Determination of Sulfadiazine Residues in Pork by Molecular Imprinted Column Coupling with High Performance Liquid Chromatography

HUANG Yun-Hong, XU Yang, HE Qing-Hua, CAO Yu-Sheng, DU Bi-Bai

1 State Key Laboratory of Food Science and Technology, Sino-German Joint Research Institute, Nanchang University, Nanchang 330047, China
2 College of Life Science, Jiangxi Normal University, Nanchang 330022, China

Abstract: A method for the quantitative monitoring of sulfadiazine (SD) residues in pork was established by molecular imprinted column coupling with high performance liquid chromatography (HPLC). The molecular imprinted column was selected as an extraction device. To obtain the optimum extraction efficiency, several parameters related to the molecular imprinted column, including column solvent, flow-rate, eluent of the sample matrix and eluent volume, were investigated. The sample solution was directly injected into the device for the extraction after simple extraction. Under the optimum conditions, the relative standard deviations (RSD) was ≤ 6.1%, and the recoveries for SD were higher than 75.6%. In comparison with the AL-SPE column, the MIP-SPE column had good reusability and extraction efficiency. This method was successfully applied to the determination of sulfadiazine residues in pork.

Key Words: Molecular imprinted column; High performance liquid chromatography; Sulfadiazine; Pork

1 Introduction

Sulfadiazine (4-amino-N-2-pyrimidinyl-benzene sulfonamide, SD) is an odorless, white or slightly yellow powder. It is stable in the solid state under exposure to air, humidity and temperature up to 100 °C for two weeks. It darkens upon exposure to light[1]. Sulfadiazine is introduced in medical therapy because it possesses antibacterial activities, and commonly used as the sulfonamide especially in veterinary practice to control bacterial infections and prevent the outbreak of diseases[2,3]. The hazard of sulfadiazine and its metabolites for people has been realized due to their potential toxic effects. The sulfadiazine is excreted as active metabolite, which increase the level of the residues in pork that is toxic for humans[4–6]. Long exposure to trace residues can lead to the increase in drug-resistant microbial strains that are difficult to control. Owing to its carcinogenicity, especially in hypersensitive individuals, the residues should be avoided or reduced to a minimum. To ascertain safe limits for human consumption, the European Union established a maximum residues limit (MRL) of 100 μg kg⁻¹ for the total amount of sulfonamides in edible tissue[7].

Several analytical methods have been developed for the determination of SD residues in pork sample[8–16]. High performance liquid chromatography (HPLC) is a feasible method in common use due to its advantages such as sensitiveness and accuracy. In the whole analytical procedure, sample preparation is important and crucial[17–20]. In general, the SD was first extracted from pork with organic solvents or the mixture of organic and aqueous solutions. Then the
extracts were centrifugated and passed a solid-phase extraction (SPE) column, such as C_{18}-SPE column and alkalinity alumina SPE column (AL-SPE column), for clean-up and concentration. The main problem associated with the use of standard SPE columns is the low selectivity of the retention mechanism. Nevertheless, this limitation can be overcome by using columns packed with materials based on molecularly imprinted polymers (MIP)[21–25]. The MIP can be selected as a particular adsorbent for they have the advantages of reusable and high stability to harsh chemical conditions. The polymer can be packed in disposable cartridges for off-line SPE or in columns for on-line SPE[26]. The molecular imprinted SPE column (MIP-SPE column) could weaken the interference target analyte from complex matrixes and improve its selectivity, thus it was applied to enrich and determine a range of analytes, such as benzimidazole[27], 2-aminopyridine[28], tetracyclines[29], and bisphenol A[23].

In this study, a method was developed for the determination of SD in pork by using the MIP-SPE column coupling with HPLC. The MIP for SD was successfully synthesized by a thermal polymerization method. And then the SPE column was packed with MIP particles. The SD in extraction solvent was preprocessed through molecular imprinted column. Finally the SD in eluent was determined by HPLC. In comparison with the AL-SPE column, the MIP-SPE column had the advantages of good reusability and high extraction efficiency.

2 Experimental

2.1 Materials and apparatuses

Sulfadiazine (SD), sulfadimidine (SM), sulfamethoxazole (SMZ) and sulfaquinoxaline (SQX) were purchased from China Institute of Veterinary Drugs Control with a purity of 100% (Beijing, China). The functional monomer acrylamide (AAM) and the free radical initiator 2,2-azobisisobutyronitrile (AIBN, > 98%) were purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). The cross-linking agent ethylene glycol dimethacrylate (EDMA) was purchased from Shanghai Hechuang Chemical, Inc. (Shanghai, China). HPLC grade acetonitrile and methanol were purchased from Tianjin Yongda chemical (Beijing, China). All the other chemicals were of analytical grade. The alkalinity alumina SPE column for SD (3 mL with 200 mg of the dry adsorbent) was obtained from Shanghai Chu Bai Laboratory Equipment, Inc. (Shanghai, China).

