MIP grafted silica by Ruckert, Sellergren and Hall [18,19]. Prepared by this surface-initiated (grafting from) living radical reaction, the polymerization in the solution can be minimized because the polymeric chain propagation takes place via the active radical attached to the silica surface, while the dormant radical is in solution. The surface grafted MIP has higher separation efficiency [20] compared with totally porous MIP material because more homogenous thin polymeric film can be formed with less mass transfer resistance. Meanwhile, block polymer with different properties for different polymer layers can be grafted and the polymer layer thickness or polymeric chain length is more controllable with iniferter technique. Moreover, composite materials with different platforms can be prepared with this method. With the advantages of controllable polymerization and living property, different "living"/controlled polymerization methods have been employed in the MIP preparation and more papers have been published in recent years [21–26].

Sulfonamides (SAs) are antibacterial agents with low cost and high cure efficiency. They are commonly used in veterinary practice to prevent infections in livestock and to treat diseases. The excessive use of sulfonamides in animal husbandry leads to the presence of sulfonamides residue in the animal-derived food products and thereby becomes a potential hazard to human health. The presence of sulfonamide residues in milk is a great concern because milk is an important nutrient source for children and adults. Different authorities around the world have established maximum residue limits (MRL) of SAs in milk. The European Union has adopted an MRL of 100 $\mu g\,kg^{-1}$ as a total of SAs in the foodstuffs of animal origin [27]. China established an MRL of 100 $\mu g\,kg^{-1}$ for the sum of SAs and an MRL of 25 $\mu g\,kg^{-1}$ for sulfamethazine only [28]. In the analysis of SAs from milk, HPLC is the most commonly used technique. Since milk consists of an emulsion with a high content of fat and proteins, multi-step pretreatment including protein precipitation and concentration [29,30] and/or off-line SPE [27,31–33] are still required for the SAs analysis in the recent published papers. Pereira and Cass have used RAM column for online clean-up of milk sample, but only sulfamethoxazole and trimethoprim were analyzed in the HPLC determination [34]. Using RAM-MIP for the sample clean-up and extraction will facilitate a direct injection and efficient analytical procedure.

In the present study, MIP grafted silica with restricted access outer-layer was synthesized using iniferter technique. Sulfamethazine, a commonly used antimicrobial agent in livestock, was used as the template. In the research, the method of synthesis was developed and conditions were optimized. The method of determination of seven SAs with column-switching HPLC and direct injection using RA-MIP-SG as pre-column was established. To our knowledge, this is the first report of preparation of restricted access-MIP using iniferter technique for sulfamethazine and application in the determination of SAs in milk by the column-switching HPLC method.

2. Experimental

2.1. Material and reagents

Silica gel (average particle size: 10 μm, pore size: 1000 Å, BET-specific surface area: 23.28 $m^2\,g^{-1}$) was purchased from Agela Technologies (Tianjin, China). 3-Aminopropyl trimethoxysilane was from Diamod Advanced Material of Chemical Inc. (Hubei, China). Sodium *N*,*N*-diethyldithiocarbamate trihydrate (NaDEDTC trihydrate) was from Guangfu Fine Chemical Research Institute (Tianjin, China). Methacrylic acid (MAA) was purchased from Damao Chemical Reagent Co. Ltd. (Tianjin, China). Glycidyl methacrylate (GMA) was obtained from Yuanji Chemical Co. Ltd. (Shanghai, China). 4-(Chloromethyl)benzoyl chloride and ethylene dimethacrylate (EDMA) were from Haiqu Chemical Factory (Shanghai, China). MAA, 4-(chloromethyl)benzoyl chloride and GMA were purified by distillation under reduced pressure before use. EDMA was washed consecutively with 10% aqueous NaOH, water and then dried with Na_2SO_4 prior to use. Sulfamethazine (SMZ), sulfamethoxazole (SMO) and sulfadizaine (SDZ) were from Alfa Aesar (Tianjin, China). Sulfaquinoxaline (SQX), sulfametoxydiazine (SMD), sulfadimethoxine (SDM) and sulfamethizole (SMT) were from Sigma–Aldrich (Shanghai, China). Bovine serum albumin (BSA) was from Solarbio Science & Technology Co. Ltd. (Beijing, China). All reagents were of analytical grade and used as received unless otherwise stated.

2.2. Preparation of iniferter bonded silica

Before the preparation, the silica gel was activated by rehydroxylation in hydrochloric acid–H_2O (1:1) under reflux for 12 h.

The preparation of iniferter bonded silica involves three steps (Fig. 1). In the first step, activated silica gel (SG, 14.0 g) reacted with 3-aminopropyl trimethoxysilane (5 mL) in dry toluene (130 mL) at 110 °C for 15 h under nitrogen protection. The resulting particles (Si-I) were Soxhlet-extracted using ethanol. In the second step, the Si-I particles were suspended in 90 mL dry DMF containing 0.4 mL pyridine. The 4-(chloromethyl)benzoyl chloride (3.7 g) dissolved in 30 mL DMF was added dropwise and the reaction was performed at 25 °C for 18 h. The Si-II particles were obtained and washed with acetone. In the third step, Si-II particles were suspended in 90 mL anhydrous ethanol and reacted with 3.0 g NaDEDTC trihydrate (dissolved in 30 mL ethanol). The reaction was performed under stirring for 8 h at 50 °C. The iniferter bonded silica (Iniferter-silica) was obtained and washed with distilled water. The particles were dried under vacuum after each reaction.

