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High-performance liquid chromatography–tandem mass spectrometry for the determination of sulfonamides in eggs

Vytautas Tamošiūnas and

Audrius Padarauskas

Department of Analytical and Environmental Chemistry, Vilnius University, Naugarduko 24, LT-03225 Vilnius, Lithuania

Daiva Babičienė and

Tomas Petrėnas

Chemistry Department, National Veterinary Laboratory, J. Kairiūkščio 10, LT-08409 Vilnius, Lithuania

High-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method was developed for the determination of ten sulfonamides (SAs) in hen eggs. Conditions for reversed-phase HPLC and electrospray ionization MS/MS in the positive ion mode were optimized monitoring two characteristic mass transitions for each analyte. The egg samples were extracted with acetonitrile and defatted with *n*-hexane. Further solid-phase clean-up on a Strata-X polymeric cartridge was performed to obtain an extract suitable for the HPLC-MS/MS analysis. The calibration curves showed acceptable linearity in the concentration range from 1 to 45 μg/kg with correlation coefficients (r^2) above 0.992. Using two labelled internal standards (sulfamethazine-$^{13}C_6$ and sulfamethoxazole-$^{13}C_6$) the mean recoveries of SAs from egg samples spiked at 10 μg/kg were in the range of 91–146%. The applicability of this technique was demonstrated by the analysis of hen eggs available from local poultry farms. Among the 10 samples analyzed, none of them were found positive for the sulfonamides studied.

Key words: high–performance liquid chromatography, tandem mass spectrometry, sulfonamides, eggs

INTRODUCTION

Sulfonamides (SAs) belong to a class of antimicrobial drugs that are widely used for food producing animals as growth promoters as well as for therapeutic and prophylactic purposes. Residues of SAs in the food chain are of an increasing concern due to their carcinogenic potency and their contribution to an increase of antibiotic resistance [1, 2]. The maximum allowable residue limit in the European Union countries for SAs in animal tissue has been established at the total level of 100 μg/kg [3]. However, no maximum residue level for SAs in eggs has been established in Europe. This means that SAs, if present in eggs, must be below the quantification limit of the analytical method used. Consequently, development of simple, rapid and sensitive analytical techniques for monitoring the SAs residues in such samples is of great significance.

Sulfonamides in food may be determined by a number of different analytical methods, based, for example, on enzyme immunoassay [4], thin-layer chromatography [5], gas chromatography [6], and reversed-phase high-performance liquid chromatography (HPLC) [7, 8]. However, the confirmation of suspect positive samples must be carried out by mass spectrometry (MS) coupled with an adequate chromatographic separation. Although gas chromatography coupled with mass spectrometry is both sensitive and selective, the derivatisation of non-volatile and thermally labile sulfonamides is required prior to the analysis [6]. This procedure significantly increases the overall analysis time and may lead to errors.

Major technical advancements made in the last decade on interfacing HPLC systems to mass spectrometers have also resulted in a number of applications of HPLC–MS-based methods for the detection of sulfonamides in food, employing various ionization techniques, such as thermospray [9], electrospray [10] and atmospheric pressure chemical ionization [11]. Liquid chromatography methods, however, do not require a derivatization step and the sensitivity of HPLC–MS approaches that of GC–MS.

Currently, the main advances in improving the sensitivity and specifity of food analyses of pharmaceutical residues are due to the application of HPLC with tandem mass spectrometric detection (HPLC–MS/MS). This more sophisticated technique allows a very effective isolation of analyte ions from the noise-producing matrix. The potential of HPLC–MS/MS has already been demonstrated in an analysis of complex food matrices, such as meat [12, 13] and honey [14, 15], and particularly in an analysis of SAs residues. To our knowledge, only one report on HPLC-MS/MS method dealing with common sulfonamides in egg matrices has been reported to date [16]. However, in that study the HPLC–MS/MS technique has been used only for the identification of SAs residues with subsequent quantification by a conventional HPLC with ultraviolet detection.

The aim of this research was to develop a sensitive and precise HPLC–MS/MS method for the quantification of common

* Corresponding author. E-mail: audrius.padarauskas@chf.vu.lt

sulfonamides in hen eggs. For this purpose, extraction and sample clean-up procedures as well as HPLC separation and MS/MS detection parameters were optimized. Finally, the suitability of the developed method was assessed by an analysis of egg samples.

