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Simultaneous Detection and Quantification of Four Sulfonamides in Buffalo Meat using RP-HPLC

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ABSTRACT

A multi-residue analysis of four sulfonamides viz., sulfadiazine, sulfadoxine, sulfamethazine, sulfamethoxazole in buffalo meat has been proposed using HPLC- UV detection method. Samples were extracted by liquid-liquid extraction using acetonitrile, n-hexane solution and the analysis was carried out using a reverse phase HPLC using UV detector. The chromatographic separation was achieved using mobile phase consisting of glacial acetic acid in acetonitrile, methanol and water (85:10:5, v/v) in an isocratic elution mode. The volume of injection and flow rate of the mobile phase were 40 µl and 1.5 ml/min respectively. UV detector was operated at a wavelength 254 nm. Specificity, linearity, recovery percentage, intra-day variation, limit of detection (LOD), limit of quantification (LOQ) were evaluated in buffalo meat matrix at drug concentrations ranging from 10-500µg/Kg. Extraction recoveries of sulfonamides viz., sulfadiazine, sulfadoxine, sulfamethazine, sulfamethoxazole were ranged to be 72-77, 79-83, 85-89 and 82-86% respectively. The LOD for Sulfonamides viz., sulfadiazine, sulfadoxine, sulfamethazine, sulfamethoxazole were 6.06, 7.63, 6.79 and 10.68 µg/Kg and the LOQ were 18.37, 23.12, 20.57 and 32.37 µg/Kg, respectively. The proposed RP-HPLC method is quite adequate for routine monitoring of residual sulfonamides in buffalo meat below Codex MRL (100 µg/Kg).

Key words: *RP-HPLC, Buffalo meat, Tissue residues, Sulfonamides, liquid-liquid extraction*

INTRODUCTION

Study of antimicrobials residues in various foods that originated from animals began way back in 1960 particularly among European countries, like Belgium and Netherlands (Nunes et al. 2018). Sulfonamides are extensively employed in aquaculture and livestock farming as therapeutic and prophylactic agent owing to their broad-spectrum antibacterial activity, high efficiency and its cost effectiveness. Non observance of withdrawal period and off label use may result in residual sulfonamides in edible tissues like meat and milk which is of paramount public health concern (Waghamare et al. 2020; Parmar et al. 2021). The subsequent consequence to exposure to sulfonamides residues is adverse effects such as allergic reactions and mutagenic effects apart from emergence of resistance to pathogenic bacteria (Xia et al. 2020). To protect human health, sulfonamides residues in for food stuffs of animal origin must

be contained below the maximum residue limit (MRL) of 100 µg/kg which was adopted by Codex Alimentarius Commission (FAO/WHO, CAC/MRL 2-2015).

Various physiochemical and immunological methods have been developed to detect and quantify sulfonamide residues in animal derived food matrices like immunoassay methods (De Keizera et al. 2008) or biosensor assay (Haasnoot et al. 2003) and capillary electrophoresis (Zhang et al. 2015).Also reports on quantification of four sulfonamides in chicken (Cheong et al.2010; Chitescu et al. 2011),two sulfonamides in eggs (Roudaunt and Garnier,2002),five sulfonamides in feed (Pietron et al.2013) and eleven sulfonamides in aquatic environment (Mahmoud et al. 2013) using HPLC with UV detector is available.LC with PDA/DAD (Biswas et al. 2007; Meena et al. 2020), LC with fluorescence detection with post or pre-column derivatization (Zotou and Christina, 2010), and LC-mass spectrometry (Junmei et al. 2020) were also documented. Most of the HPLC methods have adopted solid-phase extraction (SPE) (Rujia et al. 2021) with post or pre-column derivatization which is expensive and laborious. Considering the current state of food safety and quality assurance issues there is a need for simple and sensitive HPLC methods to generate drug residues baseline data. Therefore, the objective of the study is to optimize a simple RP-HPLC method for simultaneous detection and quantification of four sulfonamides in buffalo meat matrix that could be employed for generating baseline data on residues.

