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Article

Microextraction in Packed Sorbent for Determination of Sulfonamides in Egg Samples by Liquid Chromatography and Spectrophotometric Detection

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O presente trabalho descreve a aplicação bem sucedida da microextração em sorvente empacotado e cromatografia líquida com detecção de arranjo de diodos (MEPS/LC-DAD) para a determinação simultânea das sulfonamidas (sulfacetamida, sulfadiazina, sulfatiazol, sulfametazina sulfametoxipiridazina e sulfametoxazol) em amostras de ovos. As variáveis MEPS (pH da amostra, número de ciclos aspirar/dispensar, força iônica e procedimento de dessorção) foram otimizados para aumentar a sensibilidade analítica do método. O método apresentou linearidade na faixa de concentração de 30 ng g^{-1} (limite de quantificação, LOQ) a 300 ng g^{-1}. Este valor de LOQ é inferior ao limite máximo de resíduo (LMR) preconizado para sulfonamidas em amostras de ovos (100 μg kg^{-1}). As taxas de recuperação foram adequadas para todos os analitos (> 94%), bem como os dados de precisão inter-ensaio, com coeficientes de variação inferiores a 10%. Com base na validação analítica, a metodologia MEPS/LC é adequada para a determinação de sulfonamidas em amostras de ovos.

The present work describes a successful application of microextraction packed sorbent and liquid chromatography with diode array detection (MEPS/LC-DAD) for simultaneous determination of the sulfonamides (sulfacetamide, sulfadiazine, sulfathiazole, sulfamethazine, sulfamethoxypiridazine and sulfamethoxazole) in egg samples. The MEPS variables (pH of the sample, draw-eject cycles, ionic strength, and desorption procedure) were optimized, in order to improve the sensitivity of the proposed method. The method was shown to be linear at concentrations ranging from 30 ng g^{-1} (limit of quantification, LOQ) to 300 ng g^{-1}. This LOQ value is lower than those established as the maximum residue limit (MRL) for egg samples (100 μg kg^{-1}). The accuracy values were adequate for all analytes (> 94%), as well as the inter-day precision data, with coefficient of variation lower than 10%. On the basis of analytical validation, the MEPS/LC methodology has been shown to be a promising alternative for analysis of sulfonamides in egg samples.

Keywords: MEPS, sulfonamides, liquid chromatography, egg

Introduction

Sulfonamides (SAs) are a group of synthetic antibiotics that have played an important role as effective chemotherapeutics in bacterial and protozoan infections in veterinary medicine practice. Subtherapeutic doses of more than ten SAs are routinely used in food-producing animals for prophylactic and growth-promoting purposes. SAs residues could be potentially toxic to aquatic organisms and humans because of their carcinogenic potency and possible antibiotic resistance.[1-4] There are no sulfonamides approved for use in laying hens. The use of veterinary drugs for medicinal purposes in laying hens could result in violative drug residues in food meant for human consumption. Of all the marketed SAs, only sulfamethazine and sulfadimethoxine have been approved for use in chickens. Furthermore, this approval extends to broilers only, not laying hens.[4,5] To safeguard human health, many countries including Brazil have established safe maximum residue limits (MRLs) for SAs at the total level of 100 μg kg^{-1} in food of animal origin.

Microextraction by packed sorbent (MEPS) is a new development in the field of sample preparation. MEPS is the miniaturization of conventional solid phase extraction (SPE) packed bed devices from milliliter bed volumes to microliter volumes. MEPS can be connected online to gas chromatography (GC) or liquid chromatography (LC) without any modifications. In MEPS, approximately 1 mg of the solid packing material

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is packed as a cartridge inside a syringe (100-250 μL), between the barrel and the needle as a cartridge.[6] Several sorbent material such as silica based matrices (C2, C8, and C18), strong cation exchange, restricted access material (RAM), or molecular imprinted polymers (MIPs) can be employed.[7,8]

