Determination and validation of Sulfonamide Antibiotics in liver tissue of buffalo using QuEChERS method and LC MS/MS analysis

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Abstract

Sulfonamides (SAs) are a very important class of antibacterial compounds widely used in veterinary practice for therapeutic, prophylactic, and growth promoting purposes. Residues of SAs may remain animal tissues if adequate withdrawal time is not observed or if the SAs have been improperly administered. These residues in food are of concern because of their potential carcinogenic nature and the possible development of antibiotic resistance in humans. Therefore the aim of this study was the determination and validation of seven SAs by using QuEChERS method and LC MS/MS analysis. SAs were extracted from liver tissue in mixture of EDTA citrate buffer solution at PH 4 and acetonitrile followed by centrifugation. For quantitation and confirmation of each compound liquid chromatography mass spectrometry (LC MS/MS) with electrospray ionization (ESI) was used , the mass spectrometry was operated in multiple reaction monitoring (MRM) mode. The method performance was validated, including linearity, sensitivity, and trueness and precision, CCα and CCβ. The result have shown a high recovery ranging from 73.9-80.7%, with relative standard deviations RSD (n=48) <14%. The limit of decision (CCα) of seven SAs was ≅ 106 µg $^{kg-1}$, and the limit of detection (CCβ) was ranging from 110 to 115.5 µg ^{kg}.The limits of quantification and the limits of detection for SAs 25.0 µg $^{kg-1}$and 10.0 µg $^{kg-1}$, respectively. These parameters met the European Union criteria for method validation. This method of extraction and quantification of SAs in liver tissue was validated to be accurate, and sensitive, and precise and could be used commercially for large amount of samples.

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Key words: - Sulfonamides (SAs), EU commission, LC MS/MS, Method Validation.

1. Introduction

Sulfonamides (SAs) are antimicrobial agents most commonly used in veterinary practice to treat diseases, to control and prevent infection and to promote growth and production efficiency; they are inexpensive and offer a wide spectrum of antimicrobial activity (**Biswas *et al.*, 2007; Mamani, Reyes, &Rath, 2009**). The inappropriate or excessive use of these drugs can result in the presence of drug residue in animal tissue, which contributes to the generation of long-term health effects, including microbial antibiotic resistance, and can cause potential adverse side effects in hypersensitive individuals (**Chiaochan *et al.*, 2010**). Moreover, toxicological studies have shown that SAs can lead to blood disorders, liver damage, cancer and tumors (**Arancibia *et al.*, 2003**). Therefore, many efforts, such as monitoring veterinary drug residues to ensure the safety of food, have been made to protect consumers' health.

To limit human exposure, the European Union (EU) has set maximum residue limits (MRLs) for different food contaminants in a certain number of raw foods on the basis of toxicological data, acceptable daily intake values and the performance of current analytical technology. Within the EU, one of the main documents stating the MRLs of authorized veterinary drugs in food of animal origin is Council Regulation 2377/90/EC, which was repealed by Commission Regulation 470/2009 of the European Parliament and the Council (**European Commission, 2009a**). The pharmacologically active substances that have an MRL (permitted) are contained in Regulation 37/2010 of the Council, which provides an MRL of 100 µg kg-1 for SAs in foods of animal origin, stating that the combined total residues of all substances within the sulfonamide group should not exceed this MRL value (**European Commission, 2009b**).

Therefore, it is necessary to develop sensitive and easy analytical methods that can be used routinely, comply with current legislation and allow for the determination of residues of veterinary drugs in food of animal origin, thus ensuring the safety of the supplied products. In 2002, the European Union (EU) issued a specific 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. It was mandatory that some new parameters must be calculated, such as the limit of decision (CCα) and detection capability (CCβ) (**European Commission, 2002**).

For detecting antimicrobial residues in food, bioassay techniques are widely used in screening methods because of their simplicity and low cost (**Lamar, & Petz, 2007**). However, before samples are condemned for containing levels of antimicrobials exceeding the approved levels, it is well recognized that these methods must be supported by sufficiently selective and sensitive chemical methods (**Bogialli, & Corsia, 2009**).

