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A method for the quantification of low concentration sulfamethazine residues in milk based on molecularly imprinted clean-up and surface preconcentration at a Nafion-modified glassy carbon electrode

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Abstract

An electrochemical method for the determination of sulfamethazine at a low concentration level (25 $\mu g\,l^{-1}$) in milk is reported. The method involves sample clean-up and selective preconcentration of sulfamethazine with a molecularly imprinted polymer (MIP), and a further electrode surface preconcentration of the analyte at a Nafion-coated glassy carbon electrode (GCE). Square wave (SW) oxidative voltammetry of accumulated sulfamethazine was employed for its quantification. Sulfamethazine electrode preconcentration was carried out in 0.1 $mol\,l^{-1}$ Britton–Robinson buffer of pH 1.5, and by applying 5 min of accumulation at open circuit. A linear calibration graph was obtained for sulfamethazine at the Nafion-modified GCE over the 1.0×10^{-8} to 1.0×10^{-6} $mol\,l^{-1}$ concentration range, with a detection limit of 6.8×10^{-9} $mol\,l^{-1}$ (1.9 $\mu g\,l^{-1}$). This detection limit is remarkably better than those reported previously in the literature using electroanalytical techniques. Although the detection limit achieved was sufficient to allow the direct determination of sulfamethazine at the concentration level required in milk, a sample clean-up was shown to be necessary to obtain analytically useful SW voltammograms. This was accomplished by processing the deproteinized milk through a cartridge containing a molecularly imprinted polymer for sulfamethazine, also allowing a selective preconcentration of the analyte. Elution of the analyte from the MIP cartridges was carried out with 2 ml of a (9:1) MeOH:acetic acid mixture. Determination of sulfamethazine in milk samples was accomplished by interpolation into a calibration graph constructed with sulfamethazine standard solutions which were subjected to the same procedure than the deproteinized milk samples. Results obtained for five samples, spiked at the 25 $\mu g\,l^{-1}$ level, showed a mean recovery of (100 ± 3)%.
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Keywords: Molecularly imprinted polymers; Sulfamethazine; Nafion-modified electrode

1. Introduction

The rapid, cheap and reliable detection of antimicrobial compounds residues at low concentration levels is an important analytical challenge in dairy industry. Problems associated with residues of antiinfective substances in dairy products and other foods of animal origin include the risk of adverse health effects after consumption, increased resistance of pathogenic bacteria towards antibiotics and inhibition of starter cultures used in dairy production [1]. In this context, sulfamethazine, 4-amino-*N*-(4,6-dimethyl-2-pyrimidinyl) benzenesulfonamide (SMZ) [2] is added in combination with other feed medicaments to cattle and swine feeds because of its antibacterial activity to treat livestock diseases such as gastrointestinal and respiratory tract infections [3]. Moreover, this drug has been also employed to promote growth [4].

In order to ensure the safety and quality of foodstuffs, the European Comission adopted a maximum sulfonamide residue level (MRL) of 100 $\mu g\,kg^{-1}$ in foodstuffs of animal origin, including milk [5]. However, the Codex Alimentarius Commission established a lower maximum level in milk, being of only 25 $\mu g\,kg^{-1}$ [6]. A number of LC–MS methods have been developed for the analysis of sulphonamide residues in milk [7–9], the separation and ionization con-

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ditions affecting the precision, accuracy and sensitivity of sulfonamide determination. Although it is possible to detect sulfonamides at low concentration levels using HPLC with diode array detection [10] and fluorescence detection [11], there is still an absence of effective and rapid methods for the detection of sulfonamide residues at the concentration level mentioned above, by using analytical methodologies as simple, rapid and cheap as possible. This lack of simple methods can be attributed to problems associated with sample clean-up and purification steps.

In order to approach this matter, a method for the electrochemical determination of SMZ in milk at a concentration level of 25 $\mu g\,l^{-1}$ is reported in this article. This method involves sample clean-up and selective preconcentration of sulfamethazine by using a molecularly imprinted polymer (MIP) as a solid phase extracting agent, and a further electrode surface preconcentration of the analyte at a Nafion-modified glassy carbon electrode (GCE).

