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Govind V
Phd Scholar, Department of Livestock Products Technology (Meat Science) Madras, Veterinary College, Chennai, India

Narendra Babu R
Professor, Department of Livestock, Products Technology (Meat Science), Madras Veterinary College, Chennai, India

V Appa Rao
Professor and Head, Department of Livestock Products, Technology (Meat Science) Madras Veterinary College Chennai, India.

P Sriram
Professor and Head, Department of Veterinary Pharmacology and Toxicology, Madras Veterinary College, Chennai, India.

TMA Senthil Kumar
Professor, Department of Animal Biotechnology, Madras Veterinary College, Chennai, India

Correspondence
Govind V
Department of Livestock Products Technology (Meat Science), Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India

Determination of sulfadoxine residues in poultry meat by liquid chromatography and tandem mass spectrometry

Govind V, R Narendra Babu, V Appa Rao, P Sriram and TMA Senthil Kumar

Abstract
A study was conducted for determination of sulfadoxine residual level in poultry meat marketed in Chennai city. An LC-MS/MS analytical method has been developed and validated for the simultaneous identification, confirmation and quantitation of sulfadoxine residue. The method was validated in accordance with European commission 2002/657/EC. The target analyte from meat samples was extracted by liquid-liquid extraction with ethyl acetate and hexane. Evaporated and reconstituted samples were analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Relative standard deviation values (RSD%) for the inter-assay variation of analyte at the three levels of fortification (5,10 and 15ppb), ranged between 4.1 to 8.2. Out of 102 poultry meat samples analysed 16.7% samples had shown detectable levels of Sulfadoxine and concentration varied from the range of 1.03 ppb to 23.8 ppb which were within maximum residue level (MRL) prescribed by European Union regulations.

Keywords: Sulfadoxine, residue, LCMS/MS, poultry meat

1. Introduction
The widespread use of antibiotics in poultry and other animals leaving the residues of antibiotics in food products like meat, milk and eggs causing potential threat to public health. Further, abundant risk existed as possibility of transfer of drug resistant bacteria through food chain to consumers. Often, due to non-compliance withdrawal periods of drugs before marketing and processing of broilers, drug residues can enter the foods animal origin. In order to safeguard the public health, regulatory authorities all over the world have established maximum residue limits (MRLs) for antibiotic residues in different food matrices.

Several methods are being used for analysis of residues in foods of animal origin among them chromatographic methods coupled to tandem mass spectrometry have become more popular in recent time. HPLC MS/MS has become best choice for detection and quantification of veterinary drug residues in foods of animal origin because of its high sensitivity and separation and identification of compound based on the mass [1].

Sulfadoxine belongs to sulfonamide group which is the oldest antimicrobial group of drugs which have been widely used in both human and animal practice including poultry [2]. Chemical structure of sulfonamides shares a common p-amino benzyl ring moiety with an aromatic amino group at the N4-position and different compounds differ at substitution at N1-Position [3]. This group of drugs are most commonly used in broiler industry for treatment. Systemic human exposure of this group of antibiotics via the foods of animal origin is considerably serious problem as they have adverse effects like allergic reactions, suppression of enzyme activity, promotion of drug resistant bacterial forms, haemotoxicity and some compounds are carcinogenic too [2].

HPLC MS/MS based methods are reported for analysis of sulfonamide residues in different food matrices as either single group or along with other group of antibiotics in matrices such as pork [4], shrimp [5], beef [6], meat and baby food [7], milk[8], feed [9], eggs[10]. Different methods of extraction procedures were worked out by different authors like accelerated solvent extraction (ASE) [7], pressurized liquid extraction [11], QuEChERS EN kits [12], liquid- liquid extraction with ethyl acetate [8,13,14]. At present in India FSSAI [15] has given regulations only for sea foods for antibiotic residue and draft notification has been given for meat and meat products.