The MEPS technique has been used to extract a wide range of analytes from different matrices, such as biological fluids (plasma, blood, and urine),[7-20] water,[21,22] hair[23] and wine.[24,25] In the areas of food analysis, several published applications describe MEPS for the analysis of aflatoxin B2 and M2 metabolite trace analysis in milk, mycotoxin trace analysis in cereal, atrazine in cereal, and sulfonamide trace analysis in meat.[26]

A key factor in MEPS is that the solvent volume employed in the elution of analytes during the extraction process is of a suitable order of magnitude to be injected directly into an LC or GC system.[12]

In the present work, a LC-DAD method is described, using for the first time a sample pre-treatment by MEPS for the simultaneous determination of sulfacetamide (STD), sulfadiazine (SDZ), sulfathiazole (STZ), sulfamethazine (SMT), sulfamethoxypiridazine (SMP), and sulfamethoxazole (SMA) (Figure 1) in egg samples.

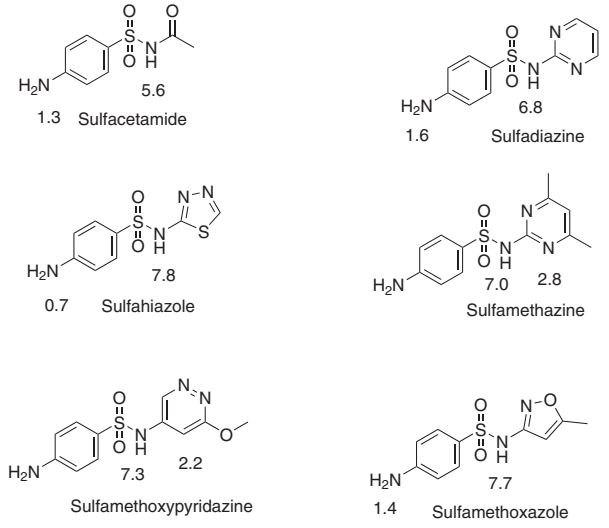

Figure 1. Sulfonamides and their corresponding pKa values.

Experimental

Reagents and analytical standards

The SAs sulfacetamide, sulfadiazine, sulfathiazole, sulfamethazine, sulfamethoxypiridazine, sulfamethoxazole, and the primidone analytical standards were supplied by Sigma Aldrich (St. Louis-USA). Primidone was used as internal standard for analysis of SAs in egg samples.

The standard solutions were prepared by diluting the stock solutions of the SAs (1 mg mL^{-1}) in methanol. These solutions were stable at a temperature of –20 ºC. The water used to prepare the mobile phase had been previously purified in a Milli-Q system (Millipore, São Paulo, Brazil). Methanol and acetonitrile (ACN) (HPLC grade) were purchased from J.T. Baker (Phillipsburg, USA); trifluoroacetic acid (TFA) (HPLC grade) was acquired from Fisher Scientific (Leics, UK), and monobasic and dibasic phosphates were provided by Merck (Darmstadt, Germany).

Chromatographic conditions

SAs were analyzed on high-performance liquid chromatographic system Varian 230 ProStar (Varian, California, EUA), detector DAD, λ = 269 nm. The separations were achieved on an analytical reversed-phase column C18 ChromSep HPLC (Varian, 250 mm × 3.0 mm, i.d.) by a linear gradient from 10% to 25% of phase A (ACN/methanol (60:40, v/v) with phase B (water pH adjusted to 4 with TFA) in 10 min, using a flow rate at 1.0 mL min^{-1}. The mobile phase had been filtered and degassed prior to use.