MATERIALS AND METHODS

This research work was carried out at ICAR –National Research Centre on Meat, Hyderabad during the year 2020.

Materials and reagents

Buffalo meat samples were purchased from local retail markets and deep frozen until analyses. The analytical standards of sulfonamides viz., sulfadiazine, sulfadoxine, sulfamethazine, sulfamethoxazole were purchased from HPC standards GmbH. HPLC-grade methanol (MeOH), acetonitrile (ACN) and water were procured from Merck (Darmstadt, Germany) were used. All standards were stored at 4 C.

Preparation of Stock and working standard solutions

Analytical grade individual standards of sulfonamides with 99.5% purity were used for this study. Stock solution of 1mg/ml concentration of sulfonamides were prepared in methanol. From this stock solution, working standards solutions of the following concentrations viz. 10, 25, 50,100,200 and 500 ng/ml were prepared by diluting with the mobile phase. All stock and working standard solutions were stored at 4 ^{o}C in the refrigerator.

Extraction Procedure

A liquid-liquid extraction protocol was employed for this purpose. Homogenized buffalo meat sample of 2.5gm was extracted using 15ml of acetonitrile, 10ml of n-hexane and 5gm of anhydrous sodium sulphate followed by centrifugation at 3000 rpm for 10min.Thereafter 5ml of n-propanol was added, then the pooled extract was subjected to nitrogen evaporation and reconstituted with acetonitrile and water (40:60) and 0.25ml n-hexane added and vortexed. Finally, the aliquot was passed through a 0.5 µ PVDF membrane filter, 40 µL of this filtrate was then injected into the column for RP-HPLC analysis.

High Performance Liquid Chromatography conditions

Residual sulfonamides were assayed by a reverse phase UFLC system equipped with UV detector (Shimadzu Corporation, Kyoto, Japan) comprised of an LC 20AD pumps, an auto injector SIL-20AC, SPD-20AV UV-VIS detector and Lab Solutions Ver. Chromatographic workstation. The separation of four sulfonamides was achieved using a C_{18} reverse phase column (Phenomenex, 4.6 x 250 mm; 5 µm) as a stationary phase. The mobile phase consists of 5% glacial acetic acid in acetonitrile, methanol and water (85:10:5, v/v) which was operated in isocratic elution mode. The flow rate was adjusted to 1.5 ml/min and the column oven temperature was maintained at 45^{o} C during the whole process. UV detector wave length was operated at 254 nm. Sulfonamides were quantified from the peak areas and their respective concentrations in the calibration curves obtained from analysis of blank buffalo meat fortified with the external standards (Table 1).

Table 1. HPLC Conditions.

System	UHPLC
Column	C_{18} 4.6 X 250 mm 5µ
Injection volume	40 µl
Oven temperature	45 °C
Mobile phase	5% Glacial acetic acid in acetonitrile, methanol and water (85:10:5, v/v)
Flow rate	1.5 ml/min
Detector	UV λmax 254nm

Method validation

Calibration curve

A six point calibration curve was constructed for quantification purpose by fortifying blank meat samples to obtain the following final concentrations of sulfonamides i.e, 10, 25, 50,100, 250 and 500 µg/kg. Residual sulfonamides were quantified in µg/ Kg based on peak area measurements using external calibration method.

Recovery

Recovery was calculated using the four sulfonamides spiked at three levels around MRL (0.5, 1 and 1.5 times MRL) and were analyzed with six replicates at each level.
% Recovery = 100 X measured content/fortification level

Sensitivity

Limits of detection (LOD) and limits of quantification (LOQ) was evaluated using the spiked samples spiked at the permitted level 100 µg/Kg. LOD and LOQ of the four sulfonamides was calculated by signal-to-noise ratio of 3 and 10 (the ratio between intensity of signal of each compound obtained and intensity of noise in a spiked sample).