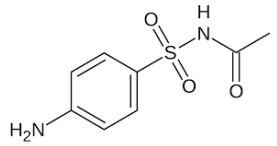
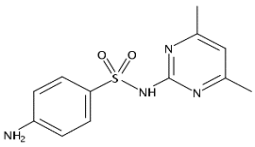
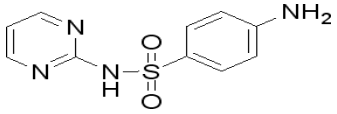
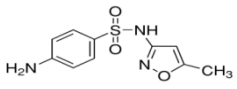
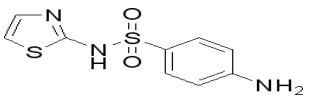
Several extraction methods have been used for SA-residue analysis, however, most are long and tedious, involving liquid-liquid extraction (LLE) (**Haller *et al*., 2002; Chico *et al*., 2008**), or solid-phase extraction (SPE) (**Pecorelli *et al*., 2004; Koesukwiwat, Jayanta, & Leepipatpiboon, 2007; Bedor *et al*., 2008**), which also include an additional step to precipitate the proteins. Therefore, A significant step forward in reducing the time to process a sample was described in 2003: the QUick, Easy, CHeap, Effective, Rugged and Safe (QuEChERS) method by **Anastassiades et al. (2003**) for pesticide analysis. The greater diversity in the chemical properties of veterinary drugs, compared to pesticides, has made combining them into large analytical suites difficult; however, this method has been used successfully by some researchers (**Stubbings, & Bigwood, 2009; Frenich *et al*., 2010, Abdullah *et al*., 2014**). For the determination of sulfonamides in tissue, gas chromatography (GC) gas chromatography/mass spectrometry (GC-MS), capillary electrophoresis (CE) (**Kowalski *et al.*, 2011**), high performance liquid chromatography (HPLC) (**Abdallah et al., 2014),** and liquid chromatography/mass spectrometry (LC-MS) (**Zhou *et al*.,2012**) have been used. GC and GC-MS need derivatization before analysis, but LC-MS has more advantages than GC, because it can offer selectivity, structural information and sensitivity without the derivatization of sulfonamides.

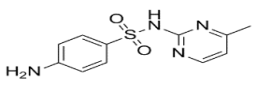
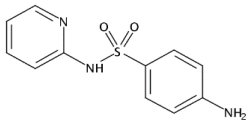
The aim of the work was to develop a method for the determination of following SAs: [Sulfamerazine, sulfapyridine, sulphacetamide, sulfadiazine, sulfamethoxazole, sulfathiazole, sulfamethazine] in liver tissue of buffalo. The method involves a simple extraction technique based on QuEChERS procedure, which implies an extraction with acetonitrile without further clean up and analytical determination by liquid chromatography coupled with tandem mass spectrometry (MS/MS). And validation of method according to 2002/657/EC European Union Commission Decision.

2. Experiment

Chemicals and materials

Table 1:- Chemical structure of seven SAs

Group	*Common Name of group*	*Compounds*	*Chemical Structure*
(B1) Antibacterial substances	**Sulfonamides**	Sulfacetamide (SAM)	
		Sulfamethazine (SMZ)	
		Sulfadiazine (SDZ)	
		Sulfamethoxazole (SMTX)	
		Sulfathiazole (STZ)	

		Sulfamerazime (SMR)	
		Sulfapyridine (SPD)	

Methanol and acetonitrile were HPLC grade and were purchased from Merck (Darmstadt, Germany). Sodium hydroxide, citric acid monohydrate, ammonium hydroxide, and formic acid were purchased from Riedel-deHaen ≥99%. Ethylenediamine Tetra Acetic Acid disodium salt dihydrate (Na2-EDTA) was purchased from Fluka ≥99%. Commercial antibiotic (SAs) standard was supplied by (Dr. Ehrenstorfer, Germany).

Buffer solution: Stock buffer (50mM Ammonium formate solution): 1.73 ml formic acid was added to 900 ml water then the PH was adjusted at 2.78 ± 0.1 with ammonia solution 10% then 50 ml methanol was added and completed to 1L with deionized water.

Dilution solvent: 25 ml methanol was added to 75 ml stock buffer to have methanol/ buffer ratio equal to (25:75).

LC mobile phase (5mM Ammonium formate solution in Methanol/Buffer (1:9): 50 ml of stock buffer was diluted with 450 ml Methanol/ Buffer (1:9), the PH was adjusted at 2.78 ± 0.1.

Standard solutions

Stock solution

100 µg/ml of references standard solution of each compound was prepared in 100 ml volumetric flask and dissolved in methanol, then was stored in -18 ˚C in dark place covered with aluminum foil, preparation of this solution is required every six month.

Mixture of working solution

4 µg/ml of reference standard solution of each compound was prepared in one mixture by diluting appropriate volume of stock solution with methanol, then was stored at -18 ˚C in dark place covered with aluminum foil. This mixture was used in spike recoveries and daily calibration mixture solution.

Calibration mixture solutions

Calibration mixture solutions were prepared by taking 1ml of the working solution mixture and complete with stock buffer to prepare standard solution at 1 µg/ml. This standard was used to prepare the calibration mixtures at concentration levels 0.025, 0.05, 0.1, 0.2 µg/ml, which was prepared by adding 12.5, 25, 50, 100 µl of 1 µg/ml mixture standard solution and the 0.5 ml vial was completed with dilution solvent. Calibration mixture solutions must be prepared daily with each set of samples. Standard in matrix was prepared one point standard in the targeted blank matrix daily (with each set of samples) at the level of interest (40 MRL) to compensate the matrix effect on the sample result. The sample result must be corrected against standard in matrix.