2.3. Photo-grafting of the SMZ-imprinted polymer on the iniferter bonded silica

The photo-grafting of SMZ-imprinted polymer was performed in a quartz flask. The Iniferter-silica particles (2.5 g) were mixed

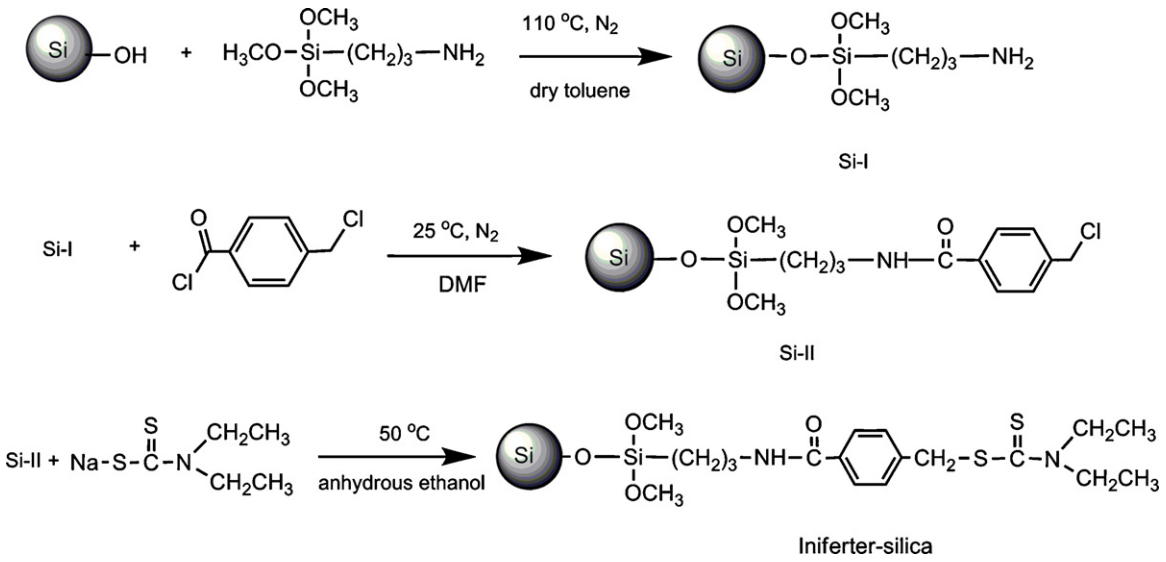

Fig. 1. Synthesis of the iniferter bonded silica.

with a solution containing SMZ (0.28 g, 1 mmol), MAA (0.69 g, 8 mmol), EDMA (7.93 g, 40 mmol) and acetonitrile (40 mL). The mixture was degassed with nitrogen for 10 min. The flask was sealed and rotated in a rotary mixer. Polymerization was initiated by UV light from a high pressure mercury lamp (150 W, Yingze Scientific Technology, China) at a distance of 10 cm. The reaction temperature was controlled by the ice-water bath. After the reaction, the MIP grafted silica (MIP-SG) was Soxhlet-extracted using CH_3OH/HOAc (90:10, v/v) and CH_3OH successively.

The non-imprinted polymer grafted silica (NIP-SG) corresponding to one MIP-SG (4h-MIP-SG) was synthesized for comparison. In the NIP-SG synthesis, the reactant composition was the same as that in the 4h-MIP-SG synthesis except the absence of the template. Because without template molecule, undesired solution polymerization appeared when the irradiation distance and time were the same as that for 4h-MIP-SG, the irradiation distance was changed to 20 cm and the reaction time was changed to 2×1 h. The material was filtered after the first 1 h reaction and the same reactant was added for the second time reaction. With this procedure, the obtained NIP-SG has the same carbon content as 4h-MIP-SG (measured by elemental analysis) to ensure that the NIP-SG and 4h-MIP-SG have the similar affinity resulting from the poly(MAA-co-EDMA) structure (non-specific interaction).

2.4. Preparation of restricted access-MIP grafted silica

Grafting of poly(GMA) chain on the MIP-SG surface was performed via living radical polymerization in a lab-made glass flask with water jacket. MIP-SG (2.0 g), GMA (30.2 mmol) and cyclohexanone (20 mL) were added into the flask. The reaction was initiated by UV irradiation from a high pressure mercury lamp at a distance of 10 cm. The polymerization was carried out with stirring at 40 °C under N_2 protection. After the reaction, the particles were extracted with acetone and then hydrolyzed with 0.1 mol L^{-1} sulfuric acid at 60 °C for 8 h. The restricted access-MIP-silica was obtained and designated as RA-MIP-SG.

2.5. Characterization of the MIP grafted silica and restricted access MIP grafted silica

After each step of reaction, the amount of grafted moiety was analyzed by the carbon increment using elemental analyzer (Elementar Vario EL, Elementar, Germany). The amount of $-CH_2Cl$ group and iniferter in the surface of silica were analyzed by the contents of chlorine and sulfur respectively with the method of oxygen flask combustion-ion chromatography.

The thickness of the imprinted polymer layer on the silica-support was estimated with the equation:

$$d = \frac{n_p \times M_p}{DS} \times 10^3 \quad \text{and} \quad n_p = \frac{m_c}{M_c} \tag{1}$$

in the equations, d is the thickness of the polymer layer and n_p is the number of polymer units in 1 g of MIP-SG. The M_p is the molar mass of one repeating unit of polymer assuming the polymer was composed by stoichiometric incorporation of MAA and EDMA. D is the weighted average density of the monomers and S is the specific surface area of the bare silica measured by BET method (Quantachrome NOVA 2000, USA). M_c is the mass of total carbon in one polymer repeating unit. m_c is the weight of carbon in the MIP polymer layer in 1 g of MIP-SG estimated by the carbon increment after the MIP grafting reaction.

The unit of the GMA in each poly(GMA) chain on the surface of the material was calculated by:

$$n_{GMA} = \frac{\Delta C}{M_r \times N_r \times n} \tag{2}$$

in the equation, ΔC is the carbon content increment in 1 g of particle after grafting of the GMA obtained by elemental analysis. M_r is the mass of the carbon atom. N_r is the numbers of carbon atom in one GMA molecule and n is the moles of the iniferter grafted on 1 g of particle.

The pore structures of the materials were analyzed by an Autopore IV 9500 V1.07 mercury intrusion porosimeter (Micromeritics, USA). The IR spectra were obtained from an AVATAR-360 FTIR instrument (Nicolet, USA). The morphology of MIP-SG and RA-MIP-SG was characterized by an SS-550 scanning electron microscope (Shimadzu, Japan).

