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LC/ESI/MS/MS method development and validation for the determination of sulfonamide antibiotic residues in shrimp sample

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Abstract

A liquid chromatography–tandem mass spectrometry (LC/ESI/MS/MS) method was developed and validated for the determination of ten sulfonamide antibiotic residues in raw shrimp meat: sulfadimethoxine, sulfachloropyridazine, sulfamethoxypyridazine, sulfamethazine, sulfamethizole, sulfamerazine, sulfathiazole, sulfamethoxazole, sulfadiazine, sulfapyridine. The procedure involves the extraction of homogenized sample into acetonitrile and diluted with mobile phase. The analysis was carried out using ODS-EP 5 micron, 100×2.1 mm column and mobile phase consists of 0.1% formic acid (A) and acetonitrile (B) in gradient mode at a flow rate of 0.5 mL/min. The final extracts were analyzed by the sensitive and selective LC/ESI/MS/MS operating in positive ion multiple reactions monitoring (MRM) mode.

Key words: sulfonamides, shrimp, liquid chromatography-mass spectrometry, method development, method validation.

Introduction

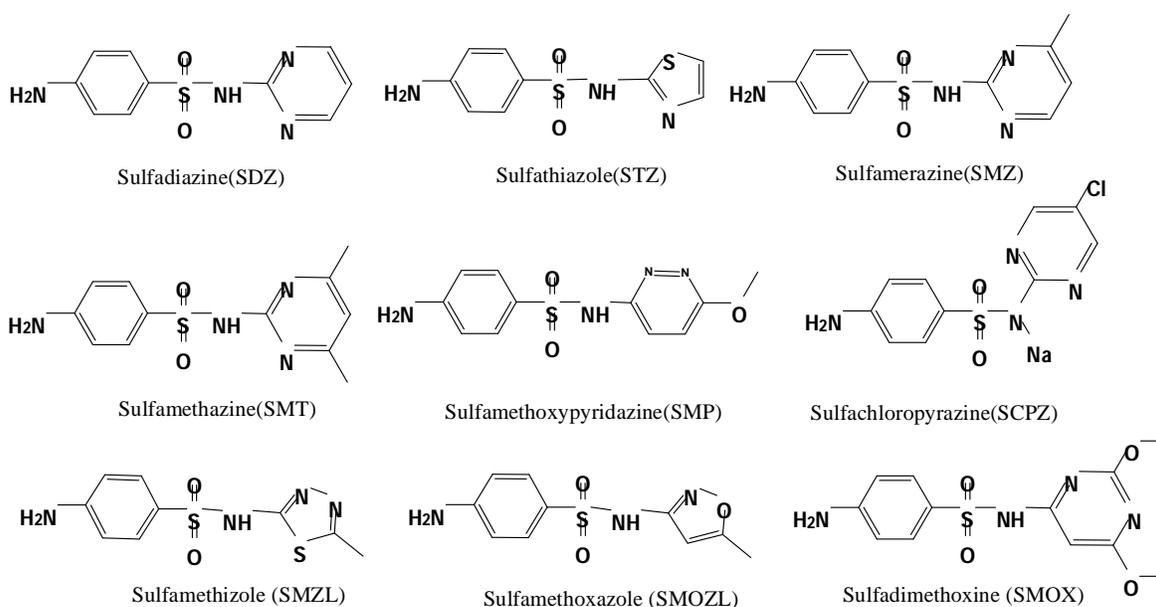
Sulfonamides (SAs) are a very important class of synthetic antibiotics, widely used worldwide to prevent and control a great variety of bacterial diseases in intensive aquaculture production¹⁻⁴. However residues of SAs may remain in animal tissues

if adequate withdrawal time is not observed or if the SAs have been improperly administered. As a consequence of their extensive usage, significant attention has been paid to the potential human health risk due to their carcinogenic potency and possible antibiotic resistance⁵⁻⁸. Therefore, to ensure the safety of the food to the consumers, regulatory agencies set

the maximum residue limits; the maximum residue limit (MRL) in the European Union countries and United States for SAs in animal muscle tissue is 100 ng/g⁹.

For the determination of sulfonamides residues in animal tissues many analytical methods like TLC, HPLC/MS, GC, and GC/MS have been described¹⁰⁻¹³. These methods utilize various extraction and clean-up techniques: liquid extraction, solid-phase extraction, and matrix solid-phase dispersion, in order to achieve the required sensitivity and specificity to detect sulfonamides at residual levels. For quantitative analysis, high performance

liquid chromatography with fluorescence or mass spectrometric detection are the most widely used techniques. LC-MS/MS is one of the most promising techniques for the analysis of antimicrobials in animal tissues, because it allows drug quantification and confirmation at trace levels¹⁶⁻²³. In this paper we describe the method development and validation of simple and sensitive LC/MS/MS method and simple sample clean-up process for the determination of nine SAs in shrimp sample the structures of studied sulfonamides are given in the **scheme.1**.