Export inspection council of India residue monitoring plan 2017-18 [16] and European Union specified maximum residue level of all sulfonamide group of antibiotics as 100 µg/kg and whereas 20 µg/kg in edible tissues as prescribed by Japan [17]. The purpose of this study is to detect or quantify the Sulfonamide group antibiotic sulfadoxine residue in chicken meat samples marketed in retail outlets of Chennai by LCMS/MS analytical method.

2. Materials and Methods

2.1 Standard, Reagents and Chemicals

Reference standard of Sulfadoxine with purity greater than 99%, acetonitrile, Formic acid and tricholroacetic acid were purchased from Sigma Aldrich (Liquid chromatographic grade). Ethyl acetate and Hexane (HPLC grade) were purchased from Thermo Fischer Scientific. Deionized water used for aqueous mobile phase was prepared in the laboratory with Millipore water system. Both the mobile phases were degassed and filtered under vacuum using mobile phase filtration unit. Stock solution of 1000ppm and 10ppm of sulfadoxine were prepared in methanol. Spiking (5ppb, 10ppb, and 15ppb) and working calibration standards at different concentration levels (0 ppb to 200 ppb) were prepared from aliquots of stock solutions with methanol and stored at 4ºC.

2.2 LCMS/MS Instrumentation and Chromatographic conditions

2.2.1 LCMS/MS System: Chromatographic separation, identification and quantification was carried out by Agilent 1260 series quaternary liquid chromatographic system (Agilent, Germany) with a Triple quadruple tandem mass spectrometer (Agilent technologies, G6460) with electron spray ionization system (ESI). It is also equipped with mass hunter software for acquisition, qualitative and quantitative analysis softwares for quantification of residues. The instrument is provided with 100 vial capacity automatic sample management system.

2.2.2 Chromatographic Conditions

The separation of sulfadoxine was accomplished with Poroshell 120EC C18 column (4.6 X 50 mm, 2.7 micron)(Agilent).The flow rate was 0.4ml/minute and injection volume 5 µl. The mobile phases used were (A) 0.1% Tricholroacetic acid in water (B) 0.1% Formic acid in Acetonitrile. The gradient elution programme was as follows

Time (Minutes)	Mobile phase (A) 0.1% Trichloroacetic acid Aqueous	Mobile phase (B) 0.1% Formic acid in Acetonitrile.
0.00	80	20
2.00	65	35
7.00	50	50
7.01	5	95
9.00	5	95

Total chromatography run time was 13 minutes including post run time of 4 minutes. To avoid the possibility of cross contamination and carryover, injection syringe was each time cleaned with 50:50 (Methanol: Water) which is pre-programmed in acquisition method developed in the Mass hunter software.

2.2.3 Mass Spectrometric Conditions

Electrospray ionization spray (Agilent Jet spray- AJS ESI) was performed in the positive ion mode for analyte. MRM transitions and LCMS/MS parameters utilized for compound sulfadoxine was given in table 1. As part of LCMS/MS method the tandem mass spectrometer (MS/MS) was operated with conditions as mentioned in list of actuals table 2. Data acquisition was performed using Agilent mass hunter software. Qualitative and quantitative analysis was performed using agilent mass hunter qualitative and quantitative software.

Table 1: LCMS/MS parameters for selected reaction monitoring of sulfadoxine

Compound name	Molecular weight	Precursor ion	Product ion	Collision energy (CE)	Fragmentor	Polarity
Sulfadoxine	251.1	251.1	156	8	96	Positive
	251.1	251.1	92.1	28	96	Positive

Table 2: Important Tandem mass spectrometer actuals used during analysis

Parameter	Condition
Ionization mode	Electron spray ionization
Polarity	Positive
Nebulizer pressure	35 psi
Collision gas flow	10l/min
Sheath gas flow	11l/min
Collision gas temperature	350º C

