P41: HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF SULFONAMIDES RESIDUES IN MILK SAMPLES

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Abstract – In this study, a highly selective molecular imprinted polymer (MIP) sorbent was used for the determination of six sulfonamides (sulfanilamide, sulfacetamide, sulfadiazine, sulfathiazole, sulfamerazine and sulfamethizole) from milk samples. MIPs particles were applied as sorbent in the solid phase microextraction (MIPSPE). The determination was achieved by an accurate and sensitive liquid chromatographic analytical method which was developed and validated according tothe European Union Decision 2002/657/EC. Several experimental parameters such as the amount of the MIP and milk, the amount and the type of the elution solvent, as well as time of absorption and extraction were investigated for the achieving optimal conditions. The study of validation is based on the investigation of the following parameters, linearity, selectivity, stability, limits of detection and quantitation, decision limit, detection capability, trueness, precision and ruggedness according to the Youden's approach. The decision limit (CCa) and the results for detection capability (CCb)in the milk were achieved from 101.9 to 113.5 μg kg^{-1} and from 114.4 to 135.4μg kg^{-1} respectively. The optimized protocol was successfully applied to milk samples from local markets as well as to breast milk sample.

Keywords: Sulfonamides, Milk, Extraction, Sample preparation, multi analyte MIP, MIPSPE

1. INTRODUCTION

In the early of twenty century, the discovery of the antibiotics was a revolution for the public health. The benefits of this use, was already obvious, so during the 1950, has started the administration of antibiotics in livestock. The widespread veterinary antibiotics drugs are tetracyclines, β-lactams and sulfonamides. The well-known drugs are given to use as therapy, disease prolepsis and growth promotion. This influences the presence of antibiotics residues in dairy products and in milk. [1, 2]

Milk is a liquid consists of water, lactose, protein, fat, minerals, and vitamins. Due to the high nutritional benefit, it is one of the most consumed foods. [2, 3] The presence of antibiotics residues in milk samples could put in danger the consumer's health. Consequently, the detection of antibiotics in milk is a necessary procedure in order to protect human health. [4]

The sulfonamides or sulfa drugs are a common category of antibacterial drugs. There are numerous sulfonamides with the same basic structure. The sulfonamides, however, differ from each other primarily by virtue of the different substituents in its R' position as shown in Fig. 1.

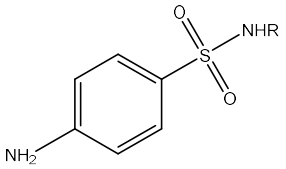

Figure 1. General sulfonamides structure.

The European Union has adopted maximum residue level (MRL) for sulfonamides in all target animal tissues (muscle, fat liver and kidney) and all types of milk (bovine, ovine and caprine), which is 100 μg/kg [5].

Molecularly imprinted polymers (MIPs). MIPs are selective materials, which are synthesized by polymerization of one or multiple functional monomers, a cross-linker, a porogenic solvent, a reaction initiator or catalyst and the target molecule (called as template). MIPs can be applied on SPE as sorbent materials.

The advantages of molecularly imprinted polymers as SPE's sorbents are summarized by this study. The selected MIP is capable for the simultaneously recognition of six sulfonamides, sulfanilamide (SN), sulfacetamide (SCM), sulfadiazine (SDZ), sulfathiazole (STZ), sulfamerazine (SMZ), sulfamethizole (SMT). The extraction of the six examined sulfonamides from milk samples is based on the molecularly imprinted polymer solid phase extraction (MIPSPE). The determination of the sulfonamides was achieved by high pressure liquid chromatography (HPLC) with diode array (DAD) detector. The developed MIPSPE method was validated according to European Union Decision 2002/657/EC.

2. EXPERIMENTAL

2.1. Preparation of standard solutions and milk samples.

Stock standard solutions of each antibiotic (100 mg L-1) were prepared in methanol and stored at 4 °C. The working standard solutions were prepared by further dilution in the mobile phase mixture. The range of their concentrations is between 0.02 and 10 mL-1. In this study, skimmed fresh milk 0% fat from local market was applied according to the protocol. For the matrix adjusted milk calibration curve was used spiked skimmed milk 0% fat. The proposed method was applied on breast milk. The breast milk that was analyzed, was obtained from a volunteer. For the performance of the experiments all milk samples, which were used, had undergone protein precipitation by adding 2 mL ACN to 1 g of milk.