2.6. Chromatographic evaluation of MIP-SG and RA-MIP-SG

HPLC was used to evaluate the MIP grafted silica and restricted access MIP-silica. A Shimadzu HPLC instrument equipped with an LC-20 AT pump, an SPD-20A UV detector was employed for the chromatographic analysis. The obtained particles were dry packed in the stainless steel columns. The detection wavelength was 268 nm for sulfonamides and 280 nm for BSA. The retention

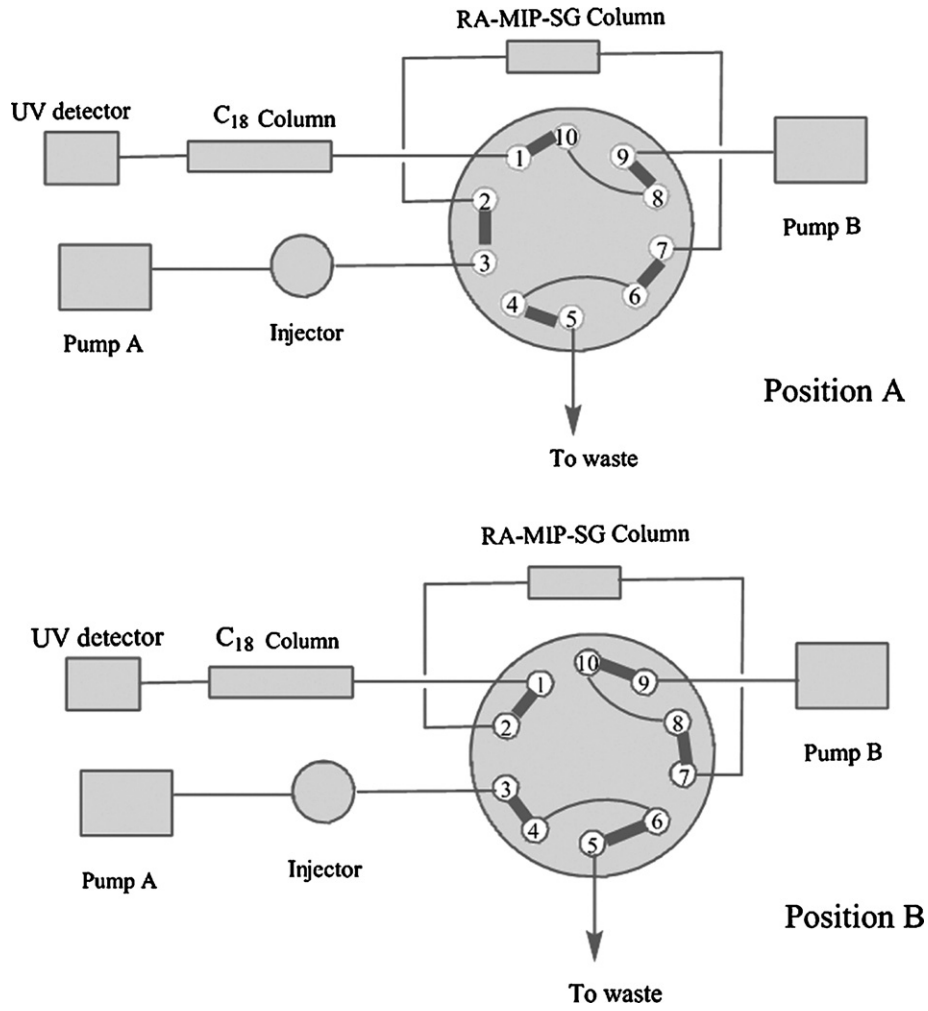

Fig. 2. The schematic diagram of the column-switching HPLC system. Position A: sample pretreatment step. Position B: analytical step.

factor (k) was calculated by $k = (t_R - t_0)/t_0$ where t_R is the retention time of the analyte and t_0 is the void time measured by potassium dichromate. Separation factor (α) was calculated by $\alpha = k_{SMZ}/k_{SMO}$, where k_{SMZ} and k_{SMO} are retention factors of SMZ and SMO respectively. The imprinting factor (IF) was determined by $IF = k_{MIP}/k_{NIP}$, where k_{MIP} and k_{NIP} are the retention factors of the template (SMZ) on the MIP-silica and NIP-silica respectively.

Recovery of BSA in HPLC process was calculated based on the peak area of BSA by taking the area obtained without a column as 100%.

2.7. Preparation of the standard solutions and sulfonamide spiked milk samples

Two stock solutions containing sulfonamides (1 mg mL^{-1} for each compound) were prepared in CH_3OH. One stock solution contained SMZ, SQX, SMD and SDM and the other contained SDZ, SMT and SMO. The standard solutions with sulfonamide concentrations: 2, 5, 25, 50, 100, 200, 400 and 1000 ng mL^{-1} were prepared by dilution of the stock solution with eluent A: (0.1 M phosphate buffer, pH 6.0)/CH_3OH (95/5, v/v).

Pasteurized bovine milk was purchased from local supermarket. The milk was centrifuged at 15,000 × g for 15 min and the central layers were withdrawn for the following sample preparation. To prepare the spiked samples, aliquots (1.0 mL) of the sulfonamide standard solution were mixed with 1.0 mL milk prepared. The solutions were ultrasonicated and agitated using a vortex mixer. The final spiked sulfonamide concentrations in the samples were: 25, 100, 500 ng mL^{-1} respectively. All solutions were stored at +4 °C after preparation.

2.8. Column-switching HPLC for the determination of sulfonamides in bovine milk

A column-switching HPLC system (Fig. 2) was used for the determination of sulfonamides in bovine milk. The system was composed of two LC-20 AT pumps, an SPD-20A UV detector and a 10-port switching valve (C2H-1000EH, VICI Valco instruments Co. Inc., USA). A Rheodyne 7725 injector with a 100 μL loop was used for sample injection. The position of the 10-port switching valve was controlled by the Vacom software (Valco instruments Co. Inc., USA). A stainless steel column (40 mm × 4.6 mm) slurry packed with RA-MIP-SG was used for sample pretreatment, coupled with a 250 mm × 4.6 mm analytical column (octadecyl Luna, 5 μm, Phenomenex, USA). In the analysis, the RA-MIP-SG was equilibrated with eluent A [(0.1 M phosphate buffer, pH 6.0)/CH_3OH (95/5, v/v)] first. After the sample injection, the RA-MIP-SG column was washed with the eluent A for 5 min at a flow rate of 1.0 mL min^{-1} (pretreatment step in Fig. 2). Then, the valve was switched to position B. The SAs were eluted from the RA-MIP-SG column by back-flush mode and analyzed on the analytical column using eluent B (for SMD, SMZ, SQX and SDM) or using eluent C (for SDZ, SMT and SMO) respectively (analytical step in Fig. 2). The eluent B was (0.1 M phosphate buffer, pH 7.0)/acetonitrile (83/17, v/v) and eluent C was