Nowadays, it is well known that MIPs constitute a clear alternative to classic methodologies for the extraction and clean-up of target analytes. The use of solid phase extraction (SPE) procedures involving MIPs (MISPE) is an attractive alternative for the analysis of organic compounds in complex sample matrices, and successful applications have been described in the literature [12–19].

On the other hand, Nafion has been widely used in last years as an electrode chemical modifier due to its attractive permselective, ion-exchange and antifouling properties [20]. The accumulation mechanism of Nafion accrues from electrostatic interactions due to the hydrophilic $-SO^{3-}$ groups, whereas its ionic selectivity for hydrophobic organic cations is achieved through hydrophobic interactions with the hydrophobic fluorocarbons of the film [21].

2. Experimental

2.1. Apparatus and equipment

Voltammetric determination of sulfamethazine was carried out using an Autolab PSTAT 10 (Ecochemie) potentiostat controlled by the GPES 4.9 software. A Metrohm 6.0805.010 glassy carbon disk electrode (3 mm in diameter), which was modified with a Nafion film, was used as the working electrode. The reference electrode was a BAS-RE-1 Ag/AgCl/KCl (3 M) electrode, and the auxiliary electrode was a Pt wire. The electrochemical cell was a BAS VC-2 10 ml cell.

The MISPE system consisted of a peristaltic pump (Perimax 12, Spetec) and a MIP cartridge prepared by packing 0.16–0.18 g of MIP in 6 ml Bond Elut containers (Scharlab) with 20 μm pore size Bond Elut 6 ml, Ø 12.7 mm PE filters (Scharlab).

A P-Selecta ultrasons ultrasonic bath, a Metrohm 728 mechanic stirrer and a P-Selecta centrifuge were also used.

2.2. Reagents and solutions

A 1.0×10^{-2} mol l^{-1} sulfamethazine (Sigma) stock solution was prepared in 9:1 methanol (HPLC-grade, SDS): acetic acid (ACS, Panreac). More dilute standards were prepared by appropriate dilution with 0.1 mol l^{-1} Britton–Robinson buffer of the desired pH which was adjusted with 2.0 mol l^{-1} NaOH or HCl. A 5% (w/v) Nafion (Aldrich) solution was employed, more dilute solutions being prepared by adequate dilution in ethanol (Panreac).

Chemicals for the MIP syntheses were sulfamethazine as the template molecule, methacrylic acid (Sigma–Aldrich) as functional monomer, ethylene glycol dimethacrylate (Sigma–Aldrich) as the cross-linker, 2,2′-azobis-isobutyronitrile (Janssen) as the initiator, and acetonitrile (HPLC-grade, SDS) as solvent. Methanol and acetic acid were used for the elution of the template.

Water used was obtained from a Millipore Milli-Q purification system and the sample analyzed was UHT milk (Solar) purchased in a local supermarket.

2.3. Procedures

2.3.1. Preparation of Nafion-modified glassy carbon electrode

Prior to coating, the GCE was polished with alumina (Metrohm, 0.3 μm) for 1 min on a polishing cloth. Then, the electrode was sonicated in deionized water for about 30 s and dried at room temperature. Modification of the GCE was accomplished by dripping 5.0 μl of a 0.5% (w/v) Nafion solution on the surface of the GCE, and allowing the solvent to dry at room temperature.

2.3.2. Preconcentration of sulfamethazine at the Nafion-modified GCE and recording of the voltammetric responses

Accumulation of sulfamethazine was performed at open circuit by immersion of the Nafion-modified GCE in the sulfamethazine solution, which was stirred at a constant rate, in 0.1 mol l^{-1} Britton–Robinson buffer of pH 1.5 for the selected period of time. Square wave (SW) stripping voltammograms were then recorded from 0.0 V towards more positive potentials using a SW amplitude (E_{sw}) of 25 mV, a potential step (ΔE_s) of 8 mV and a frequency (f) of 30 Hz.