2.3 Sample collection and preparation for LCMS/MS analysis

250 grams of each 102 poultry meat samples (Deboned) were collected in different retail outlets of corporation zones of Chennai over a period of one year. Deboned meat samples were thinly sliced and homogenized using meat homogenizer (York scientific instruments). Sample extraction procedure was done as per the method described by earlier workers [13, 14] with slight modifications. The representative sample of 2 ±0.1 gram homogenized poultry meat weighed and placed into 50ml polypropylene centrifuge tube. Samples were homogenized for 2 minutes using meat homogenizer with 10ml ethyl acetate. Resultant mixture was vortexed for 30 seconds. Then samples were centrifuged at 10000rpm for 10 minutes. The supernatant was collected and transferred to 10ml test tubes, dried and concentrated under nitrogen gas using Turbovap® (Kemi scientific) at 50ºC. To the concentrate, 1ml of 50:50 mobile phase: water was added. To this 1ml of n-hexane was added, vortexed and supernatant hexane layer was removed. Resultant mixture was filtered through 0.22µ PVDF 13mm diameter syringe filter into LC auto sampler vial.

2.4 Analytical method validation studies

As part of method validation, linearity calibration curve, precision, selectivity, limit of quantification (LOQ) and limit of detection (LOD), recoveries (%) and specificity were performed. LOD and LOQ were performed by analysing the signal to noise ratio of peaks obtained from chicken meat sample fortified at known lower concentration levels and analysing the method response for the same. The selectivity of the method was analysed by injecting the blank sample (n=15) and absence of the signal above the signal to noise ratio of 3 at the retention time of sulfadoxine in comparison with the different concentrations of standard. Specificity is confirmed based on the presence of transition ions (Quantifier

and qualifier) at the correct retention time of sulfadoxine. Linearity of calibration curve had been drawn using different level of concentrations (0 ppb to 200 ppb). Accuracy of the method was determined by studying the recovery percentages previously analysed blank meat samples spiked at three different levels. Recovery values were measured by subtracting recovered concentration from spiked concentration and expressed as percentage. For intra-day variation measurement of concentration set of samples at three different levels was measured with six replicates. To determine inter-day variation 3 sets with three different levels with six repetitions measured over next 2 days (Table 3).

3. Results and Discussion

3.1 Analytical validation study

Selectivity of the method measured in blank poultry tissue matrices in which no signal above the signal to noise ratio of 3 was found at sulfadoxine retention time in turn indicates no interference in the method. Specificity of the method based on the ratios of quantifier and qualifier ions of three spiked levels are within the prescribed range specified by European commission decision (53.4-80.1) as observed with working standards. The least squares linear regression analysis was carried out by plotting the abundance of ions versus different calibration concentrations and correlation coefficient (R^2) was higher than > 0.999 (Figure 1). Possible detection levels of sulfonamide group of antibiotic residues in different studies reported earlier were at very low levels, hence in this study the accuracy studies were done at 10 times below the level of Prescribed MRL. Recovery and reproducibility were evaluated by fortifying the sulfadoxine standard at three different levels 5, 10, 15 ppb levels and intra-day precision and inter day precision RSD values were measured. The results for accuracy and precision were shown in table 3, different spiked levels recovery levels were within the acceptable range fixed by Codex alimentarius commission (CAC) [18] and European union commission decision [19] where prescribed acceptable recovery range for 1 to 10 ppb spiking concentrations was 70 to 110% and for the spiking concentrations of level more than 10 ppb should be 80% to 110% and RSD% values less than 15. The limit of quantification and limit of detection of the method described were found to be 1.13 and 0.28 ppb after analysis as determined by signal to noise ratio method after fortifying the matrix at low concentration levels.

3.2 Application to actual samples

The LCMS/MS method was applied to the determination of sulfadoxine residue in 102 poultry meat samples collected from different retail outlets distributed across different greater corporation regions of Chennai city. Out of 102 poultry muscle tissue analysed 16.6% samples (17 samples) had shown detectable levels of Sulfadoxine and concentration varies from the range of 1.03 ppb to 23.8 micrograms per kg. Positive samples of sulfadoxine detected range, fall within Maximum residue limit (100 microgram per kg) prescribed by CAC [18], Export inspection agency of India [16] and European Union commission[19]. The chromatogram of the extracted sulfadoxine from marketed poultry meat sample was shown in Figure 2. Solvent ethyl acetate used in the sample preparation had shown good recovery as observed in this study and results suggests, it can be extended for the determination of other compounds in sulfonamide group. The meat sample is a complex matrix which provides interferences like fat and to remove fat interference, effective cleaning with hexane was performed in this study which might helped in good recovery.