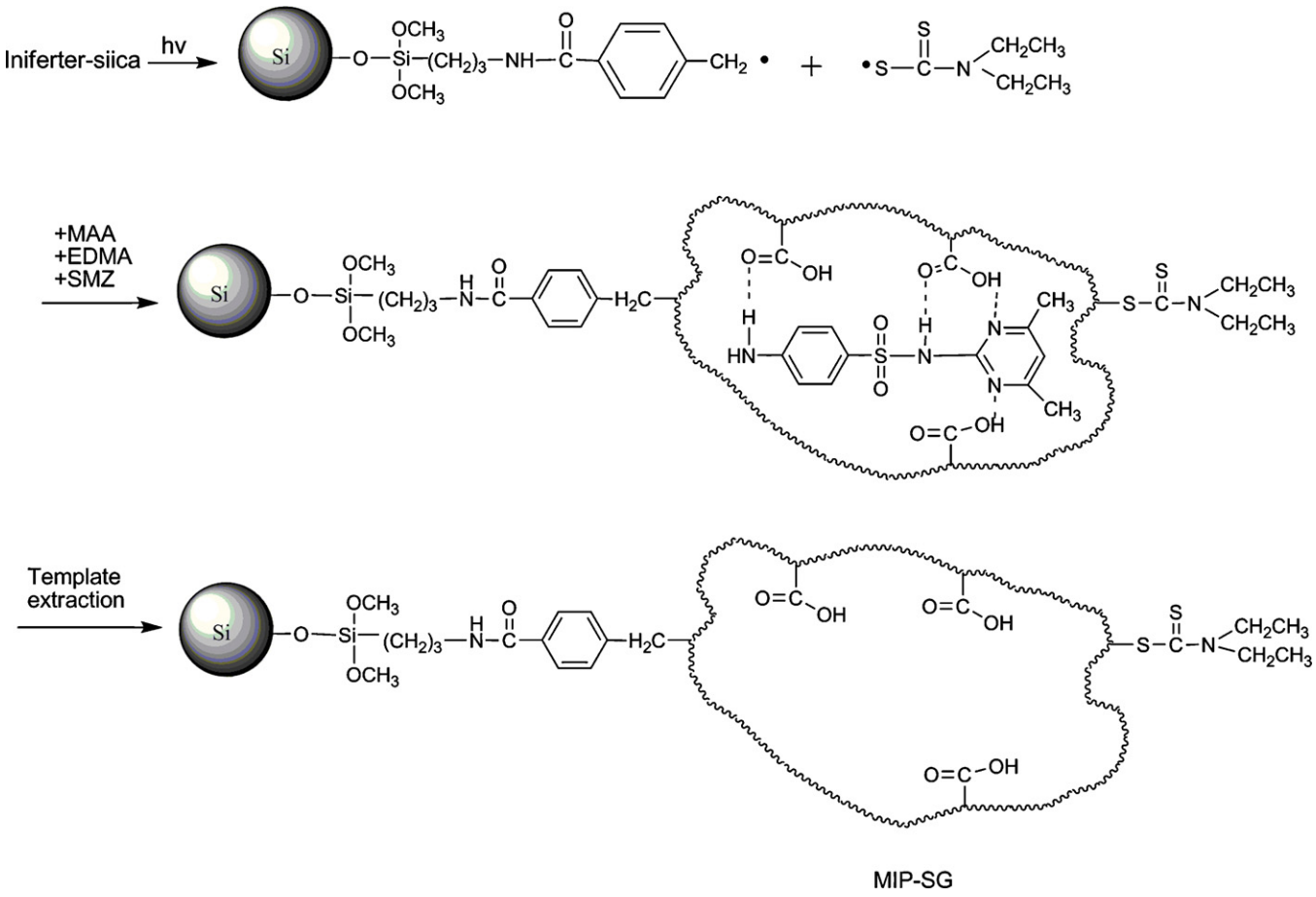

Fig. 3. The schematic illustration of grafting of the MIP layer on the surface of silica via iniferter technique.

(0.1 M phosphate buffer, pH 8.0)/acetonitrile (94/6, v/v). Flow rate was 1.0 mL min^{-1}. The SAs were detected at 268 nm. The mobile phases for SA separation in analytical column were the same as the eluting solution.

The linearity was obtained with the same conditions and procedures for the sample analysis using standard solutions. The method validation was performed with the spiked milk samples.

3. Results and discussion

3.1. Grafting of iniferter on the surface of silica

To perform a surface initiated "grafting from" iniferter polymerization, the selected iniferter group (benzyl *N*,*N*-diethyldithiocarbamate, BDC) has to be grafted on the support. The method for introducing BDC on the silica in this study was different from our previous work [35]. Higher grafting density was obtained by the present strategy. The procedure for the preparation of iniferter (benzyl *N*,*N*-diethyldithiocarbamate) bonded silica is illustrated in Fig. 1. In the synthesis, silica gel was reacted with 3-aminopropyl trimethoxysilane to introduce amino group, followed by the reactions with 4-(chloromethyl)benzoyl chloride and sodium *N*,*N*-diethyldithiocarbamate successively. The amount of aminopropyl group grafted on the silica (Si-I) was 0.19 mmol g^{-1} derived from the elemental analysis. The content of $-CH_2Cl$ group and iniferter immobilized on the silica-based support were 90.5 μmol g^{-1} (3.88 μmol m^{-2}) and 39.5 μmol g^{-1} (1.69 μmol m^{-2}) respectively, determined by oxygen combustion-ion chromatography.

3.2. Synthesis of SMZ imprinted polymer grafted silica via iniferter technique

After making the iniferter bonded silica, the molecularly imprinted polymer was synthesized on the surface of the silica under UV irradiation. The reaction for the grafting of SMZ imprinted polymer on the silica surface by iniferter technique is demonstrated in Fig. 3.

The MIP layer was formed using MAA as the functional monomer and EDMA as the cross-linker. The molar radio of template/monomer/cross-linker was 1/8/40, which is the optimized number from our previous research [35]. The reaction temperature was controlled by the ice-water bath to stabilize the template-monomer complexation. Retention of the SMZ and separation of SMZ and its structural analog SMO on the MIP grafted silica (MIP-SG) were used to evaluate the affinity and selectivity of the material respectively. MIP-SGs with different polymer thickness were created by variation of the polymerization period to study the relation between the properties of the material and the thickness of the MIP layers.