2.3.3. Preconcentration of sulfamethazine using MIP cartridges

The MIP for sulfamethazine was synthesized as described earlier [22]. The MIP cartridges were firstly washed with appropriate volumes of MeOH:HAc (9:1) to remove residual contamination. Samples and standard solutions were processed in the MISPE system at a flow rate of 0.4 ml min^{-1} [22]. Retained sulfamethazine was then eluted with 2 ml of MeOH:HAc (9:1) at a flow rate of 0.1 ml min^{-1}, and directly collected in the electrochemical cell. The eluate was led to dryness under a gentle stream of nitrogen at room tempera-

ture and the dry residue was dissolved in 5.0 ml of 0.1 mol l^{-1} Britton–Robinson buffer solution of pH 1.5. Voltammetric analysis of these solutions was carried out as described in Section 2.3.2.

2.3.4. Determination of sulfamethazine in milk

Twenty millilitres of UHT milk spiked with sulfamethazine at the 25 μg l^{-1} level, were transferred to a 30 ml centrifuge tube. Then, 2 ml of 15% trichloroacetic acid (Panreac) were added to the tube, and this was thoroughly shaken for 1 min to achieve proteins precipitation. After centrifugation at 3000 rpm for 10 min, the obtained buttermilk was filtered and collected in a volumetric flask (a volume of approximately 16 ml was collected). Next, the buttermilk was passed through the MISPE system, then eluted with 2 ml of MeOH:HAc (9:1) and the eluate was subjected to the same procedure described in Section 2.3.3. Determination of sulfamethazine was accomplished by SW voltammetry as described above, by interpolation into a calibration graph constructed with sulfamethazine standard solutions which were subjected to the same whole procedure than the buttermilk obtained from milk samples.

3. Results and discussion

Sqware wave (SW) oxidative voltammetry of sulfonamides at a glassy carbon electrode has demonstrated to be a useful and sensitive method for the determination of this type of drugs [23,24]. However, the detection limits achieved when this technique is applied are not low enough to permit the direct determination at the concentration level required by legislation in foodstuffs such as milk. Consequently, preconcentration of the analytes is required for this purpose. A useful strategy to achieve this goal consists of suitable modification of the electrode surface. Considering, the attractive properties of Nafion for the accumulation of different organic cations [25–29], we decided to use SWV at a Nafion-modified GCE in order to decrease sufficiently the limit of detection for sulfamethazine to allow the desired application to be performed in a simple and reliable way.

Fig. 1 shows SW voltammograms obtained at a bare GCE and at a Nafion-modified GCE, with no accumulation period and when an accumulation period of 5 min at open circuit under continuous stirring was applied. As can be seen, no oxidation response for 1.0×10^{-6} mol l^{-1} sulfamethazine was observed in both cases at the bare GCE. However, a small sulfamethazine oxidation peak was obtained at the Nafion-modified GCE even without accumulation and a very well defined symmetric peak was produced when 5 min of accumulation were elapsed prior the voltammetric scan. This simple experiment showed fairly well that accumulation of sulfamethazine on Nafion-modified GCE can be used as an effective preconcentration step before quantitative measurements. Moreover, no significant dependence of the SW net peak current on the accumulation potential was found, and

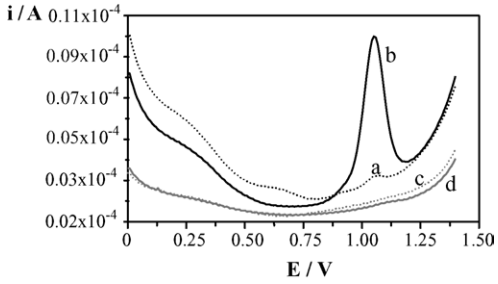

Fig. 1. SW voltammograms for 1.0×10^{-6} mol l^{-1} sulfamethazine in 0.1 mol l^{-1} Britton–Robinson buffer of pH 1.5 at a Nafion-modified GCE (a, b) and at a bare GCE (c, d), with no accumulation period (voltammograms a and c) and with 5 min of accumulation at open circuit under constant stirring (voltammograms b and d); E_{sw} = 25 mV, AE_s = 8 mV, f = 30 Hz.

therefore we decided to carry out the accumulation step at open circuit, which also contributed to the simplicity and rapidity of the proposed methodology.