EXPERIMENTAL

HPLC analyses were performed using a Waters Alliance 2795 Separations Module (Milford MA, US) equipped with a quaternary solvent delivery system, autosampler, and a column heater. HPLC separation was achieved using a Phenomenex Aqua C18 column (150 mm × 2.0 mm, i.d., 3 µm particle size, Waters), maintained at 40 °C with a mobile phase flow rate of 0.4 mL/min. The injection volume was 20 µL.

The detection was performed using a Quattro Premier XE tandem quadrupole mass spectrometer (Waters, Manchester, UK). The instrument was operated using an electrospray source in the positive mode with multiple reaction monitoring (MRM). The ionisation source parameters were the following: capillary voltage was 2.5 kV, source temperature was 120 °C and desolvation gas temperature was 350 °C. Data acquisition and processing were performed using MassLynx 4.0 with TargetLynx.

Purified water was obtained with a Milli-Q apparatus (Millipore, Bedford, MA, USA). Acetonitrile, ethyl acetate, *n*-hexane, formic acid, acetic acid and trifluoroacetic acid were HPLC grade and used as received (Merck, Darmstadt, Germany). Standards of sulfonamides were purchased from Sigma (St. Louis, MO, USA). Chemical structures of the SAs used in this study are shown in Fig. 1. Isotope-labelled internal standards (sulfamethazine-$^{13}C_6$ and sulfamethoxazole-$^{13}C_6$) were obtained from Cambridge Isotope Labs. (Andover, MA, USA). Stock solutions of sulfonamides and internal standards at the concentration of 0.1 mg/mL were prepared in acetonitrile and stored at –20 °C, protected from light. Working solutions used for spiking blank samples were obtained by an appropriate dilution.

RESULTS AND DISCUSSION

HPLC-MS/MS method development

Sulfonamides are known to possess amphoteric character due to the presence of nitrogen functions in their structures, which because of their positions in the structure can protonate/deprotonote depending on the pH of the environment. For this reason, most SAs are positively charged at acidic conditions, neutral in the pH range 3–6 (approximately) and negatively charged under alkaline conditions. Consequently, the pH of the mobile phase plays a predominant role in their separation. In addition, considering the characteristics of electrospray ionization, the mobile phase composition and additive may significantly affect the detection sensitivity. Usually, the separation of SAs is performed by HPLC in a reversed-phase mode under acidic conditions [7, 8, 15].

In order to optimize the chromatographic separation, a series of preliminary experiments was performed testing mobile phases consisting of water, acetonitrile and methanol with different additives (formic acid, acetic acid and trifluoroacetic acid) to obtain acidic conditions. Initial separations were performed under gradient conditions with two mobile phases: mobile phase A contained 0.05% of appropriate acid in water and mobile phase B was 90% acetonitrile with 10% methanol. The gradient program employed was the following: 0–2.0 min 98% A, 18.0–20.0 min 70% A, and 21.0–25.0 min 98% A. The best resolution of SAs and the highest detection sensitivity were achieved using formic acid. Next, different formic acid concentration levels ranging from 0.02% to 0.10% were investigated. The results showed that the resolution and the detection sensitivity of the analytes increased with an increase in the formic acid concentration from 0.02% to 0.05%. However, higher HCOOH concentrations resulted in the loss of the detection sensitivity, most likely due to the analyte ionization suppression. Thus, 0.05% formic acid was chosen as the mobile phase additive. Under optimized HPLC conditions, all the SAs were successfully separated in less than 20 min.

Mass spectra of all the analytes were acquired in a full scan mode with cone voltage of 25 V, using positive and negative electrospray ionization. All the compounds produced higher signal-to-noise ratios in the positive ion mode. As expected, sulfonamides showed relatively simple mass spectra. For all the SAs except two internal standards, the most intense fragments in the positive ion mode were detected at *m/z* 156, 108, and 92, although the abundance was different depending on the variable compounds. Cone voltages were optimized for maximum signal intensity of typical ions during an injection of single compounds into the mass spectrometer. The selected MRM transitions as well as the individual cone and collision energy voltages applied for the analytes and internal standards are summarized in Table 1.