The morphology of sample was analyzed by SU-1510 scanning electron microscopy (SEM, Hitachi). The sulfadiazine in samples was analyzed by Agilent-1100 HPLC-UV system (Agilent Co., USA) with a C_{18} column (250 mm × 4.6 mm, i.d., 5 μm, Agilent Co., USA). The mobile phase consisted of the mixture of acetonitrile-water containing 0.01% phosphoric acid (20:80, V/V). The flow rate was 1 mL min^{-1} and the monitoring wavelength was 270 nm. The injection volume was 20 μL and column temperature was held constant at 25 °C. All solutions used for HPLC were filtered through a 0.45-μm filter before use.

2.2 Synthesis of MIP

The molecularly imprinted polymers were synthesized using bulk polymerization. The pre-polymerization mixture consisted of 1 mmol template SD and 4 mmol AAM was dissolved in 10 mL of acetonitrile prepared in a 50-mL borosilicate glass bottle with a screw cap. The mixture above was pre-polymerized for 30 min. Then 4 mmol EDMA and 1 mmol AIBN were added. The pre-polymerization mixture was purged with argon for 5 min to remove oxygen. Thermo-polymerization was initiated by placing the borosilicate glass bottle in a conduction oil bath at 60 °C for 24 h. The non-imprinted polymers (NIP) were synthesized under the same conditions but without the addition of the template.

The bulk polymers obtained were crushed, ground and sieved. The particle size fraction from 0.18 to 0.28 mm was collected. The resulting particles were placed in a Soxhlet extraction apparatus and washed with mixture of methanol and acetic acid (9:1, V/V) to remove the template molecules until SD was no longer detected in the eluent. Then, the products were dried under vacuum at 50 °C for further work.

2.3 Sample preparation

Pork obtained from local market was deep-frozen at −4 °C until analyzed. These samples were homogenized by the blender. About 5.0 g of pork sample and 4 g of anhydrous sodium sulfate were directly put into a 50-mL centrifuge tube and then extracted with 25 mL of acetonitrile by vibrating for 6 h. The sulfadiazine was extracted for twice. The supernatant liquid obtained by centrifugation was transferred into a 250-mL separatory funnel including 30 mL normal hexane. The acetonitrile was transferred into a 100-mL heart left bottle including 5 mL normal propyl alcohol. The resultant solution was evaporated to dryness by reduced pressure concentration at 50 °C and finally stored at 4 °C in a refrigerator for subsequent experiment. The residues were re-suspended in a 5-mL column solvent and mixed thoroughly before analysis.

2.4 Solid-phase extraction experiments

A 2.5-mL injector (5.8 cm × 1.0 cm, i.d.) packed with 100.0 mg of MIP was used as solid phase extraction column, in which a small portion of sieve plate was packed to prevent the loss of adsorbent during sample loading. After the MIP-SPE column was pre-treated with 3 mL of acetonitrile and 3 mL of distilled water, the sample solution containing SD was passed
through the column at a flow rate. Then, the column was eluted with the mixture of acetonitrile and water solution. The eluate was analyzed by the HPLC.

3 Results and discussion

3.1 Characteristics of MIP

The structures of MIP and NIP particles were observed by SEM. It was necessary to cover the surface of the polymer with a thin, electrically conductive layer by gold sputtering prior to the SEM observation of the samples because polymers were insufficiently conductive on their own. It was found that the SEM images of NIP and MIP had appreciable differences in the morphology. The surface of the MIP exhibited a more porous structure than that of the NIP (Fig.1). The regular structure of the imprinted polymer was due to the fact that specific binding sites had been created for the polymer. The cavities in the MIP were probably caused by the structure of the target molecule, sulfadiazine. The phenomenon showed that the MIP could be successfully synthesized using bulk polymerization. Also these provided a guarantee of a sufficient extraction performance of the MIP for SD.

The adsorption experiments were carried out with a batch method. The dry MIP particles (100 mg) were immersed in a 10-mL sample solution containing 10 mg L⁻¹ of SD, SM, SMZ or SQX, respectively. The mixture in flask was incubated at 25 °C on a rotary shaker at 200 rpm for 2 h. Following centrifugation, the supernatant was analyzed by HPLC to quantify the concentration of residual sulfonamides. The NIP particles were done under the same conditions.