Preparation of spiked egg sample

Organic egg samples were purchased from retail markets. Preliminary analyses showed they were analyte-free. 0.5 g of these blank egg samples (yolk and white) was spiked with an internal standard (IS) (primidone, 15 μL, 30 μg mL^{-1}), and standard solutions of the SAs that resulted in a concentration level of 100 ng g^{-1}. After this procedure, 1.0 mL ACN was added to the samples and the mixture vortex-mixed for 20 s. The samples were then centrifuged at 25 °C for 25 min, at 3500 rpm. Aliquots of 500 μL of the supernatant liquid were transferred to a centrifugal filter device (Amicon Ultra 4, 2/PK, MILLIPORE, Bedford, MA) and centrifuged again for 20 min, for additional protein exclusion. The supernatant was dried and reconstituted with 500 μL of the 0.05 mol L^{-1} buffer phosphate solution pH 3. Blank samples were prepared in the same way as above, but without the compound spiking step. These spiked egg samples were used for preparation of calibration curve and validate the analytical method.

MEPS procedure

The MEPS syringe (250 μL syringe, C8 and strong cationic exchange sorbent, 2 mg) was donated by SGE (Melbourne, Australia). This sorbent has irregular particles

with an average size of 50 µm and nominal porosity 60 Å. Before being used for the first time, the sorbent had been manually conditioned with 250 µL methanol, followed by 250 µL water. After this procedure, the spiked egg samples were diluted with 500 µL phosphate buffer solution pH 3, and 250 µL of these samples were manually drawn through the sorbent and ejected in the same vial, four times (preconcentration of the analytes). The washing step was also evaluated with different solvents (methanol, mobile phase, water, and 0.05 mol L^{-1} phosphate buffer solution pH 3.0), in order to ensure removal of unwanted weakly retained interferences from the sorbent. The analytes were then desorbed (eluted) directly into the LC system with the mobile phase (100 µL - mixture of 20% phase A and 80% phase B). The sorbent was reused 60 times approximately. This procedure takes about 4 min.

The packed syringe was used several times. Between injections, the sorbent phase was washed with 500 µL of methanol and with 500 µL of water.

The MEPS variables, such as pH of the sample, draw-eject cycles, ionic strength, and desorption conditions (solvent and solvent volume) were optimized, in order to improve the sensitivity of the proposed method.

Analytical validation

Analytical validation of the MEPS/LC method was carried out with egg samples free of SAs, spiked with standard solution of the analytes at different concentrations, taking into consideration the safe maximum residue limits (MRL). The linearity was evaluated by calibration curves constructed by using linear regression of the ratio between the SAs and internal standard (Y) peak areas *versus* the nominal water and egg concentrations of SAs (X, ng mL^{-1}). These sample concentrations ranged from 30 to 300 ng g^{-1} for determination of SAs in egg samples.

Accuracy and inter-day precision values were determined by calibration curves, by means of quintuplicate MEPS/LC assays of the blank samples spiked with the analytes. Accuracy values were calculated by comparison between the concentrations of SAs added to the samples with sulfonamides concentrations determined by the calibration curves.

Results and Discussion

MEPS procedure

The MEPS variables, such as pH of the sample, ionic strength, draw-eject cycles, and desorption conditions (solvent and solvent volume) were optimized, in order to establish the partition equilibrium of the SAs in shorter analysis times.

The egg samples (prepared as previously described) were diluted with 0.05 mol L^{-1} phosphate buffer solutions using different volumes (0.5, 1.0, 2.0 and 3.0 mL) and different pH values (3.0, 4.0, 5.0, and 6.0). The best MEPS/LC analysis results were obtained with samples diluted with 500 µL of the 0.05 mol L^{-1} phosphate buffer solutions at pH 3.0 (Figure 2a and 2b).