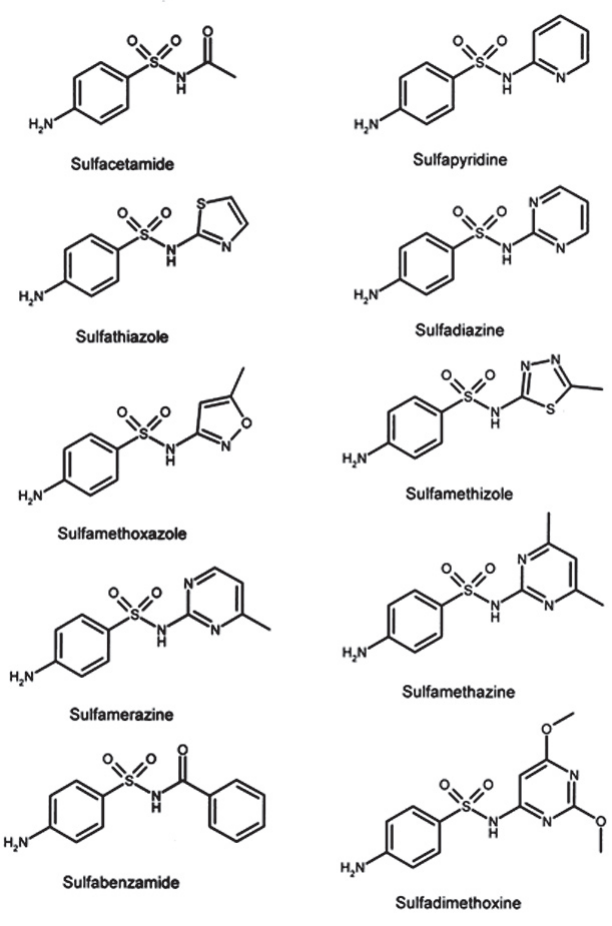

Fig. 1. Chemical structures of the sulfonamides investigated

Table 1. **MRM transitions and MS operating parameters selected for the analysis of sulfonamides**

Compound	MRM transitions (m/z)	Cone voltage (V)	Collision energy (eV)
Sulfacetamide	215 > 156	17	8
	215 > 108	17	17
Sulfapyridine	250 > 156	26	17
	250 > 108	26	25
Sulfadiazine	251 > 156	24	14
	251 > 108	24	24
Sulfathiazole	256 > 156	25	16
	256 > 108	25	24
Sulfamerazine	265 > 156	28	16
	265 > 108	28	28
Sulfamethoxazole	254 > 156	25	16
	254 > 108	25	23
Sulfamethoxazole-$^{13}C_6$	260 > 162	25	18
Sulfamethizole	271 > 156	20	14
	271 > 108	20	18
Sulfamethazine	279 > 186	31	18
	279 > 108	31	26
Sulfamethazine-$^{13}C_6$	285 > 186	27	15
Sulfabenzamide	277 > 156	16	13
	277 > 108	16	22
Sulfadimethoxine	311 > 156	31	20
	311 > 108	31	30

Extraction and sample clean-up

Common sample preparation strategies for SAs in food samples involve an extraction with organic solvent (e. g., acetonitrile, methanol, ethyl acetate) followed by lipid removal with *n*-hexane [7, 12, 13, 16]. Thus, preliminary studies were conducted to optimize the solvents and extraction conditions. The extraction efficiency of SAs from spiked (at a 100 µg/kg fortification level) blank samples was studied using two solvents: acetonitrile and ethyl acetate. The obtained results showed that acetonitrile provided slightly higher recoveries of SAs from the spiked blanks. Based on these results, acetonitrile was selected as an extractant for the further studies.

In the next step, the extraction of the SAs from the spiked blank samples was optimised by varying the volume of acetonitrile from 5 to 15 mL. However, acetonitrile volumes greater than 10 mL provided neither cleaner extracts nor higher analyte recoveries. Furthermore, the influence of the number of repeated extractions was tested by performing one, two and three consecutive extractions. The obtained results indicated that the volume of 10 mL of the solvent and two consecutive extractions with a subsequent defatting provided absolute (without internal standard) recoveries between 50% and 70% of all the analytes, except sulfathiazole and sulfamethazine, for them the obtained recoveries were about 23%.