Statistical analysis

The recovery and precision data were evaluated with an in-house statistical software program making use of Snedecor and Cochran concepts (Snedecor and Cochran, 1989).

RESULTS AND DISCUSSION

The proposed procedure entails extraction of buffalo meat samples with liquid-liquid extraction as discussed in extraction method. Instrumental analysis was performed using liquid chromatography with UV detector operated at 254 nm in line with Cheong et al. (2010) and Chitescu et al. (2011). Good chromatographic separation was exhibited for sulfonamides viz., that was achieved using 5% glacial acetic acid in acetonitrile, methanol and water (85:10:5, v/v) as the mobile phase (Table.1). The whole analytical run was accomplished with in a total run time of 16 minutes. LODs and LOQs studied by spiking at permitted limit. The LOD (µg/Kg) for sulfonamides viz., sulfadiazine, sulfadoxine, sulfamethazine, sulfamethoxazole were 6.06, 7.63, 6.79 and 10.68, respectively. The LOQ (µg/Kg) for sulfonamides viz., sulfadiazine, sulfadoxine, sulfamethazine, sulfamethoxazole were 18.37, 23.12, 20.57 and 32.37, respectively. The obtained LOQ values were substantially lower than the Codex Alimentarius Commission MRL (100 µg/kg) established for the analyte of our interest (FAO/WHO CAC, MRL 2-2015).

Following that validation of the performance parameters to demonstrate that this RP-HPLC method complies with the criteria applicable for the relevant performance characteristics was carried out. The performance parameters demonstrated the complete adequacy of the method for detecting and quantifying the residues of sulfonamides, in the buffalo meat keeping in view CD 2002/657/EC and CXG 90-2017 guidelines (Table 2 and 3).

The validity of specificity was demonstrated beyond doubt by running twenty blank samples and checked for any interference at the retention times (RTs) of the four antimicrobials of our interest. Analysis of blank muscle samples demonstrated that there were no interfering compounds at the RTs of antimicrobials of our interest (Fig.1), demonstrating the selectivity of the method in compliance with EC regulation (CD 2002/657) and the RTs of sulfadiazine, sulfadoxine, sulfamethazine, and sulfamethoxazole were found to be 3.9, 7.3, 11.7 and 12.8 minutes, respectively (Fig. 2). Linearity accuracy was studied by constructing a 6-point calibration curve in the range 10 to 500 µg/ Kg that corresponds to 0.1 to 5 times the maximum permissible level. The assay was linear from 10 to 500 µg/ kg (Fig.3 and 4). The coefficients of determination (R^2) values of the calibration curves were higher than 0.99, complying the guidelines.

Table 2. Performance parameters.

Performance parameter	Sulfadiazine	Sulfadoxine	Sulfamethazine	Sulfamethoxazole
Selectivity	No co-eluting interference found			
Linearity Range (µg/ kg)	10 - 500	10 - 500	10 - 500	10 - 500
Co-relation coefficient	0.9998	0.9992	0.9934	0.9903
LOD (µg/Kg)	6.06	7.63	6.79	10.68
LOQ (µg/Kg)	18.37	23.12	20.57	32.37

Table 3. Mean Recovery spiked at three different levels in buffalo meat.

Spike levels(µg/Kg)	Mean recoveries (n=6) at each spike levels							
	SDZ%	CV%	SDX%	CV%	SMT%	CV%	SMZ%	CV%
50 (0.5 MRL)	74	3.7	79	6.0	89	5.5	82	3.5
100(1.0 MRL)	77	4.9	83	5.5	88	5.9	85	4.7
150(1.5 MRL)	72	5.5	80	4.3	85	4.6	86	3.9

(SDZ-Sulfadiazine; SMZ-Sulfamethoxazole; SDX-Sulfadoxine; SMT-Sulfamethazine)