The protonated molecule that is the precursor ion chosen for identification and fragment ion m/z 156 representing sulfanyl ring is used for quantification, and this particular fragment ion said to be quantifier ion for majority of the sulfonamides [20]. The advantage of using MS/MS for quantification of antibiotic residues is that complete chromatographic separation of target analyte from target matrices is not necessary for selective detection as target analyte was identified based on m/z ratio. Short HPLC C_{18} column (4.6 X 50mm) with particle size less than 3 microns have widespread use in analytical methods having advantages like screening of antibiotics for single or multiresidue, increased resolution and good separation in short time that is speeding up of analysis etc. [2].

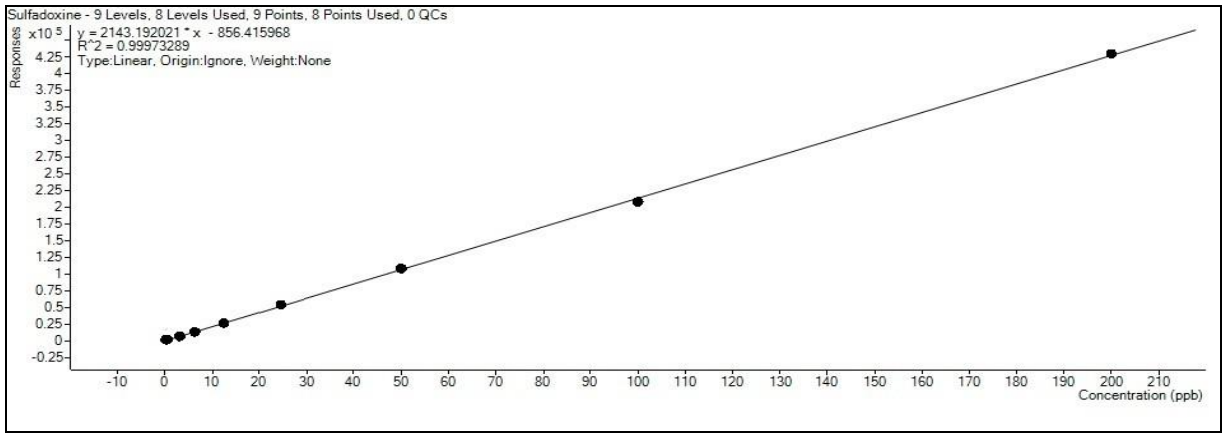

Fig 1: Linear calibration curve of sulfadoxine (µg/kg)

Table 3: Matrix matched calibration-Recovery and method intermediate precision as RSD (%) –Method intermediate precision (Intraday and Inter day) measured at 3 spiking levels (5ppb, 10ppb, 15ppb).

Compound	Accuracy		Precision		
	Concentration (µg/kg)/ppb	Recovery (%) (RSD %)	Concentration (µg/kg) /ppb	Intraday assay (RSD %)	Inter-day assay (RSD %)
Sulfadoxine	5	87.6 (6.8)	5	4.1	6.5
	10	88.5 (5.6)	10	6.2	8.2
	15	89.2 (4.8)	15	5.2	7.4

4. Conclusion

The results of analysis of marketed poultry meat samples in the present study showed that the presence of sulfadoxine residues were within the prescribed limits. The residual level of drugs in tissues or eggs will normally be based on time of usage of drug during rearing period in compliance to withdrawal period. Although detected levels were not thought to be able to cause adverse effects, from a food safety perspective there is every chance transmission of drug resistant bacteria which is a concern. Nowadays as there is increasing awareness among the consumers regarding the antibiotic residues being allergic, mutagenic or carcinogenic and also the evolution of antibiotic resistant microbes, it has become ever more necessary to restrict their levels in the meat being consumed or exported. Regular monitoring and surveillance of the other commonly used antibiotic drug residues in the meat with sensitive instruments like LCMS/MS might help in their traceability in food chain and further development of risk based monitoring programmes to minimize the antibiotic residues in the meat.