2. Materials and Methods

2.1. Chemicals and materials :

Sulfadimethoxine, Sulfachloropyridazine, Sulfamethoxypyridazine, Sulfamethazine, Sulfamethizole, Sulfamerazine, Sulfathiazole, Sulfamethoxazole, Sulfadiazine, Sulfapyridine, methanol and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, USA). Acetonitrile, ethylacetate were purchased from Merck. ODS-EP 5 micron, 100 x 2.1mm HPLC column was obtained from GLSciences Inc. All the other inorganic chemicals and

organic solvents were of reagent grade or higher. Shrimp samples were collected from local market and stored at -20°C before the analysis procedure.

2.2. Preparation of stock solution (standard solution)

The standard stock solutions were prepared by accurately weighed 10 mg of each standard is transferred into 10mL volumetric flask and made up to the mark with HPLC grade methanol and stored at 2-8°C. After proper dilution these stock solutions were used to prepare calibration curve and spike into the blank shrimp samples.

2.3. Equipment :

The Agilent LC/ESI/M/MS system was equipped with 1290 model HPLC and 6460 model triple quadrupole mass spectrometer. The instrument is under the control of Mass Hunter software, Version B.06.00. The typical source parameters were: Gas Temperature:

350⁰C, Gas flow: 10.5 L/min, Nebulizer: 53psig, Sheath Gas Temperature: 350⁰C, Sheath Gas flow: 11 L/min, V Cap: 4000V, Nozzle Voltage: 0V. The analysis is carried out in Dynamic MRM (Multiple reaction monitoring) mode with positive ion mode. The dynamic MRM conditions were given in the table 1.

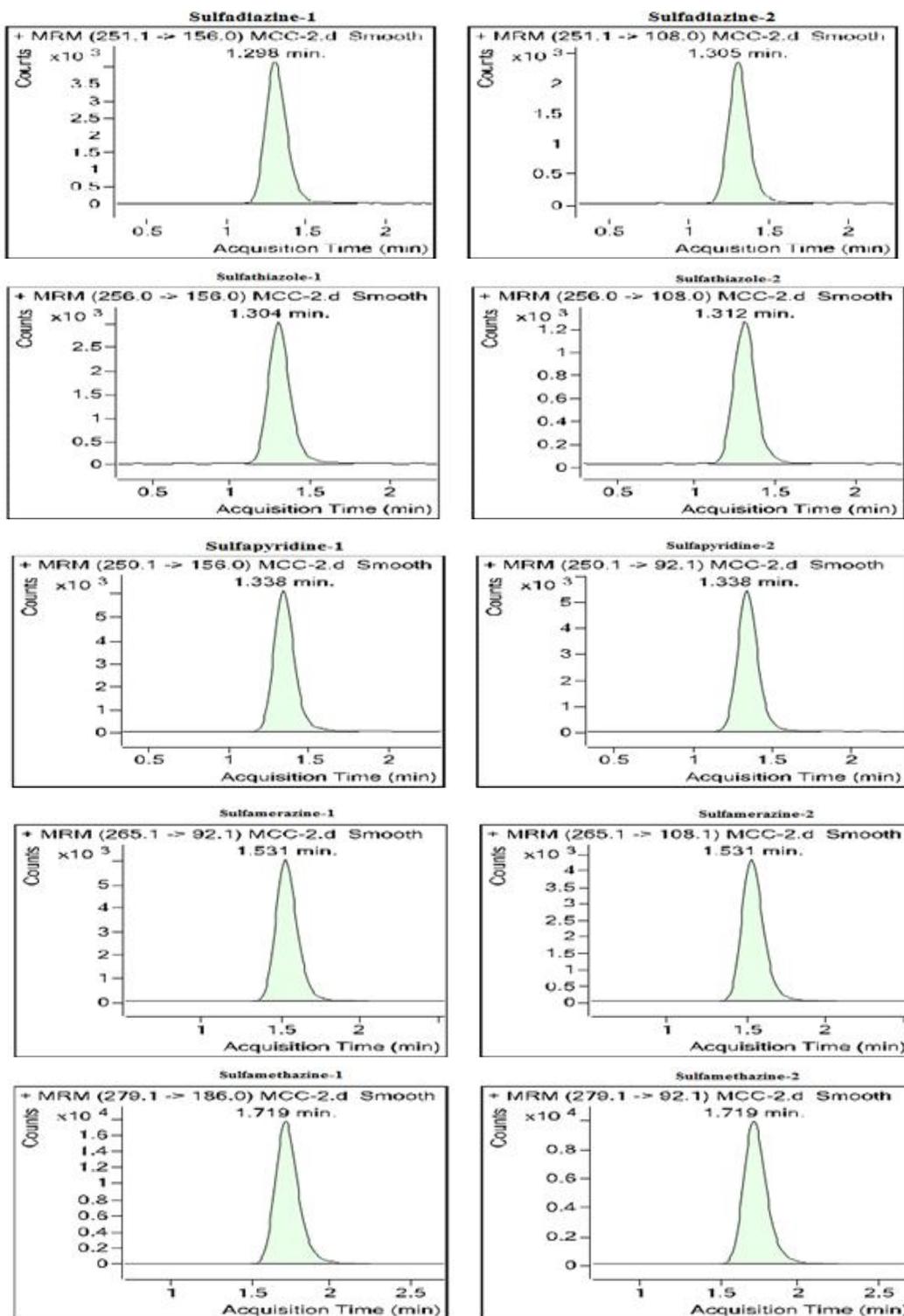
Table 1: MRM transitions (Dynamic MRM) of sulfonamides