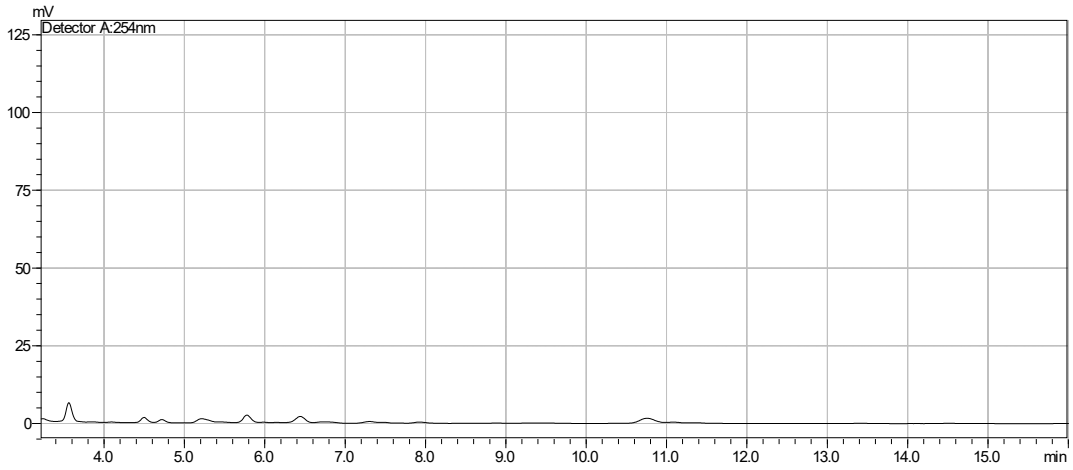

Fig. 1: LC Chromatogram of blank buffalo meat matrix

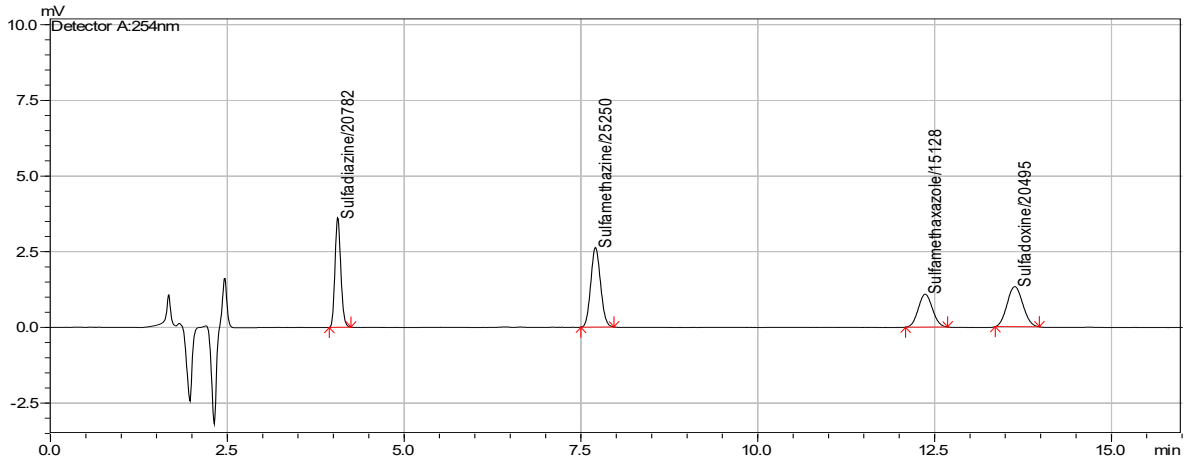

Fig. 2: LC Chromatogram of Sulfonamides spiked in matrix blank at 100 µg/Kg

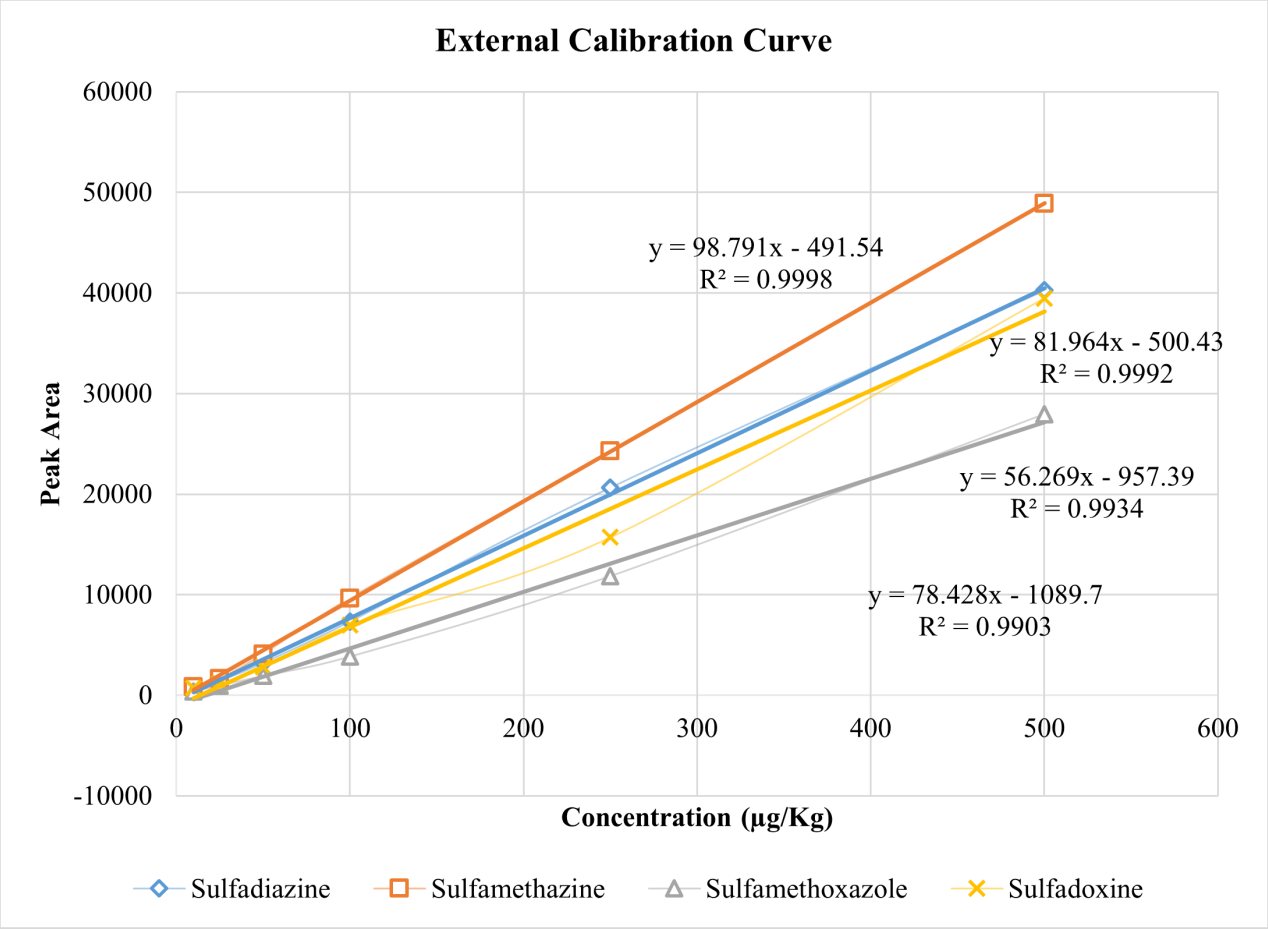

Fig. 3: External calibration curve in the concentration range of 10-500 µg/Kg

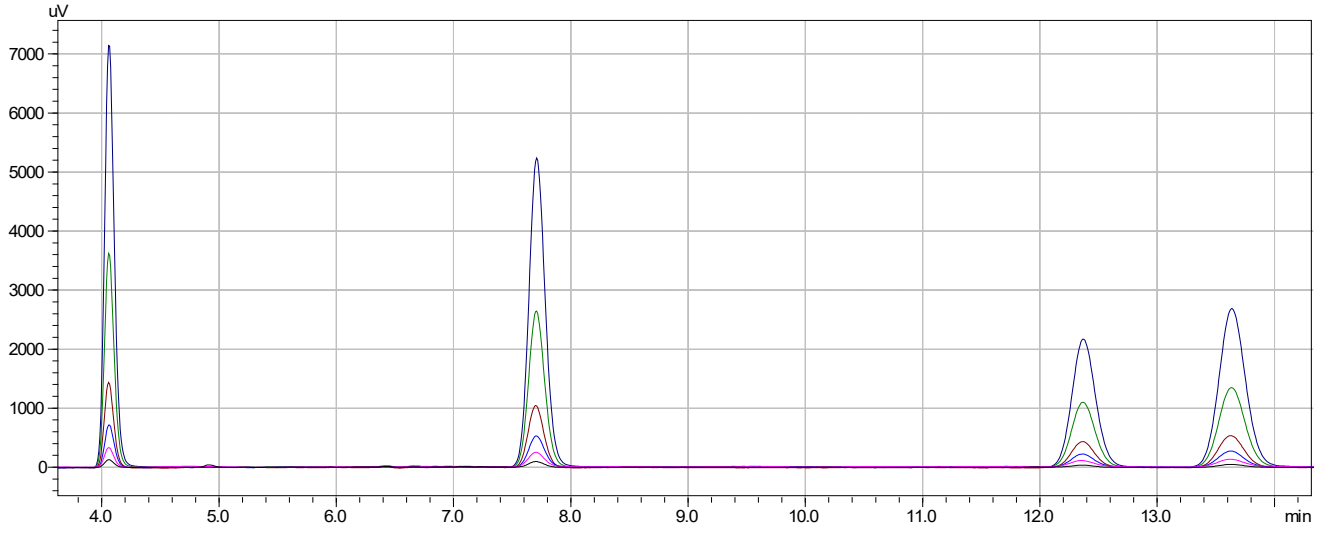

Fig. 4: Linearity overlay in the concentration range of 10-500 µg/kg

Also the recovery percentage was investigated by fortifying buffalo meat at three levels that corresponds to 0.5,1 and 1.5 times the permitted limit (100µg/Kg) and by analyzing six replicates at each level. The percentage recovery in buffalo meat matrix was quite adequate for quantitation purpose, they