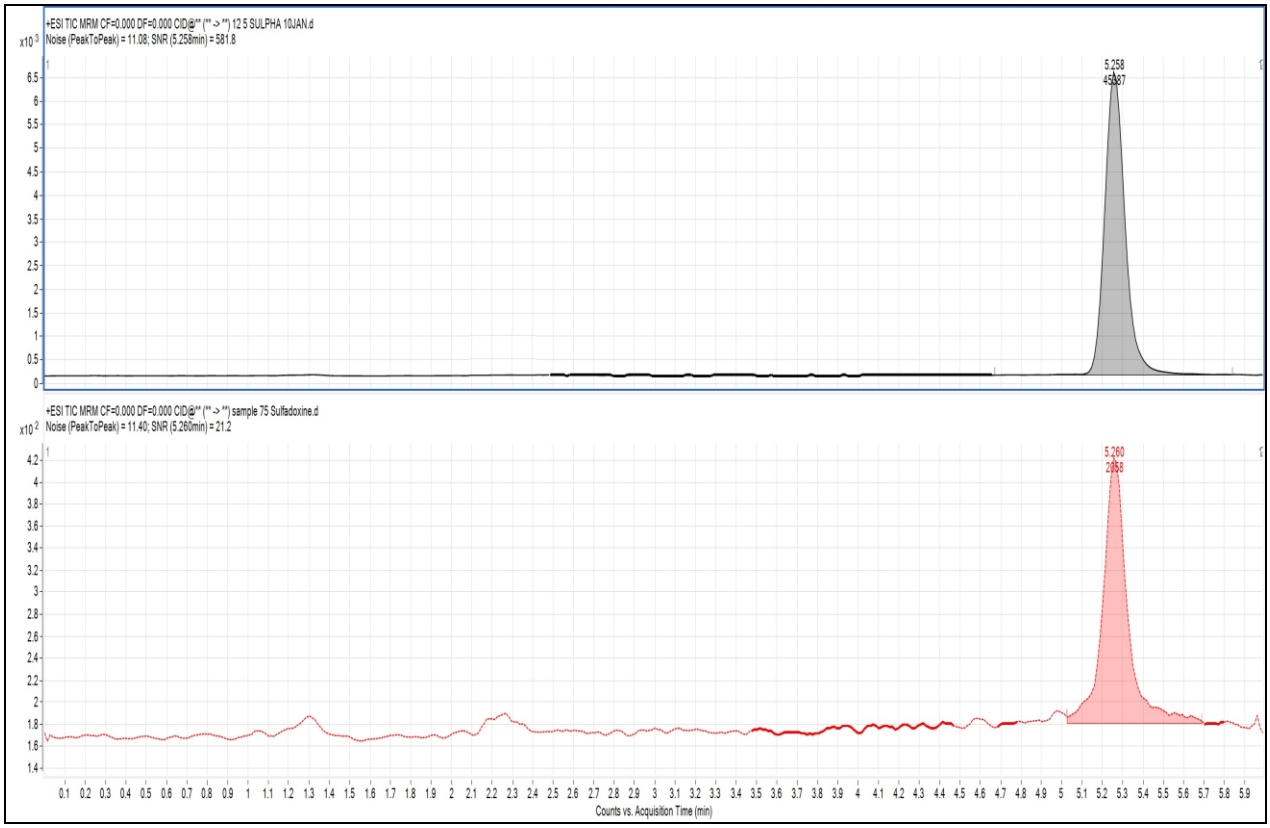

Fig 2: LCMS/MS chromatogram for a sulfadoxine positive sample in comparison with Sulfadoxine standard 12.5 ppb level

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