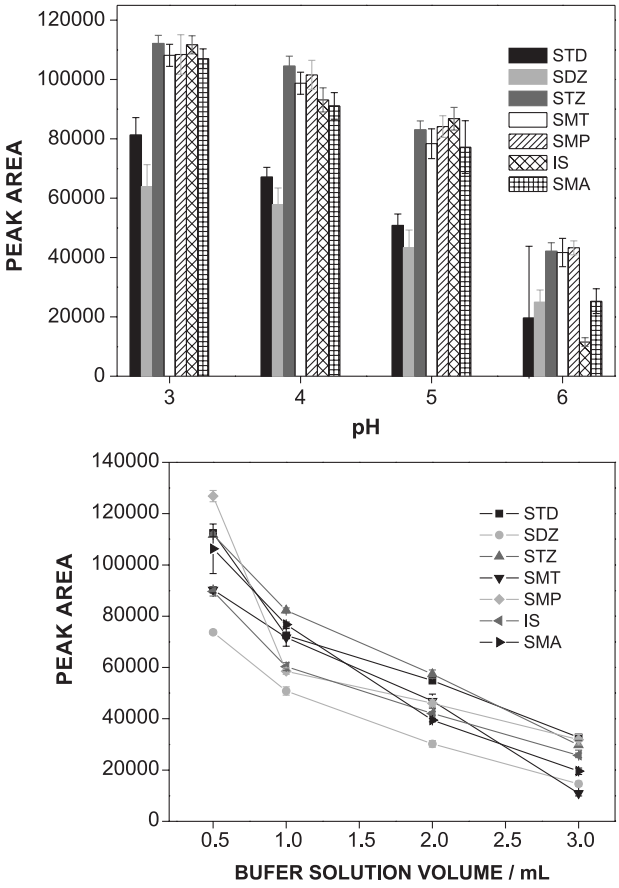

Figure 2. The effect of the pH egg samples (2a) and buffer solution volume (2b) on MEPS performance.

The pH of the extraction mixture is important for drugs containing a pH-dependent dissociable group. A low pH value favored the sorption between the C8/SCX phase and the SAs. The pH values lower than 3 could damaged the silica sorbent.

The number of draw-eject cycles was evaluated to establish the sorption equilibrium. The peak areas of the drugs increased from one up to four cycles (4 × 250 µL, draw-eject) (Figure 3). However, after this value the extraction efficiency decreased, probably because of partial desorption of the drugs during each eject step.

The addition of NaCl (ionic strength) to the egg samples was also evaluated. The addition of NaCl to egg samples

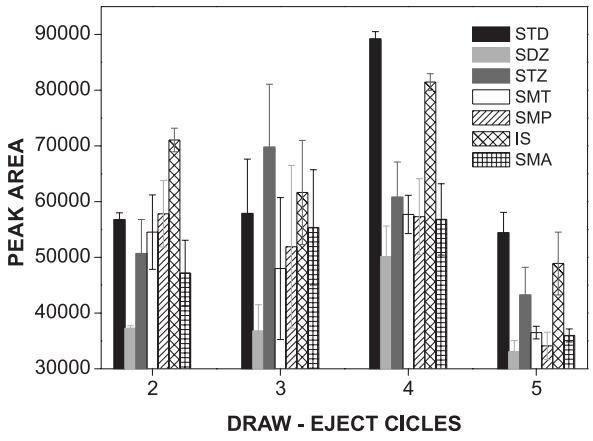

Figure 3. The effect of draw-eject cycles on MEPS performance.

decreased the extraction yield. Probably, the presence of endogenous egg compounds (like proteins, lipids), in the sample solution, promotes interaction between salt molecules and drugs, thereby reducing their ability to move into the extraction phase.[27] Another factor to be considered is the extraction phase used (M1, a mixture of C8 and SCX). Thus, in addition to the retention mechanism of sulfonamides by reversed phase (C8), the analytes are also adsorbed by cation exchange (SCX). Therefore, NaCl (Na^+) excess in the sample could interfere in sulfonamides retention on the surface of extraction phase by exchange process:

$$R^- Na^+ + SA^+ \rightleftharpoons R^- SA^+ + Na^+$$

R^- = cation exchange extraction phase ; SA^+ = sulfonamides charged positively.