Apparatus:

LC-MS/MS System, Agilent 1200 series liquid chromatography system equipped with Applied Biosystem (API 4000 Qtrape) tandem mass spectrometers with electrospray ionisation (ESI) interface. Separation was performed on a C18 column ZORBAX Eclipse XDBC18 4.6mm x 150 mm, 5 µm particle sizes. The injection volume was 25 µl. A gradient elution program was at 0.3ml/min flow rate, in which one reservoir contained 10 mM ammonium formate solution in MeOH: H2O (1:9, v: v) and the other contained methanol The ESI source was used in the negative mode, and Nitrogen was used as nebulizer gas, heater gas and collision gas according to manufacturer's settings; source temperature was 300ºc, ion spray potential 5500V, decluster potential and collision energy were optimized using Harvard apparatus syringe pump. The multiple Reaction Monitoring Mode (MRM) was used in which two MRM was used for quantification and other was used for confirmation.

Methodology

Sample preparation and processing

Buffalo liver was provided by the laboratory. It was then washed and chopped into small pieces. The chopped liver sample was homogenized thoroughly with a food grinder then stored at -20^{0}C to reduce degradation of antibiotics. Before analysis, liver tissue were thawed at room temperature for treatment and analysis.

Extraction of SAs

SAs were extracted from liver using an extraction procedure based on QuEChERS methodology. Briefly the procedure was as follows: 2 g of sample was weighed in a polypropylene centrifuge tube (40 mL), 50 µl of standard in matrix (40 MRL) was added, 1 ml

from both I M Sodium citrate buffer at PH 4 and 0.5 Na2EDTA were added then vortexed for 1 min, 10mL of acetonitrile was added, and the mixture was homogenized for 2-3 mins using Ultra Turax. The mixture was centrifuged at 4500 rpm for 10 min at -4 ˚C for phase separation and the supernatant was transferred in 100 ml round- bottomed flask. The extraction step was repeated with another 10 ml acetonitrile, and then vortex for 3 mins at 900 rpm followed by centrifugation again as previously described and combined the supernatant at the same 100 ml round-bottomed flask, then evaporated at 40 ˚C and avoid the back suction. Finally, 2mL of dilution solvent was added and 5µL were injected onto the HPLC–MS/MS system under the optimized conditions.

Method validation:

For the validation study, most of the performance characteristics were determined following the procedures described in 2002/657/EC Commission Decision. Blank liver tissue was used as negative control. The procedure was validated using the blank samples fortified 6 replicate with the working mixed solution, at (**25, 50, 100 and 200µg kg−1**) for two days.

3. Results and Discussion

The aim of this study was the development of an effective method for determination of SAs in liver tissue, where the extraction procedure is the key factor for successful determination. In our study the extraction procedure was based on liquid-liquid extraction where the extraction solvent was acetonitrile, which have shown a high recovery up to 80 % as shown in (Table 2). This result goes in accordance with **Li, et al (2016**), which also have chosen acetonitrile as extraction solvent for determination of SAs in fish sample, and the result have shown a high recovery 85%, who also have mentioned that acetonitrile not only extracted the SAs, but also denatured the protein ingredient in fish samples to make the matrix solution clean. Where this point was also supported by **Dasenaki and Thomaidis. (2010)**. the same study made a comparison with Ethyl acetate as an organic solvent, however the result have shown a lower recovery than acetonitrile 60%.

Matrix effect

Owing to the complexity of liver tissue, ME was investigated to evaluate our method. The results of ME were shown in Fig 1. An obvious suppression of signal was observed for SMTX, STZ and SMZ with ME >20%. A slight suppression of response was observed for SMR, SPD, SAM and SDZ with ME in the range of 6–20%. These results showed that the suppression effect was observed for most of the SAs in liver. Owing to the ME, matrix-matched calibration was used to compensate for the loss of signal. Thus, a matrix-matched calibration curve was applied for the determination of SAs in fish tissue.