The result has shown that when the polymerization time was changed from 3 h to 4 h, the thickness of the MIP layer on the silica was increased from 3.6 to 6.2 nm and the separation factor of the materials was increased from 1.6 to 2.3 (Table 1). While the thickness of the MIP layer and separation factor of the MIP-SG did not have obvious change when the reaction time was increased further. To find out the reason for this situation, the content of iniferter in the 4h-MIP-SG (38.5 μmol g^{-1}) was determined by oxygen flask combustion-ion chromatography. The similar iniferter content before and after the MIP grafting suggested that the loss

Table 1
The effect of the polymerization time on the affinity and selectivity of the material evaluated by HPLC analysis.[a]

MIP-SG	Polymerization time (h)	Thickness of MIP layer (nm)[b]	Retention factor (k)		Separation factor (α)
			SMZ	SMO	
3h-MIP-SG	3	3.6	1.4	0.9	1.6
4h-MIP-SG	4	6.2	1.6	0.7	2.3
8h-MIP-SG	8	6.5	1.8	0.7	2.6

[a] HPLC column size was 150 mm × 4.6 mm. Acetonitrile was used as mobile phase with flow rate of 1.0 mL min^{-1}. The detection wavelength was 268 nm.
[b] The thickness of the polymer layer was calculated from Eq. (1) which is based on the carbon content increment from the elemental analysis.

of iniferter in the MIP grafting process is negligible. Based on this result, we attribute the less increase of MIP layer in the 8 h reaction to the less flexibility of the surface iniferter in the cross-linked MIP layer. The MIP-SG synthesized from 4 h reaction, designated as 4h-MIP-SG, was used in the subsequent study. The NIP-SG corresponding to 4h-MIP-SG was synthesized. The retention factor (k) for SMZ on the NIP-SG was 0.6. The imprinting factor (IF) of 4h-MIP-SG, determined by $IF = k_{MIP}/k_{NIP}$ was 2.7.

The chemical composition of the grafted layer on the silica was studied with Infrared spectroscopy (Fig. 4). The absorption peak at 1730 cm^{-1} in the spectrum of MIP-SG (4h-MIP-SG) was assigned to ester carbonyl stretching vibrations, which indicated MAA and/or EDMA were bonded in the material.

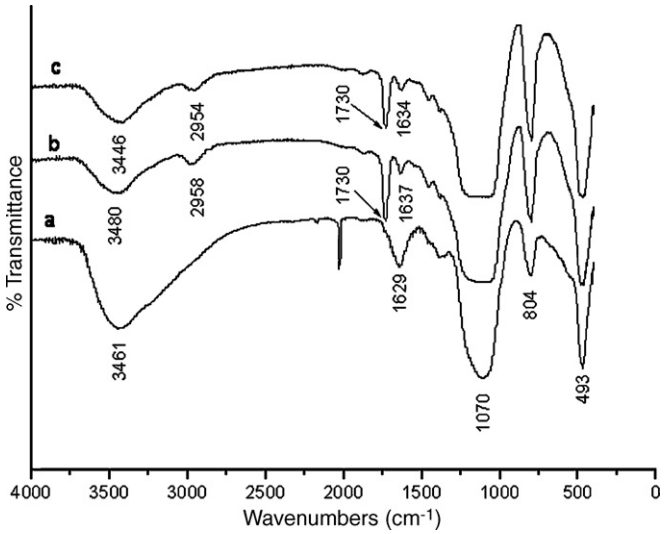

Fig. 4. FTIR spectra (KBr) of Iniferter-silica (a), MIP-SG (b) and RA-MIP-SG (c).

3.3. Surface grafting of hydrophilic layer on the MIP-silica

In the construction of the hydrophilic polymer chain, GMA was chosen as monomer because it can be easily converted to the glycerol monomethacrylate (GMMA) possessing diol groups, which has biocompatibility and has often been used to cover the surface of restricted access material (RAM). The process of the hydrophilic chain grafting is demonstrated in Fig. 5. After grafting poly(GMA) on the surface of MIP-SG using iniferter polymerization, the epoxide rings were opened by hydrolysis to form hydrophilic structure [poly(GMMA) chain]. The resulting material (RA-MIP-SG) with hydrophilic and MIP layers has similar IR spectrum as MIP-SG (Fig. 4).

It is assumed that the hydrophilic poly(GMMA) chains prevent protein from being adsorbed in the internal MIP layer. The percent coverage of the inner surface by the hydrophilic chain depends on the poly(GMMA) length and density of the iniferter. In the GMA grafting process, the GMA concentration and reaction temperature were optimized. The reaction time was used to control the length of the poly(GMA) chain. The recovery of BSA from the RA-MIP-SG

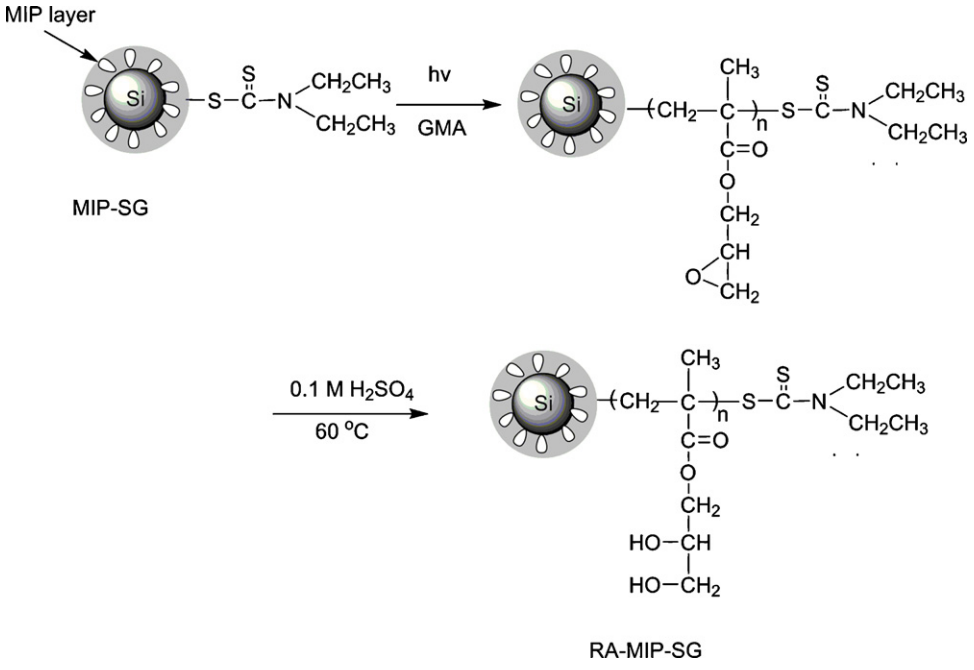

Fig. 5. Surface grafting of hydrophilic layer on imprinted polymer grafted silica (MIP-SG).