The pH value of the solution sample is acidic, pH 3, which favors protonation of the amino group of sulfonamides.

The washing step with different solvents was also evaluated. However, leakage of SAs was significant during this process, diminishing the analytical sensitivity of the method, mainly when the organic solvent percentage in the washing solution was increased. Consequently, the washing step was not incorporated in MEPS procedure. The egg endogenous compounds (interferences) did not co-elute with the analytes (Figure 4).

The desorption process (elution of drugs) was evaluated with different solvents (mobile phase, phosphate buffer solution pH 3, water, and methanol) and different solvent volumes (70, 100, 150, and 200 µL). The best results were obtained with the mobile phase (mixture of 20% phase A and 80% phase B), which eluted the drugs in the smallest volume possible, 100 µL (Figure 5).

The sorbent phase in the MEPS was easily and effectively washed (500 µL of methanol and 500 µL of water) between injections to reduce the possibility of carry-over.

The MEPS sorbent (C8/SCX) was reused more than 60 times for egg samples, with minimum loss of the extraction efficiency. Based on previous studies, MEPS analysis with new different cartridges presented similar values of accuracy and precision for all studied cartridges.

Analytical validation of the MEPS/LC-DAD method

The specificity of the developed method was demonstrated by representative chromatograms of reference egg blank sample, and reference egg blank sample spiked with analytes (300 ng g^{-1}), Figure 4. This chromatogram evidence the ability of the method to measure the analytes in the presence of endogenous egg components.

The linearity of the MEPS/LC - DAD method ranged from 30 ng g^{-1} (LOQ) to 300 ng g^{-1} for egg samples. The regression equations and the corresponding correlation coefficients obtained for all the SAs in egg samples are given in Table 1.

The LOQ values were determined as the lowest concentration in the calibration curve in which the coefficient of the variation (CV) was lower than 10%, based on a signal-to-noise ratio of about 10.

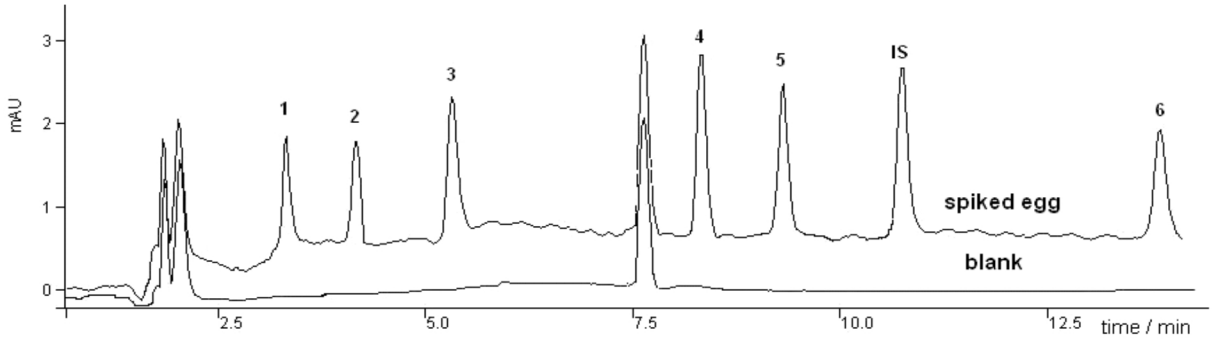

Figure 4. Representative chromatograms of reference egg blank sample, and reference egg blank sample spiked with analytes (100 ng g^{-1}). 1, sulfacetamide; 2, sulfadiazine; 3, sulfathiazole; 4, sulfamethazine; 5, sulfamethoxypitidazine; 6, sulfamethoxazole. Primidone was used as internal standard.