2.2. Optimization of molecularly imprinted solid phase extraction conditions.

Two different types of MIP sorbent media were studied: molecularly imprinted polymer for sulfathiazole (MIP-STZ) and molecularly imprinted polymer for six sulfa drugs (sulfanilamide, sulfacetamide, sulfadiazine, sulfathiazole, sulfamerazine and sulfamethizole), (MIP-6). Initially, the MIP-STZ was extensively investigated for the extraction of sulfathiazole in milk samples. Then the optimum conditions of this study were applied to the other sorbent, MIP(6-sulfa), with some significant changes.

Extraction columns were prepared by packing 30 mg of MIP(STZ) and NIP particles in 2 mL empty syringe barrels, one frit was placed to the bottom of the particles while the top was free of every stopper type. The frits were obtained from commercial SPE cartridges. These homemade cartridges were applied to the SPE system.

For the first extraction protocol with the use of MIP(STZ) was investigated several extraction parameters for the imprinted and non-imprinted polymer. Fifteen elution systems were applied, as it is seemed in Fig. 2. Sample loading time as well as extraction time were checked at 0, 2, 5, 10, 15 and 20 min. In both cases, best results were achieved by 15 min. The effect of the volume of eluent solution was checked in 0.5, 1 and 2 mL, by passing 500 mL of a 5 ng μL^{-1} solution to a 30 mg sorbent and measuring the amount of sulfathiazole eluted into the liquid phase by HPLC-DAD.

The multi analyte MIP was synthesized in order to be used for the six sulfa drugs. In these drugs belongs the sulfathiazole. An extraction study had already completed for STZ. As one drug is the same (sulfathiazole), we observe that both of sorbents present the same behavior during the course of the experiment. So, the optimized conditions of the first study were applied with the minimum modifications for further study of the MIP(6-sulfa). The MIP(6-sulfa) was applied on SPE as sorbent and on an epperndorf, as a modification of dSPE (with and without sonication at sample loading and extraction step, respectively).

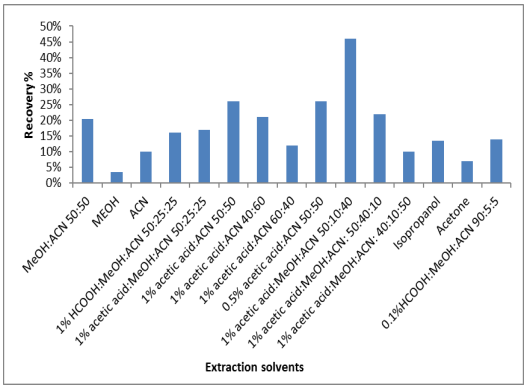

Figure 2. The examined elution solvents and the recovery results for the STZ molecule.

3. RESULTS AND DISCUSSION

3.1. Chromatography.

The separation of six analytes was achieved on the Merck-Lichrospher RP18e (5 µm 250 × 4 mm) analytical column, that was operated at room temperature. The isocratic eluent program applied for the effective separation with a mobile phase

consisted of 80% formic acid 0.1%, 3% ACN and 17% MeOH, at a flow rate 1.0 mL min^{-1}. The operating pressure was 167 bar, the wavelength was chosen at 265 nm and the total time of analysis was almost 15.30 min. Retention times were 2.801 min for SN, 4.856 min for SCM, 5.967 min for SDZ, 7.318 min for STZ, 8.134 min for SMT and 13.387 min for SMT.

3.2. Optimization of MIPSPE procedure.

The following studies were performed in order to achieve the optimum conditions for the final protocol and to evaluate the effect of each parameter.

Firstly, the selected MIP(6-sulfa) was applied as SPE sorbent and on an eppendorf (with and without sonication at sample loading and extraction step, respectively). These three approaches were investigated for standard solutions and milk samples. The results are represented on Table 1. According to the data the MIP(6-sulfa) as SPE sorbent has better recovery results.

Table 1. Recovery results.

Compound	Recovery % Values: standard-spiked milk samples		
	SPE	Eppendorf	Eppendorf supported by sonication
SN	**13.6-6.8**	9.2-4.2	11.3-5.6
SCM	**17.5-5.3**	17.9-5.8	24.0-2
SDZ	**14.2-4.4**	10.5-3.6	10.1-5.8
STZ	**12.5-5.9**	9.05-3.8	12.8-2.2
SMZ	**13.1-6.2**	8.3-4.2	9.5-2.5
SMT	**44.7-10.3**	53.0-10.5	38.8-10.7

3.3. Effect of type, volume and flow rate of the elution system for the extraction of 6 SAs.

Taking into consideration the above description, various elution systems were applied and evaluated for the effective extraction of the sulfathiazole from the MIP particles, MIP(STZ). In Fig. 2 it is shown that 1% CH_3COOH-MeOH-ACN-50:10:40% v/v was provided the highest rate. This elution solvent was chosen for further experiments which performed for the extraction of six SAs.