Complete sample extraction and lipid removal procedure optimized for the analysis of SAs residues was as follows: A 2 g of egg sample and 10 mL of acetonitrile were placed into a beaker and the mixture was agitated on a minishaker for 10 min. This extraction procedure with acetonitrile was repeated once more and the combined extracts were evaporated to dryness at 50 °C under nitrogen flow. The residue was dissolved in 20 mL of 0.2 mol/L ammonium acetate buffer (pH 5.3) and defatted with 5 mL of *n*-hexane by centrifugation for 10 min at 4000 rpm. After the centrifugation, the aqueous phase was ready for a clean-up procedure.

An extract from a biological sample contains many diverse compounds in addition to possible traces of the target analytes. To exclude these interfering substances, a number of clean-up methods have been developed for the analysis of sulfonamide residues. In most published methods for the determination of SAs in food of different origin, at least one solid phase extraction (SPE) step with polar [13], non-polar [7, 8, 16] or strong cation-exchange sorbent materials [12] is included. The advantage of an SPE-step prior to the LC–MS/MS analysis is that in some cases the suppression from the matrix components is decreased, and in most cases the detection limit of the method is decreased. Strata-X cartridges (200 mg/6 mL) from Phenomenex were tested for the clean-up of the egg samples. According to the manufacturer, unique surface properties of this surface-modified styrene-divinylbenzene polymeric stationary phase offer numerous retention mechanisms, including hydrophobic, hydrogen bonding and π-π interactions. Aliquots (20 mL) of blank egg extracts spiked at 100 µg/kg were processed through all the steps of the SPE in order to monitor the recoveries. For each extraction, the Strata-X cartridge was preconditioned with 5 mL methanol followed by 5 mL 0.2 mol/L ammonium acetate buffer (pH 5.3). After sample loading, washing was performed with 5 mL of water and then the cartridge was dried under vacuum. Next, we investigated the volume of acetonitrile to completely elute the SAs from the cartridge. In this experiment, the adsorbed analytes (2 µg each) were eluted from the cartridge as 1 mL fractions for the determination of SAs in each fraction. The obtained results showed that a complete desorption of the analytes requires at least 7 mL CH_3CN. Higher acetonitrile volume did not result in higher SAs recoveries. This optimized SPE procedure yielded satisfactory high (≥80%) absolute recoveries for all the SAs from the spiked blank extracts.

Analytical performance

Method validation was carried out according to the criteria described in [17]. The parameters taken into account were the following: response linearity, decision limit, detection capability, trueness and precision.

Usually the quantification of drug residues is performed using a matrix-matched calibration curve made from fortified blank samples prepared in the same matrix as the real samples. To test the linearity of the calibration curve, six standards of SAs in the blank egg matrix were analyzed. The calibration curves showed acceptable linearity in the concentration range from 1 to 45 µg/kg with correlation coefficients (r^2) above 0.992.

The EU decision [17] introduces the concepts of a decision limit (CCα) and a detection capability (CCβ) for a chemical analytical method. These method parameters are to be used instead of the more familiar limit of detection and limit of quantification. The definition of the CCα for a forbidden compound is: "The limit at and above which it can be concluded with an error probability of 1% that a sample is noncompliant." The definition

Table 2. **Performance data of the HPLC-MS/MS method for the analysis of sulfonamides**

Analyte	Method limits, µg/kg		RSD, % (n = 4)				Recovery at 10 µg/kg, %
	CCα	CCβ	5 µg/kg	10 µg/kg	20 µg/kg	40 µg/kg	
Sulfacetamide	13	34	25	26	31	34	146
Sulfadiazine	12	18	43	15	18	21	95
Sulfathiazole	11	16	23	12	14	16	101
Sulfapyridine	9.0	13	20	12	12	13	113
Sulfamerazine	9.3	12	12	10	9.6	9.1	103
Sulfamethazine	8.2	10	15	8.2	7.0	7.4	102
Sulfamethizole	10	14	23	12	9.1	8.3	91
Sulfamethoxazole	7.4	9.3	11	5.2	3.6	4.7	100
Sulfabenzamide	26	>40	78	47	33	25	95
Sulfadimethoxine	14	21	36	20	11	6.7	110

of the CCβ for a forbidden compound is: "The lowest concentration at which a method is able to detect truly contaminated samples with an error probability of 5%." The CCα and CCβ were obtained using the calibration graph approach [17]. Blank material was fortified at 5 different concentrations (n = 20) and the standard error of the *y* intercept was calculated. The decision limits (CCα = 2.33 × standard error of the *y* intercept) and the detection capabilities (CCβ = CCα + (1.64 × standard deviation of 20 spikes at CCα)) for SAs are listed in Table 2.