The selectivity of the MIP was evaluated by comparing the equilibrium adsorption ability to the template with that to the structural analogues. The adsorption amounts of SD, SM, SMZ and SQX on MIP and NIP are shown in Fig.2. There was a little difference between the adsorption amounts of mentioned sulfonamides on NIP. The adsorption amounts of SD on MIP were 870 mg g⁻¹ and were the highest than that of the other sulfonamides on MIP. This difference demonstrates that MIP exhibiting the enhanced affinity for SD was due to the imprinting effect produced in the presence of SD as the template. The results showed that the MIP as an adsorbent could be used to separate the SD from sample.

3.2 Optimization of solid-phase extraction

A preconcentration process was necessary because of the relative low amount of the analytes in the food. The objective of the optimization procedure was to obtain maximum analyte recovery. The parameters such as column solvent, flow-rate, eluent of the sample matrix and eluent volume were investigated. Optimization experiments were performed in triplicate.

3.2.1 Effect of column solvent on MIP–SPE column

The analytes were dissolved by the weaken solvent as far as possible under the conditions that the analytes could be dissolved in the SPE process so that it could retain well in the column. The certain proportion solvent prepared with organic solvent and water was used as column solvent because the solubility of SD was poor in the water. The solution for SD was made of the different proportions of acetonitrile/water that used as solvent. The MIP-SPE columns were added into a 1.0-mL sample containing 5 mg L⁻¹ of SD, then eluted with 3 mL acetonitrile. The concentration of the SD was determined by HPLC.

The column solvent impacted the productivity of the MIP-SPE column. As seen in Fig.3, the different column solvents hold the different recoveries of SD. The recovery of SD deceased rapidly with the increase of the volume fraction of acetonitrile. The SD can’t soluble in water and is slight soluble in acetonitrile. With the increase of the volume fraction of acetonitrile, the solubility of SD in the column...
solvent increased, thus the productivity of the MIP-SPE column deceased. The recovery of SD was 95% when the volume fraction of acetonitrile was 2.5%. So, the acetonitrile with 2.5% of the volume fraction was selected as the column solvent.

3.2.2 Effect of flow-rate on MIP-SPE column

The flow-rate of column solvent was crucial for the recovery of SD. The MIP-SPE column was added into a 1-mL sample containing 5 mg L\(^{-1}\) of SD, and then was eluted with 3 mL of the acetonitrile. The column solvent was flowing at 1.0, 2.0, 3.0 and 4.0 mL min\(^{-1}\), respectively. As shown in Fig.4, the recovery of SD decreased rapidly when the flow-rate was increased. The recovery of SD was more than 95% when the flow-rate was lower than 2.0 mL min\(^{-1}\). The recovery of SD was only 80% at 4 mL min\(^{-1}\). Both SD and MIP particles in MIP-SPE column interacted. And the MIP particles did not completely adsorb the SD, whereas the flow-rate of column solvent increased so that the recovery of SD was low. Consequently, the flow-rate of column solvent was 2 mL min\(^{-1}\) for further study when the efficiency of the column was considered.

3.2.3 Effect of eluent on MIP-SPE column

The eluent is a key factor to increase the productivity of the MIP-SPE column. The MIP-SPE column was added into a 1-mL sample containing 5 mg L\(^{-1}\) of SD and then eluted with 3 mL of the mixture of acetonitrile and water (1:4, 3:7, 2:3, 1:1, 3:2, V/V) solution respectively. As shown in Fig.5, the result indicated that the recovery of SD increased rapidly when the volume fraction of acetonitrile increased. The result was in accordance with the result in Section 3.2.1. Extraction and elution are reversible processes. The solubility of SD in the eluent increased when the volume fraction of acetonitrile increased, so that the productivity of the MIP-SPE column increased. In conclusion, the mixture of acetonitrile and water (2:3, V/V) solution was the optimum eluent for the following study.

3.2.4 Effect of eluent volume on MIP-SPE column

To determine the optimum eluent volume, the sample spiked with 5 µg SD was concentrated with the MIP, and different volumes (1.0, 2.0, 3.0 and 4.0 mL) of the eluent were applied in the washing step. As can be seen from Fig.6, for 1.0 mL of eluent solvent, the recoveries were lower than 65.1%. However, for 3.0 mL of eluent, the recoveries were up to 99%. So, 3 mL of eluent was selected as the optimal eluent volume.

3.3 Reusability of MIP-SPE column

One of the advantages of the MIP adsorbent is their reusability. To test this performance of the MIP-SPE column, these experiments were carried out under the optimized conditions. The MIP-SPE column was washed respectively by 5 mL acetonitrile and 5 mL water after elution. The results showed that the recovery of SD was about 98% and experienced no significant decrease after ten times of reuse of the MIP-SPE column. It showed that the MIP-SPE column had good reusability and could be applied to preprocess SD in the pork.