Table 2
Effect of the GMA polymerization time on the poly(GMA) chain length and exclusion ability of the material.[a]

Material[b]	Reaction time for GMA grafting (h)	Calculated number of the GMA unit in poly-GMA chain[c]	Recovery of BSA (%)	
			5 mg mL^{-1}	10 mg mL^{-1}
4h-MIP-SG	–	–	4.4	4.6
4h-RA-4h-MIP-SG	4	2.8	4.9	5.6
6h-RA-4h-MIP-SG	6	17.1	99.0	101.4

[a] HPLC column size was 150 mm × 4.6 mm. The mobile phase was 0.1 M phosphate buffer (pH 7.0)/THF/isopropanol (84/6/10, v/v). BSA solution (20 μL) was injected and detected at 280 nm.
[b] The 4h-MIP-SG is the MIP-silica from 4 h imprinting reaction. The 4h-RA-4h-MIP-SG and 6h-RA-4h-MIP-SG are the materials after hydrophilic chain modification on the 4h-MIP-SG.
[c] The number of the GMA unit in grafted poly-GMA chain was estimated from the carbon content increment after reaction.

Table 3
Pore structures of the materials measured by mercury intrusion method.

Material[a]	Average pore diameter (nm)	Total intrusion volume (mL g^{-1})	Total pore area (m^2 g^{-1})
SG	98.2	0.85	34.5
4h-MIP-SG	61.5	0.66	42.7
6h-RA-4h-MIP-SG	55.6	0.43	31.2

[a] SG is the silica gel without any modification. The 4h-MIP-SG is the MIP grafted silica gel (4 h grafting reaction). The 6h-RA-4h-MIP-SG is the material after MIP grafting (4 h grafting reaction) and hydrophilic chain modification (6 h reaction).

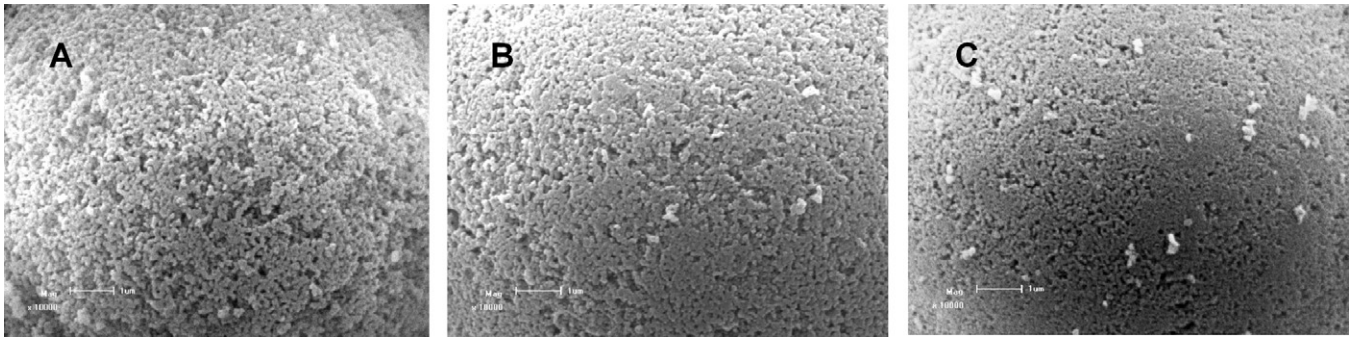

Fig. 6. Scanning electron micrographs of SG (A), MIP-SG (B) and RA-MIP-SG (C).

column was used to evaluate the protein exclusion efficiency of the material. The relation between the poly(GMMA) chain length and the recovery of BSA from the RA-MIP-SG column was studied. The result demonstrated that a good BSA recovery was achieved on the 6h-RA-4h-MIP-SG with longer poly(GMA) chains from 6 h reaction (Table 2), whereas the recovery of BSA on the 4h-MIP-SG (without hydrophilic chain) in HPLC was less than 5% (Table 2). It further proved that the hydrophilic layers in RA-MIP-SG prevent the irreversible adsorption of the BSA. Because undesired gelation in solution was found in the 8 h synthesis, 6 h was selected as reaction time.

The influence of grafted polymer on the pore structure of the material was investigated by mercury intrusion method. The average pore diameter in RA-MIP-SG is 55.6 nm (Table 3), which is much larger than the hydrodynamic diameter of BSA (about 8 nm at neutral pH [36]). It indicated that the poly(GMMA) chain in the RA-MIP-SG created a chemical diffusion barrier for protein exclusion. The pore diameter and pore volume of the material decrease after surface modification by MIP and poly(GMA), indicating that polymer is in the inner surface of the silica pores. The comparison of the scanning electron micrographs of SG, MIP-SG and RA-MIP-SG (Fig. 6) has shown that the particle surface became smoother after each polymer grafting process, which further demonstrated the immobilization of the polymers on the silica.

Table 4
Retention of SMZ and SMO and separation factors on the MIP-SG, RA-MIP-SG and NIP-SG.[a]

Material[b]	Retention factor (k)		Separation factor (α)
	SMZ	SMO	
MIP-SG	2.6	1.2	2.2
RA-MIP-SG	1.2	0.6	2.0
NIP-SG	1.2	1.0	1.2

[a] HPLC column size was 150 mm × 4.6 mm. The mobile phase was (0.1 M phosphate buffer, pH 7.0)/CH_3CN (80/20, v/v). The detection wavelength was 268 nm.
[b] The data for MIP-SG and RA-MIP-SG were from the 4h-MIP-SG and 6h-RA-4h-MIP-SG respectively. NIP-SG is the non-imprinted polymer grafted silica correlated to the 4h-MIP-SG.

3.4. Evaluation of the recognition ability of the RA-MIP-silica

To evaluate the recognition property of the material, separations of SMZ and SMO in HPLC using MIP-SG and RA-MIP-SG as the stationary phase were studied respectively. The separation factors on the RA-MIP-SG and MIP-SG are similar (Table 4), which indicated that the imprinted recognition sites remained in the restricted access-MIP-silica. The decreased retention of SMZ on the RA-MIP-SG demonstrated that the hydrophobic interaction favors the retention.