The accuracy and precision of the method were determined by spiking blank egg samples with SAs at four concentration levels (5, 10, 20 and 40 µg/kg) and four samples per concentration level. Two labelled internal standards (sulfamethazine-$^{13}C_6$ and sulfamethoxazole-$^{13}C_6$) at 5 µg/kg were used to compensate SAs losses during the extraction and clean-up procedures. The accuracy was expressed in terms of recovery rates and the precision was expressed as relative standard deviation (RSD). These results are also summarized in Table 2. According to the EU guidelines for approval of a method for drug residue analysis, the average recovery of quantitative methods at analyte concentrations higher than 10 µg/kg should be 80 to 110% and the RSD should not exceed 25%. The results presented in Table 2 indicate that all the analytes, except sulfacetamide and sulfabenzamide, meet these criteria.

Finally, the HPLC–MS/MS method was applied to hen egg samples available from local poultry farms. Fig. 2 shows the typical chromatograms of the egg sample (a) and the same sample spiked at 5 µg/kg of SAs (b). Among the 10 samples analyzed, none were found positive for the sulfonamides studied.

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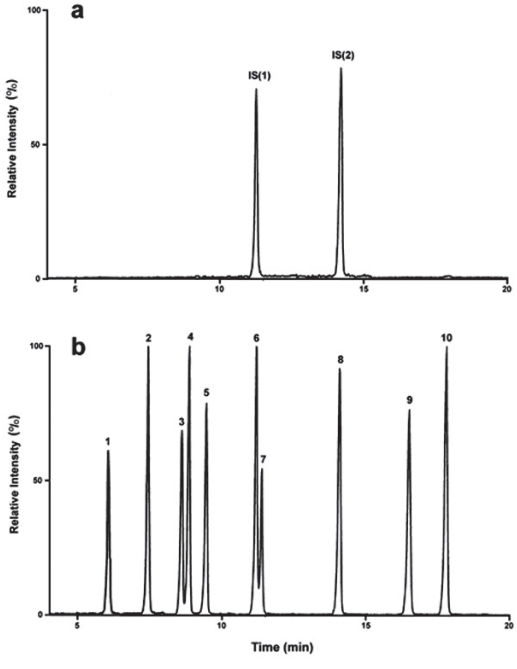

Fig. 2. Chromatograms of a blank egg sample (a) and an egg sample spiked with 5 µg/kg of sulfonamides (b). Peaks: IS(1) – internal standard (sulfamethazine-$^{13}C_6$), IS(2) – internal standard (sulfamethoxazole-$^{13}C_6$), 1 – sulfacetamide, 2 – sulfadiazine; 3 – sulfathiazole, 4 – sulfapyridine, 5 – sulfamerazine, 6 – sulfamethazine, 7 – sulfamethizole, 8 – sulfamethoxazole, 9 – sulfabenzamide, 10 – sulfadimethoxine

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Vytautas Tamošiūnas, Daiva Babičienė, Tomas Petrėnas, Audrius Padarauskas

SULFONAMIDŲ NUSTATYMAS KIAUŠINIUOSE EFEKTYVIOSIOS SKYSČIŲ CHROMATOGRAFIJOS–DVIGUBOS MASIŲ SPEKTROMETRIJOS METODU

S a n t r a u k a

Optimizuotas efektyviosios skysčių chromatografijos–dvigubos masių spektrometrijos metodas sulfonamidams vištų kiaušiniuose nustatyti. Sulfonamidai iš kiaušinio mėginių ekstrahuojami acetonitrilu, ekstraktas nuriebalinamas *n*-heksanu ir papildomai išvalomas kietafazės ekstrakcijos būdu leidžiant ekstraktą per polimerinį Strata-X adsorbentą. Išmatuotos kalibracinės kreivės tiesinės, esant 1–45 µg/kg koncentracijai ($r^2 \geq 0{,}992$). Vidutinės sulfonamidų standartinių priedų (10 µg/kg) kiaušinio matricoje išgavos siekia 91–146%. Ištyrus 10 kiaušinių mėginių, nei viename iš jų sulfonamidų liekanų neaptikta.