3.1. Optimisation of the sulfamethazine electrode surface preconcentration

As it is obvious considering the electrostatic interactions in which the accumulation mechanism on Nafion films was based, the pH value of the analyte solutions had a dramatic effect on the modified electrode voltammetric response. The dependence of the peak current and peak potential for 1.0×10^{-6} mol l^{-1} sulfamethazine in Britton–Robinson buffer solutions with pH values ranging from 1.5 to 10.0 was checked. An accumulation period of 5 min at open circuit was employed in all cases. As expected, the higher SW peak currents occurred at the more acidic pH values tested, with a noticeable decrease in i_p as the pH value increased. Taking into account the pKa_1 value of sulfamethazine, 2.65, the cationic form of this sulfadrug was predominant in solution at pH values lower than 2.65 and, consequently, the electrostatic interactions with the negatively charged Nafion film were then favoured. Obviously, for pH values higher than pKa_2 of sulfamethazine (7.0), no voltammetric response was obtained as a consequence of the negative charge on the analyte molecule, and the subsequent repulsion with the modifier film. The plot of E_p versus pH displays two well defined linear portions with significantly different slopes, the intersection of these portions allowing the obtaining of the apparent pKa_1 value of the accumulated sulfamethazine (2.68). Accordingly to these results, a pH value of 1.5 was chosen for sulfamethazine accumulation.

Concerning the amount of modifier onto the electrode surface, different volumes of a 0.5% Nafion solution were deposited onto the GCE and the SW voltammetric response for 1.0×10^{-6} mol l^{-1} sulfamethazine checked. An increase in the Nafion amount deposited would increase the film thickness and, consequently, the ion exchange capacity, but, conversely, a too thick film may decrease the mass transfer rate and therefore the current obtained. The sulfamethazine peak current increased with the volume of 0.5% Nafion solution

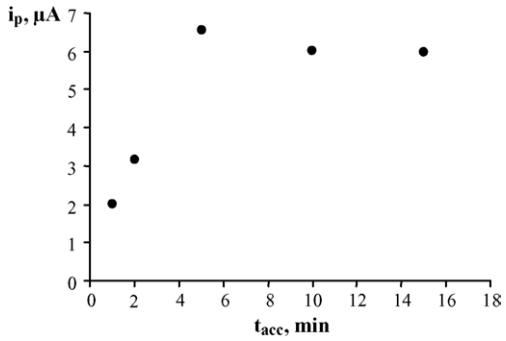

Fig. 2. Effect of the accumulation period at open circuit under constant stirring on the SW voltammetric anodic peak current for 1.0×10^{-6} mol l^{-1} sulfamethazine. Other conditions as in Fig. 1.

deposited up to 5.0 μl, following which a decrease in i_p was observed for larger volumes, as a consequence of the effects commented above [30]. Therefore, 5.0 μl of a 0.5% Nafion solution was used to modify the GCE. Finally, the effect of the accumulation period on the sulfamethazine SW response was evaluated (Fig. 2). As it can be observed, the peak current increased with the accumulation period up to approximately 5 min, a levelling off occurring for longer periods of preconcentration; 5 min were then employed for sulfamethazine accumulation in further work.

3.2. Analytical characteristics

The reproducibility of the voltammetric measurements was evaluated by repeating 10 times the whole preparation of the Nafion-modified GCE and the accumulation procedure. A relative standard deviation (R.S.D.) value of 6.2% was obtained for 1.0×10^{-6} mol l^{-1} sulfamethazine peak currents, which demonstrated a good reproducibility in film deposition on the electrode surface, as well as in the analyte preconcentration step.