The HPLC profiles of SMZ, SMO and BSA on the columns of MIP-SG and RA-MIP-SG are shown in Fig. 7. The mobile phase: (0.1 M phosphate buffer, pH 7.0)/THF/isopropanol (84/6/10, v/v) was used for better BSA elution. Although three compounds were well separated on the MIP-SG column, the recovery of BSA from the column was very poor (4.6%, Table 2). On the other hand, complete recov-

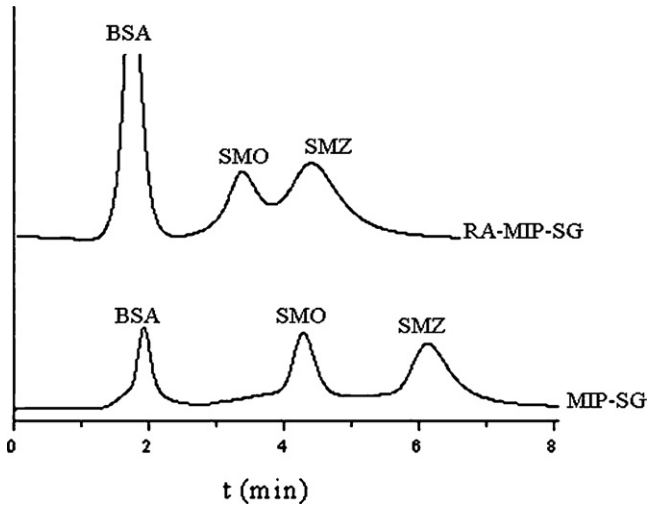

Fig. 7. Chromatographic separation profiles of SMZ, SMO and BSA on the RA-MIP-SG and MIP-SG columns respectively. HPLC column size was 150 mm × 4.6 mm. The 6h-RA-4h-MIP-SG was used for RA-MIP-SG and 4h-MIP-SG was used for MIP-SG respectively. The mobile phase was 0.1 M phosphate buffer (pH 7.0)/THF/isopropanol (84/6/10, v/v). The flow rate was 1.0 mL min^{-1}. The analytes were detected at 280 nm.

Table 5
Retention of sulfonamides and BSA on the RA-MIP-SG column.[a]

Sulfonamides	Retention factor (k)
SQX	24.5
SDM	21.7
SMD	12.5
SMZ	10.0
SMO	3.8
SDZ	2.4
SMT	1.3
BSA	0.1

[a] HPLC column size was 150 mm × 4.6 mm. The mobile phase was (0.1 M phosphate buffer, pH 7.0)/CH_3OH (90/10, v/v). The detection wavelength was 268 nm. The 6h-RA-MIP-SG was used for RA-MIP-SG.

ery of BSA was achieved from the RA-MIP-SG column, while the separation factor for SMZ/SMO was only 0.2 less than that on the MIP-SG.

3.5. Column-switching HPLC conditions for the determination of sulfonamides in bovine milk

In bio-analytical HPLC methods, the sample preparation is often considered to be the most time consuming procedure. An increasing number of studies have used RAM columns to reduce sample handling and preparation time. In this research, a method of using RAM-MIP for online sample pretreatment in the determination of SAs from bovine milk with direct HPLC injection was developed.

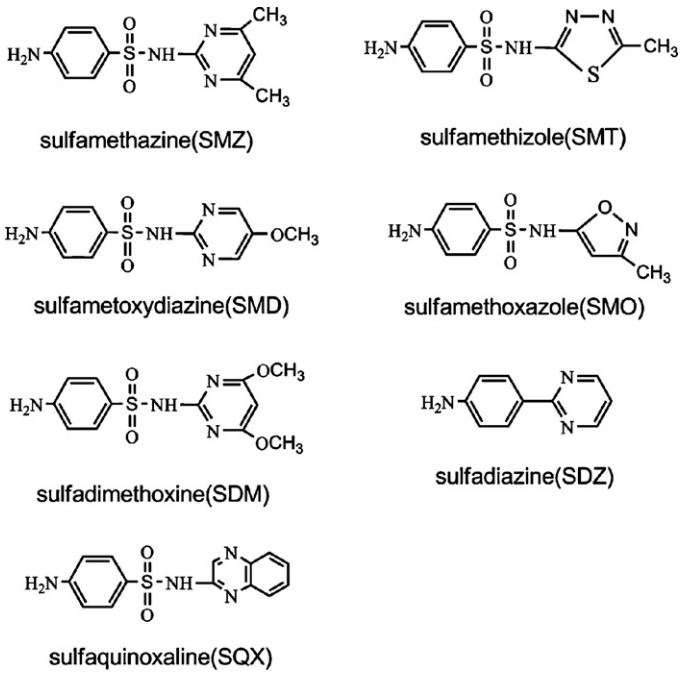

Fig. 8. The structures of sulfonamides used in the study.

Table 7
Accuracy and precision of column-switching HPLC method for the determination of sulfonamides in bovine milk (n = 5).

Sulfonamides	%Recovery (%RSD)		
	25 ng mL^{-1}	100 ng mL^{-1}	500 ng mL^{-1}
SMD	94.5 (2.8)	100.3 (7.9)	101.5 (1.7)
SMZ	99.5 (7.1)	107.1 (2.1)	97.1 (1.6)
SQX	97.9 (4.9)	103.4 (3.3)	98.7 (1.6)
SDM	95.6 (3.5)	105.6 (2.0)	99.9 (1.9)
SDZ	100.6 (4.9)	93.9 (1.6)	97.8 (3.4)
SMT	95.4 (6.8)	93.4 (0.9)	96.8 (2.0)
SMO	95.3 (2.9)	93.5 (1.8)	98.8 (0.9)

To find out if the RA-MIP-SG material can be used for more sulfonamide determination in milk, the retentions of seven sulfonamides on the RA-MIP-SG were studied and compared with the retention of BSA (Table 5). The structures of the sulfonamides are shown in Fig. 8. Because some SAs have better solubility in methanol than in the acetonitrile, CH_3OH was used as organic modifier in the mobile phase. The results showed that the compounds: SQX, SDM and SMD, whose structures are similar to the template molecular (SMZ), have higher retention than SMZ on the RA-MIP-SG when higher phosphate buffer content (90% in volume ratio) was used in mobile phase (Table 5). In this mobile phase condition, BSA was eluted in void volume and can be well separated from SAs. It demonstrated that the RA-MIP-SG can be used for milk sample clean-up and SAs extraction using direct HPLC injection.