Analyte(Transition)	Parention (Q ₁)	Production (Q ₃)	Fragmentor (Volts)	CE (Volts)	CAV
Sulfadimethoxine-1	311.1	156	125	15	7
Sulfadimethoxine-2	311.1	108	125	28	7
Sulfachloropyridizine-1	285	156	100	8	7
Sulfachloropyridizine-2	285	108	100	22	7
Sulfamethoxypyridizine-1	281.1	156	125	10	7
Sulfamethoxypyridizine-2	281.1	108	125	24	7
Sulfamethazine-1	279.1	186	120	12	7
Sulfamethazine-2	279.1	92.1	120	29	7
Sulfamethizole-1	271	156	100	10	7
Sulfamethizole-2	271	92.1	100	29	7
Sulfamerazine-2	265.1	108.1	120	23	7
Sulfamerazine-1	265.1	92.1	120	26	7
Sulfathiazole-1	256	156	100	9	7
Sulfathiazole-2	256	108	100	21	7
Sulfamethoxazole-1	254.1	156	100	10	7
Sulfamethoxazole-2	254.1	92	113	24	7
Sulfadiazine-1	251.1	156	100	8	7
Sulfadiazine-2	251.1	108	100	22	7
Sulfapyridine-1	250.1	156	113	10	7
Sulfapyridine-2	250.1	92.1	113	25	7

2.4. Chromatographic conditions :

The samples were separated by Inertsil ODS-EP 5 micron, 100 x 2.1mm HPLC column, using the mobile phase consists of 0.1% formic acid (A) and

acetonitrile (B) in gradient mode with a flow rate of 0.5mL/min. The gradient programme was given in the table 2. The resultant MRM chromatograms are given in the figure 1.