Under the optimised conditions mentioned above, a linear calibration graph was obtained for sulfamethazine at the Nafion-modified GCE over the 1.0×10^{-8} mol l^{-1} to 1.0×10^{-6} mol l^{-1} concentration range ($r = 0.996$), with slope and intercept values of $(7.3 \pm 0.2) \times 10^6$ μA mol^{-1} l and (0.3 ± 0.1) μA, respectively. A detection limit of 6.8×10^{-9} mol l^{-1} was calculated according to the $3s_b/m$ criterion, where m is the slope of the linear calibration graph, and s_b was estimated as the standard deviation ($n = 10$) of the signals from 1.0×10^{-8} mol l^{-1} sulfamethazine. This detection limit which corresponds to 1.9 μg l^{-1} is well below the maximum level permitted for sulfamethazine in milk. Moreover, it is also remarkably better than those reported previously in the literature using electroanalytical techniques, which involved SWV at poly (3-methylthiofene)-coated GCE (LOD 3.7×10^{-7} mol l^{-1}) [23], amperometric detection at a carbon-disk electrode (LOD 1.0×10^{-6} mol l^{-1}) [31] and at a diamond electrode (LOD 50 nM) [32], as well adsorptive-stripping voltammetry at a mercury electrode (LOD 10 μg l^{-1}) [33].

3.3. Determination of sulfamethazine in milk

The proposed methodology involving preconcentration of sulfamethazine at a Nafion-modified GCE coupled to SW voltammetric quantification, was applied to the determination of this sulfonamide in milk spiked at the maximum level permitted by the Codex Alimentarius Commission, 25 μg l^{-1} (9.0×10^{-8} mol l^{-1}). In principle, since the detection limit achieved was sufficient to allow for the direct determination of sulfamethazine, the Nafion-modified GCE was immersed in the buttermilk obtained after milk deproteinization (see Section 2), whose pH value was adjusted to 1.5, and an accumulation time of 5 min at open circuit was applied. The subsequent SW voltammogram recorded from 0.0 V towards more positive potentials, showed no significant oxidation response for sulfamethazine at the concentration level considered. This was attributed to a strong matrix effect, and, consequently, a sample clean-up step was necessary. In order to do this, and also to achieve a selective preconcentration of the analyte, the spiked buttermilk was processed through a MISPE system which had been previously optimised in our laboratory [22]. This system involved the use of cartridges containing a synthesized MIP for sulfamethazine, which was prepared using sulfamethazine as the template molecule, methacrylic acid as the functional monomer and ethylene glycol dimethacrylate as the cross-linking monomer in the presence of acetonitrile as the solvent. The synthesized MIP exhibited recognition sites which are mainly complementary to the template in terms of size and shape, although other substances with molecular structures similar to sulfamethazine (sulfamerazine, sulfamethoxazole, sulfadiazine and sulfathiazole) showed some ability to be retained in the MIP but not in a quantitative and reproducible way. Elution of the analyte from the MIP cartridges was accomplished with 2 ml of a (9:1) MeOH: acetic acid mixture. Moreover, as it can be deduced from Section 2.3.4, the elution-reconstitution procedure applied to the sample gave rise to a preconcentration factor slightly higher than 10, which permitted to work with higher analytical concentrations in the electrochemical cell, and, therefore, to obtain electroanalytical responses which can be measured with a better accuracy.

Prior to the analysis of the spiked milk samples, calibration curves for sulfamethazine standard solutions which were passed through the MISPE system, eluted with 2 ml of MeOH:HAc (9:1) and subjected to the same procedure, including accumulation at the Nafion-modified GCE, than the milk samples (see Section 2.3.4), were constructed. Fig. 3 shows the comparison of one of these calibration curves with that obtained for sulfamethazine standard solutions which were not processed through the MISPE system (i.e. that corresponding to the results commented in Section 3.2) over the 2.0×10^{-7} mol l^{-1} to 1.0×10^{-6} mol l^{-1} concentration range (this range was that used for the further determination of the analyte in the milk samples). As it can be easily seen, although a linear relationship between the anodic peak current and sulfamethazine concentration, is maintained, the

Table 1
Determination of sulfamethazine in spiked milk samples after preconcentration and solid phase extraction using MIP cartridges and accumulation at a Nafion-modified GCE