Table 6
The linearity, LOD and LOQ of the column-switching HPLC method developed for the determination of sulfonamides in bovine milk.

Sulfonamides	Linear equation[a]	r	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
SMD	$Y = 453.49x + 1227.59$	0.9999	0.5	1.8
SMZ	$Y = 488.50x + 5053.13$	0.9994	0.4	1.3
SQX	$Y = 527.80x + 12{,}508.28$	0.9991	0.2	0.7
SDM	$Y = 552.98x + 10{,}269.28$	0.9987	0.2	0.7
SDZ	$Y = 42.17x + 417.35$	0.9998	0.4	1.3
SMT	$Y = 448.22x + 339.04$	0.9995	0.8	2.7
SMO	$Y = 4224.98x + 427.97$	0.9981	0.7	2.3

[a] The linearity was measured in the SA concentration range of 2–400 ng mL^{-1}.

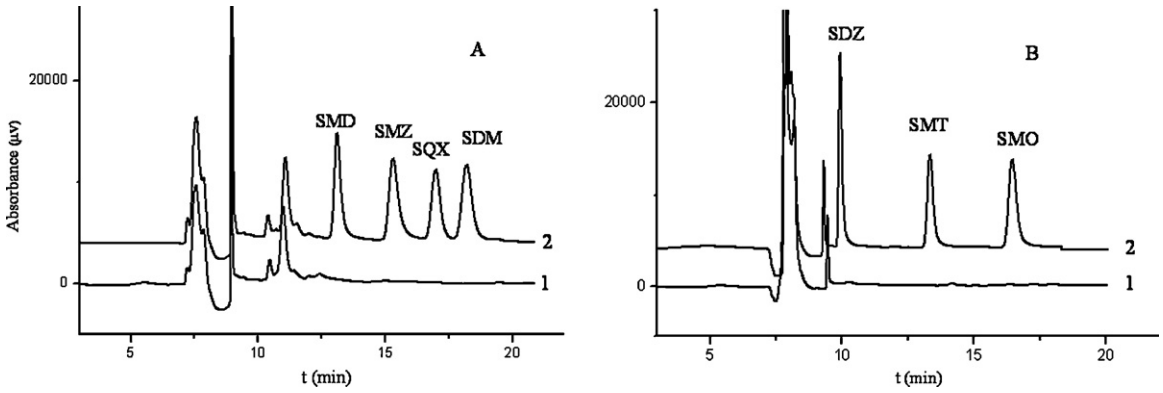

Fig. 9. The HPLC profiles of the blank milk (trace 1) and the milk sample spiked with sulfonamides (trace 2). The column-switching HPLC condition is in Section 2. A is for the determination of SMD, SMZ, SQX and SDM. The eluent B [(0.1 M phosphate buffer, pH 7.0)/acetonitrile (83/17, v/v)] was used in the analytical step. B is for the determination of SDZ, SMT and SMO. The eluent C [(0.1 M phosphate buffer, pH 8.0)/acetonitrile (94/6, v/v)] was used in the analytical step.

To separate SAs after the RA-MIP-SG extraction, a C_{18} column was used. The column-switching HPLC system for the analysis is shown in Fig. 2. To choose a better condition for protein exclusion and the analyte extraction, the influence of the mobile phase on the retention of SAs and protein on the RA-MIP-SG was studied. Different solutions with variation of pH, buffer salt concentration and ratio of buffer/CH_3OH were evaluated as eluent in the study. The mobile phase: 0.1 M phosphate buffer (pH 7.0)/THF/isopropanol (84/6/10, v/v) cannot be used because the proteins in milk sample have longer elution time due to the higher concentration, which resulted in a overlapping of the protein and SA peaks. The eluent A: [(0.1 M phosphate buffer, pH 6.0)/CH_3OH (95/5, v/v)] at flow rate 1.0 mL min^{-1} was finally selected for loading and washing (pretreatment) step. Under this condition, the matrix components in milk including protein were eluted within 5 min and all the SAs were retained in the RA-MIP-SG column.

The HPLC conditions for eluting the SAs from the RA-MIP-SG and separation on the analytical column were also selected and optimized by a series of experiments. Because suitable peak shape and analytical time cannot been obtained if seven SAs were analyzed simultaneously by one mobile phase, SAs were divided into two groups and analyzed separately under different mobile phases. In the study, eluent B (mobile phase B) was used for SMZ, SQX, SMD and SDM, which have higher retention on the RA-MIP-SG, while eluent C (mobile phase C) was used for SDZ, SMT and SMO respectively. Best separations have been achieved under these two conditions. Two groups of SAs do not interfere each other if they existed in the same sample.

3.6. Method validation

The HPLC method was validated for each analyte. The calibration curves, obtained from peak area of the analytes versus the concentration, are linear with a correlation coefficient greater than 0.998 in the SA concentration range of 2–400 ng mL^{-1} (Table 6). The limit of detection (LOD) and limit of quantification (LOQ) were defined as 3 times and 10 times of the noise of HPLC profile respectively. The highest LOQ is 2.7 ng mL^{-1} (Table 6), which is far below the MRL established in European and China.

The accuracy of the method was determined by recovery test. The fortified milk samples with three SA concentration levels (25, 100, 500 ng mL^{-1}) were analyzed. The recoveries were from 93% to 107% with the RSD less than 8.0% (Table 7).

The chromatograms of blank and spiked milk samples by the column-switching HPLC procedure are shown in Fig. 9. No leaking of the template molecules from RA-MIP-SG has been found. The results have shown that there is no interference from the endogenous compound for the detection of sulfonamides, indicating that the method provided adequate clean-up of milk samples. The accurate determination of trace amounts of sulfonamides in bovine milk can be attained by this method.

4. Conclusion

A RAM-MIP grafted silica was synthesized using surface-initiated iniferter technique. SMZ imprinted polymer internal layer and hydrophilic external layer were immobilized on the surface of silica successfully. The material has the properties of MIP and RAM and can be used in selective extraction and sample clean-up in the SAs analysis in milk. A simple direct-injection HPLC method was established using the RAM-MIP grafted silica for sample online pretreatment. Good accuracy and precision were obtained, indicating that the method can be applied in the determination of sulfonamides in milk with good reliability. This research demonstrated that the iniferter technique provides a way of synthesizing the restricted access-MIP. This multifunctional material can be used in the determination of drug residues in the biological sample with higher analytical efficiency.

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