The volume of the elution system was checked to the MIP-6 particles, at 0.5, 1 and 2 mL. Finally, 2 mL, as it is shown in Fig. 3 given the highest recovery.

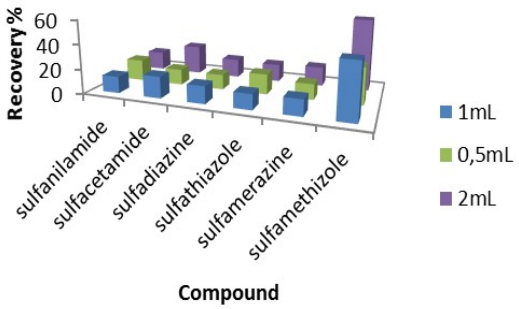

Figure 3. The effect of volume of the elution solvent.

3.4. Effect of sample loading and extraction time

The results were adopted for the MIP(STZ) study. It is mentioned above that, 15 min. were selected by the equilibrium and extraction time with the highest recovery. These conditions were applied to MIP(6- sulfa) study.

3.5. Effect of the MIP amount.

Furthermore, the amount of MIP(6-sulfa) particles was investigated with tests at 20, 30 and 40 mg, with their results shown on Figure 4. Each sulfa drug appears the highest recovery by using 30 mg of the MIP(6-sulfa) particles.

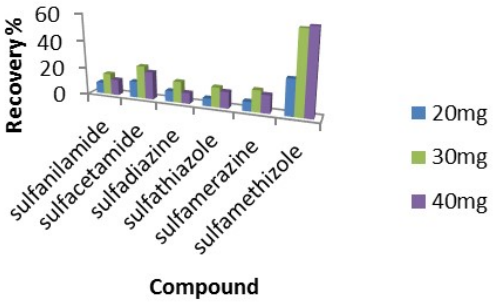

Figure 4. The effect of the MIP amount.

3.6. Fat and protein removal from milk samples.

Deproteinization tests were applied in order to eliminate the impact of fat and proteins to recovery during the sample preparation procedure. Precipitation was carried out with the use of acetonitrile, trifluoroacetic acid 10% v/v, tris-HCl 20% v/v and acetone. The highest recoveries were provided by using acetonitrile.

3.7. Validation of MIP(6-sulfa) method.

3.7.1. Selectivity.

The effect of the matric was checked and as it is observed on Fig. 5 there are not interactions at the same time with the analytes.

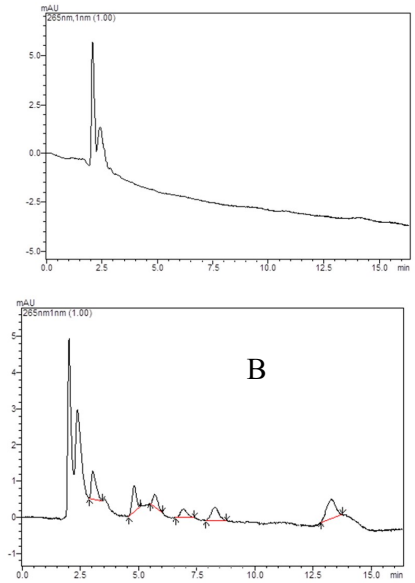

Figure 5. Chromatograph of a (A) blank milk sample and (B) spiked milk sample at MRL (100 μg/kg).

3.7.2. Method validation's results.

The developed MIPSPE method was validated according to the performance criteria of European Commission 2002/657/EC using spiked milk samples. These criteria were represented in the Table 2.

Table 2. Validation parameters of the MIPSPE method for the determination of sulfonamides in milk samples.

Validation parameters	***value***
Linear range μg/kg	50-500
Linearity R^2	>0.9325
LOD=3.3S/N	1.9-13.3 μg/kg
LOQ=10S/N	5.6-42.2 μg/kg
CC_a	101.9-113.4 μg/kg
CC_b	114.4-135.4 μg/kg
Intra assay precision at 3 concetrations levels, n=4	85.8-115.7% RSD%<13.4%
Inter assay precision at 3 concetrations levels, n=4×3	85.8-109.2% RSD%<9.9%
Youden Test	Stable method

4. CONCLUSIONS

The developed MIPSPE method was validated according to the European Union Decision 657/2002/EE. This method is capable of the quantitative determination and the identification of sulfonamides in milk samples which exist in lower concentrations than the maximum residue limit of 100 μg/kg. The suggested method is highly selective, simple in usage, sensitive and gives repeatable results. Therefore, this method can be characterized as suitable for the desired purpose.

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