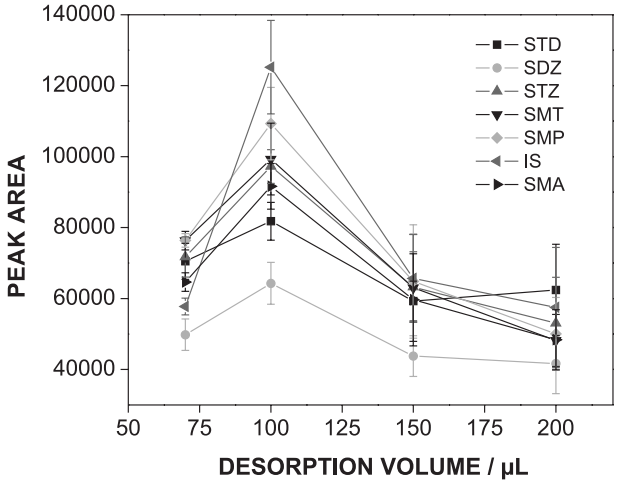

Figure 5. Effect of desorption solvent volume (mobile phase - mixture of 20% phase A and 80% phase B) on MEPS performance.

Table 1. Linearity of the MEPS/LC-DAD method for analysis of SAs in egg sample

Sulfonamides	Linear regression (LOQ: 300 ng g $^{-1}$)	r^2
sulfacetamide	Y = 0.04082 + 0.00741X	0.998
sulfadiazine	Y = 0.01704 + 0.00472X	0.998
sulfathiazole	Y = –0.06302 + 0.00825X	0.998
sulfametazine	Y = –0.09395 + 0.00893X	0.999
sulfamethoxypiridazine	Y = –0.07723 + 0.00964X	0.998
sulfamethoxazole	Y = –0.00647 + 0.00794X	0.998

MEPS methods can be used in manual or automatic mode with different chromatographic systems. However, the variation coefficients of manual MEPS methods have been higher than automatic, due to the difficulty in maintaining exactly the same conditions (*i.e.*, sample flow rate through the sorbent).

The inter-day precision presented CV values ranged from 2.2% to 9.5% (Table 2). The CV values obtained in this work are in agreement with manual extraction MEPS methods. The MEPS/LC-DAD method developed presented adequate precision and accuracy for analysis of SAs in egg samples (Table 2).

The methodology developed for determination of SAs in egg samples compared with other published methods,[28,29] that used spectrophotometric detection presented similar or better sensitivity.

Conclusions

The proposed method (MEPS/LC-DAD) presented the following advantages: the robustness of the sorbent extraction phase (C8/SCX) that was reused more than 60 times with minimum loss of extraction efficiency, the reduced sample extraction time (about 4 min), the small egg sample (0.5 g), and the small organic solvent consumption (desorption, 100 µL of the mobile phase). Moreover, this method allows desorption and injection steps online in LC chromatographic system using the MEPS syringe.

Table 2. Inter-day precision (coefficient of variation, CV) and accuracy of the MEPS/LC-DAD method for determination of SAs in egg samples

Sulfonamides	Added concentration / (ng g $^{-1}$)	CV / (%) (n = 5)	Accuracy / (%) (n = 5)
sulfacetamide	150	4.9	96
	100	4.3	110
	50	2.2	99
sulfadiazine	150	2.5	97
	100	3.8	104
	50	9.5	97
sulfathiazole	150	3.6	99
	100	3.6	111
	50	7.7	98
sulfametazine	150	3.5	94
	100	2.9	100
	50	6.1	104
sulfamethoxypiridazine	150	8.3	94
	100	7.8	96
	50	4.8	104
sulfamethoxazole	150	2.6	98
	100	5.1	94
	50	6.1	102

The egg matrix effect (endogenous compounds) decreases the analytical sensitivity of the MEPS/LC-DAD method for determination of SAs. However, this method presented LOQ values smaller than those established as MRLs (100 µg kg^{-1}) in food of animal origin. Therefore, the MEPS/LC methodologies developed can be used for determination of SAs in eggs samples, considering the evaluated analytical validation parameters.

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