ranged from 72-77% (sulphadiazine), 79-83% (sulfadoxine), 85-89% (sulfamethazine) and 82-86% (sulfamethoxazole). The recovery percentage is well within the acceptance range (70-120% with RSD values ≤20%) as mentioned in Codex guidelines CXG 90-2017.The intra-day variation was studied by arriving at the coefficient of variation (CV %) of the mean yield fortified at 0.5,1 and 1.5 times MRL (Table 3.) was found not exceeding 16% which is on par with the results obtained by Cheong et al. (2010). The applicability of the optimized RP-HPLC method was also studied in real samples. Our results demonstrated that this optimized RP-HPLC method is quite adequate for the routine monitoring of four sulfonamides residues in buffalo meat at and below Codex MRL.

CONCLUSION

The proposed method established a high specificity and sensitivity with acceptable recovery percentage for analyzing four sulfonamides in buffalo meat using RP-HPLC. The performance parameters of this method satisfies the requirements for detection and quantification of residual sulfonamides. The LOQ obtained in this method is substantially lower than the established Codex MRL, therefore it may be employed for regular monitoring of sulfonamides at and below Codex MRL (100µg/Kg) in buffalo meat matrix.

COMPETING INTERESTS

The authors do not have any competing interests among themselves or others related to this research work.

ETHICS STATEMENT

Not applicable

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