Milk sample	Sulfamethazine added ($\mu g\,l^{-1}$)	Sulfamethazine found ($\mu g\,l^{-1}$)	Mean recovery (%)	R.S.D. (%)	CCα ($\mu g\,l^{-1}$)	CCβ ($\mu g\,l^{-1}$)
1	25	25.0				
2	25	23.9				
3	25	24.9	100 ± 3	2.8%	26.1	27.3
4	25	25.2				
5	25	25.8				

slope value of the calibration curve was lower in the case of MISPE-processed solutions. In fact, both slopes values were statistically different when they were compared using the Student's *t*-test method for a significance level of 0.05. Although the reason for this difference in the slope values is not clear at present, it cannot be attributed to a low recovery of the compound from the column, since we demonstrated previously [22] that this was of $98 \pm 9\%$. Therefore, some effect on the preconcentration step once the analyte was eluted from the MISPE system should occur. According to these results, the calibration curve obtained after processing of the analyte solutions through the MISPE system, was further employed for quantification of sulfamethazine in milk samples. Moreover, four different calibration curves for sulfamethazine, constructed after passing SMZ standard solutions through different MIP cartridges, showed no significant differences in their slopes values ($(1.7 \pm 0.2) \times 10^6\ \mu A\ mol^{-1}\ l$), thus demonstrating a good reproducibility of the methodology used.

Following the simple procedure described in Section 2.3.4, sulfamethazine was determined in five milk samples which were spiked at the $25\ \mu g\,l^{-1}$ level. It is important to remark that the pH value measured in the buttermilk after deproteinization with trichloroacetic acid was the same (4.0) than that used for the sulfamethazine rebinding in the MIP cartridges [22], and, therefore, no change in the buttermilk pH was necessary before processing it through the MISPE system. The results obtained in the analysis of the five milk samples are summarized in Table 1, the confidence interval being calculated for a significance level of 0.05. A non spiked aliquot of milk was also subjected to the same whole procedure that the spiked samples. The absence of oxidation signals at the potential values for sulfamethazine confirmed that this drug was not initially present at detectable levels in the analyzed milk. As it can be observed in Table 1, a mean recovery of $(100 \pm 3)\%$ was achieved for sulfamethazine. The CCα and CCβ values, according to the regulation decision (2002/657/EC) concerning the performance of methods and the interpretation of results in the official control of residues in products of animal origin [34], are also given in Table 1. These new parameters are defined as the limit of decision (CCα) and detection capability (CCβ). The CCα value, where $\alpha = 0.05$, was calculated from the MRL value ($25\ \mu g\,l^{-1}$ in this case) plus 1.64 times the standard deviation of the fortified samples at the MRL. The CCβ is obtained adding to CCα 1.64 times the same standard deviation [10]. All the results indicate the suitability of the methodology developed for the determination of this analyte in complex samples such as milk even at such allow concentration level in a simple and rapid way.

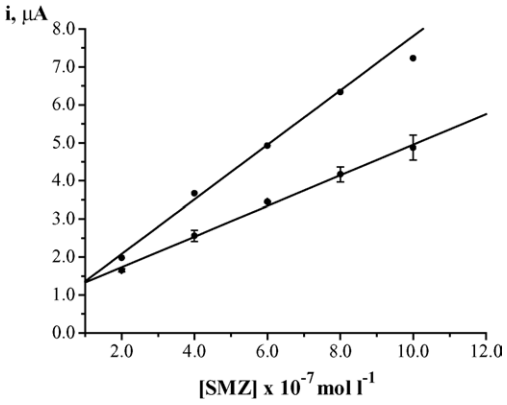

Fig. 3. Calibration curves obtained by SWV for sulfamethazine at a Nafion-modified GCE with (▲) and without (●) processing through the MISPE system. Other conditions as in Fig. 1.

4. Conclusions

A simple and highly sensitive electrochemical method for the determination of sulfamethazine residues in milk has been developed by coupling sample clean-up and selective preconcentration of the analyte at a sulfamethazine molecularly imprinted polymer, with electrode surface preconcentration at a Nafion film-coated glassy carbon electrode, and square wave voltammetric quantification. The proposed methodology allows the determination of sulfamethazine in a complex sample as milk is, in a simple, reproducible, efficient and acceptably rapid (around 2 h for the whole procedure excluding the MIP synthesis) way, even for concentrations as low as the maximum level permitted by the Codex Alimentarius Commission.

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