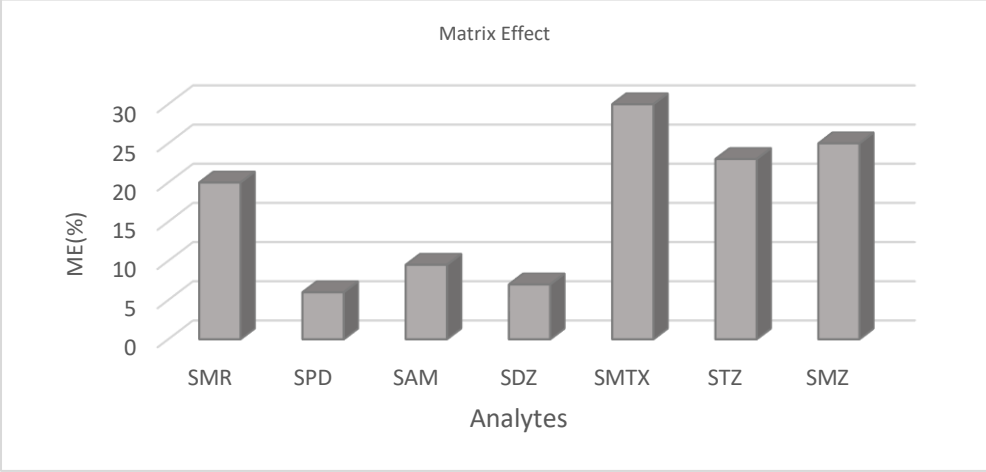


Figure 1:- Matrix effect of seven sulfonamide antibiotics (SAs) in liver tissue.

Method validation

The method performance was validated, including linearity, sensitivity, and trueness and precision, CCα and CCβ. Liver tissue spiked with standard mixture solutions of SAs were used for the validation study.

Linearity

Liver samples spiked with serials standard solutions were used for the determination of linearity. Good linearity was obtained for all SAs, matrix calibration curves of SAs were achieved with the correlation of determination (R^2) >0.99. The sensitivity was evaluated with LOD and LOQ, which were defined as a signal-to-noise ratio of 3 and 10.0, respectively. LODs were 10.0 µg $^{kg-1}$. LOQs were 25.0 µg $^{\mathbf{kg-1}}$**.**

Trueness and precision

The trueness of our method was tested by performing recovery experiments. Repeatability was tested to evaluate the precision values and expressed as the relative standard deviation (RSD). Blank liver sample, in which no interference was detected, was

spiked at four levels (25, 50, 100 and 200 µg $^{kg-1}$). Six replicates were performed on in two days with the same conditions for the determination of RSD. The recoveries of SAs were determined based on a matrix-match calibration curve. The results showed that average recoveries were in the range from 73.9 to 80.7%. As shown in Table 2, the values of RSD were <14.0%. The results confirmed that the presented method was reliable and sensitive to quantify SAs in liver tissue.

Decision limits (CCα) and Detection capabilities (CCβ):

The EU decision introduces the concepts of decision limit (CCα) and detection capability (CCβ) for a chemical analytical method. These 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 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%". CCα and CCβ were calculated at MRL (100µg/kg) in matrix liver. For CCα and β calculation following equations was used.

CCα = MRL+1.64*SD of 12 fortified blanks at MRL

CCβ = CCα+1.64*SD of 12 fortified blanks at CCα

Our result as illustrated in table 3, had shown that the limit of decision (CCα) of seven SAs were ≅ 106, and the limit of detection (CCβ) were at range from 110-115.5.

Table 2: Average Recoveries of SAs from liver samples fortified at 25, 50, 100 and 200 µg/kg (n =48) and overall RSD (The trueness and precision of the method for SAs in spiked liver samples (*n* = 48)

Analytes	Average Recoveries %				Overall Relative Standard Deviation (RSD %)			
	25(µg/kg)	50(µg/kg)	100(µg/kg)	200(µg/kg)	25(µg/kg)	50(µg/kg)	100(µg/kg)	200(µg/kg)
SMR	78.65	77	73.9	74.25	13.2	8.3	4.4	4.1
SPD	78	78.35	74.4	74.25	13.3	7.2	5.1	4.4
SAM	79	78.5	74.332	76.125	12.7	6.4	4.4	3.2
SDZ	77.35	78	73.7	74.75	9	8	5	10
SMTX	79.65	78.2	73.9	74.25	11.1	7.0	4.4	4.1
STZ	78.65	77	73.9	74.25	13.2	8.3	4.3	4.1
SMZ	80.7	77.5	74	74.46	11.1	7.6	4.7	4.3

Table 3: Calculation of CCα and CCβ of SAs by fortification at the MRL (100 µg/kg) (n=24)

Analytes	CCα	CCβ
SMR	106	113
SPD	106.03	115.5
SAD	105.772	112.2
SDZ	105.729	110.3
SMX	105.652	113
STZ	105.161	112
SMZ	105.68	111

4) Conclusion

In this study, QuEChEr method for simultaneous determination of seven SAs in liver tissue was developed in combination with HPLC-MS/MS. The results showed that satisfactory performance was achieved with fast analysis speed (~20 min) and reasonable recovery (73.9 - 80.7%). From the result of validation it can be concluded that the studied method is very sensitive, accurate, precise and selective to analyze SAs in liver. It require less run time and extraction time this method can be used commercially for the analysis of large quantity of samples**.**

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