3.4 Validation of method

The method for the determination of SD was validated by determining its performance characteristics regarding linearity, repeatability, and precision. To test the SD response linearity, a series of standard solutions of SD in the concentration range
of 50–4000 μg kg⁻¹ were analyzed (at least eight samples covering the whole range were used) by comparing with blank sample extraction solvent. The relationship between peak area (y) and sample concentration (x) was linear for SD according to the equation: \( y = 0.063x + 0.0294 \) \( (R^2 = 0.9998, n = 3) \). The limit of detection (LOD) and the limit of quantification (LOQ) (the signal to noise ratio was 3 and 10, respectively) were 10.0 and 33.3 μg kg⁻¹, respectively.

The chromatograms of SD standard solutions and spiking pork samples before and after treated by the MIP-SPE column in comparison with the AL-SPE column are shown in Fig.7. The results show that the peak time of SD was about 4.8 min. There are many peaks of the interfering substances due to matrix complexity in pork samples. There were similar peaks from the pork samples treated by the MIP-SPE column and the AL-SPE column. It can be seen that the significant interference from the pork samples treated by the MIP-SPE column and the AL-SPE column was weaken. The sensitivities of SD in spiked pork sample were greatly enhanced with the MIP-SPE column and the AL-SPE column in comparison with sample untreated. The quantification of the SD could be successfully achieved by the MIP-SPE column coupling with HPLC.

The spiking concentration for SD was set with two levels of 80 and 120 μg kg⁻¹ with a standard addition method, and the recovery of SD in the pork samples are shown in Table 1. It can be seen that the recovery of SD in the spiked pork samples treated by the AL-SPE column and the MIP-SPE column are in the range of 70.2%–89.1%, 75.6%–96.4%, respectively, and the RSD values of them were found to be less than 8.0%. The extraction efficiency of the MIP-SPE column for SD was a little higher than that of the AL-SPE column. The AL-SPE column adsorbs the SD through physical surface adsorption. However, the MIP-SPE column adsorbs the SD by means of molecular interaction except physical surface adsorption. The results indicated that the MIP-SPE column coupling with HPLC was a reliable method to determine traces SD in pork samples with high accuracy and repeatability.

### 3.5 Determination of SD in pork samples

The 30 pork samples bought from Nanchang market at random were determined by the MIP-SPE column coupling with HPLC and the AL-SPE column coupling with HPLC, respectively. As shown in Fig.8, it could be seen that the relationship between the measured values by the AL-SPE column coupling with HPLC (y) and by the MIP-SPE column coupling with HPLC (x) was fitting linear according to the equation: \( y = 1.0437x + 0.1496 \), \( (R^2 = 0.9994) \). These results confirmed that the MIP-SPE column prepared in the present work could be effectively applied to pretreat and enrich SD in pork samples.

### 4 Conclusions

The MIP for SD were successfully synthesized by a thermal polymerization method using acrylamide as functional monomer, ethylene glycol dimethacrylate as cross-linker, 2,2-azobisisobutyronitrile as free radical initiator, acetonitrile as porogenic solvent, and sulfadiazine as template. The molecular imprinted column was selected as an extraction device. To obtain the optimum extraction efficiency, several parameters related to the molecular imprinted column, including column solvent, extraction flow-rate, eluent of the
Table 1  Recoveries and intraday and interday precisions of SD in pork (n = 3)

<table>
<thead>
<tr>
<th>Methods</th>
<th>Added (μg kg⁻¹)</th>
<th>Recovery (%)</th>
<th>Intraday RSD (%)</th>
<th>Interday RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIP-SPE column</td>
<td>80</td>
<td>75.6</td>
<td>5.4</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>96.4</td>
<td>4.4</td>
<td>5.4</td>
</tr>
<tr>
<td>Al- SPE column</td>
<td>80</td>
<td>70.2</td>
<td>7.1</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>89.1</td>
<td>6.5</td>
<td>7.6</td>
</tr>
</tbody>
</table>

Fig.8  Correlation between Al-column and MIP-column by HPLC results

sample matrix and eluent volume, were investigated. Under the optimum conditions, the reproducibility of the method was found by intraday and interday precisions, yielding the relative standard deviations (RSD) of ≤ 6.1%. The recoveries for SD were higher than 75.6%. The MIP-SPE column coupling with HPLC was reliable to determinethe traces SD in pork samples with higher recoveries than that of other previous methods. These results confirmed that the MIP-SPE column coupling with HPLC could be effectively applied to determine the SD in pork samples.

References