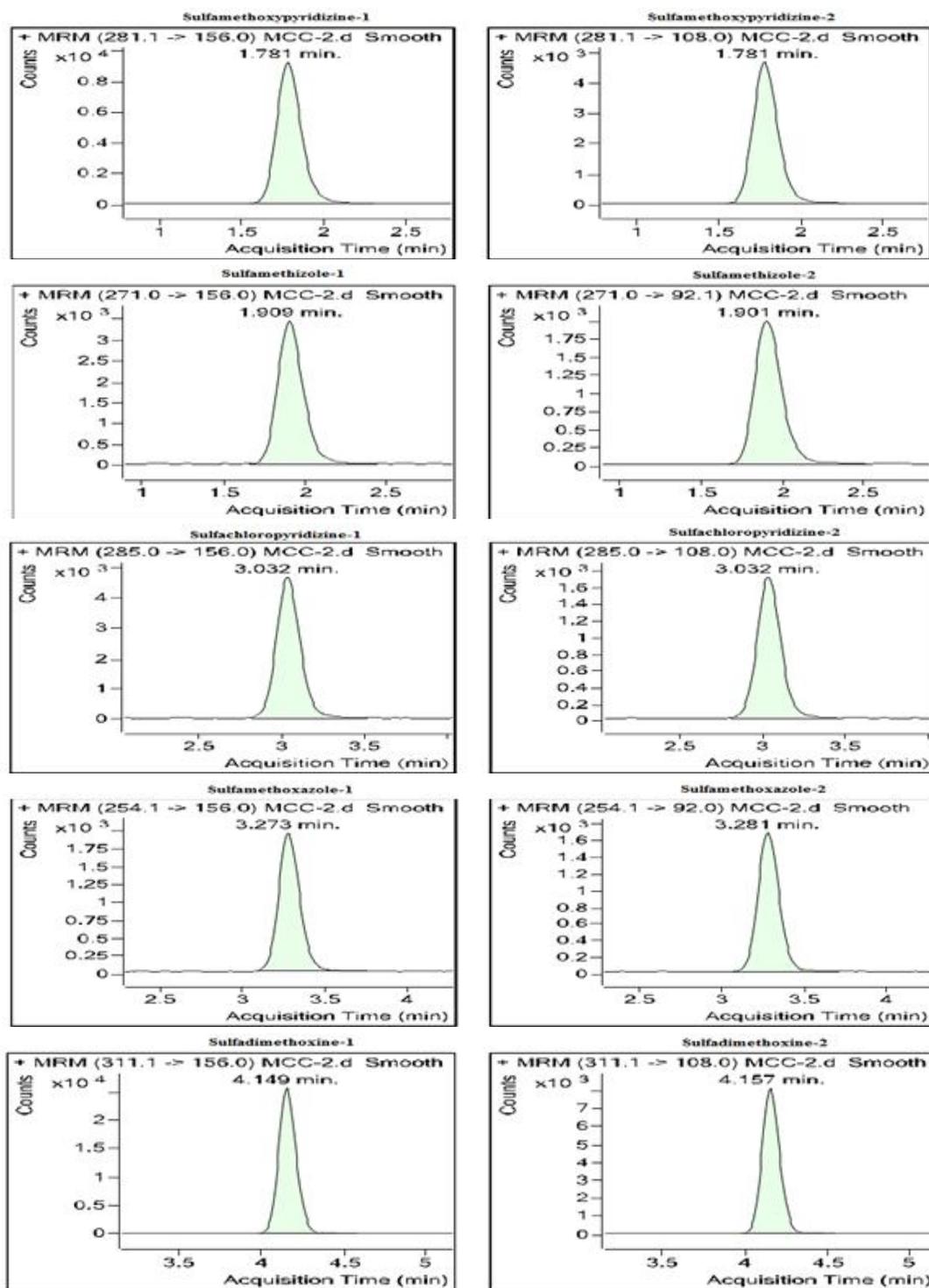


Figure 1: MRM Chromatograms of studied sulfonamides

Table 2: Gradient Program

S.No.	Time (min)	% Composition of A	% Composition of B
1	0	80	20
2	1.5	50	50
3	5.0	0	100
4	7.0	0	100
6	8.5	80	20
7	10.0	80	20

3. Results and Discussion

3.1. Sample preparation :

Weighed 5 g of homogenized sample and taken into 50mL centrifuge tube, to this 3mL of water is added, vortexed for 2 min, then extracted the sample with 7mL acetonitrile by vortexing for 10 min followed by centrifugation at 4000rpm for 10min at 4°C. Filtered the upper layer through 0.2micron filter into 2mL vial and injected into LC/MS/MS.

3.2. Method validation :

3.2.1. System suitability :

System suitability was performed by injecting six replicates of known concentration of standard solution. The results were within the acceptance criteria i.e. % RSD of area was less than 20%. The results have been tabulated in table 3.

Table 3: System suitability

S. No.	Analyte Name	Rep-1	Rep-2	Rep-3	Rep-4	Rep-5	Rep-6	Mean	SD	%RSD
1	Sulfadiazine	873729	1272255	1281316	1253816	1235152	1209565	1187638.83	155943.81	13.13
2	Sulfathiazole	618909	923137	935652	920012	884254	849866	855305.00	119995.43	14.03
3	Sulfapyridine	1793706	2453254	2479207	2456876	2416549	2360321	2326652.17	264388.31	11.36
4	Sulfamerazine	1160487	1740563	1770406	1733782	1692228	1639039	1622750.83	230993.47	14.23
5	Sulfamethazine	3357679	4537925	4590634	4517726	4397774	4248598	4275056.00	465815.12	10.90
6	Sulfamethoxypyridazine	1617127	2234180	2267609	2195688	2134727	2090379	2089951.67	240463.01	11.51
7	Sulfamethizole	708925	1050448	1081812	1055803	1031852	990123	986493.83	139351.67	14.13
8	Sulfachloropyridazine	646281	1066036	1095715	1094916	1041377	986751	988512.67	172478.48	17.45
9	Sulfadimethoxazole	563795	891781	930919	917126	896165	857690	842912.67	138997.63	16.49
10	Sulfadimethoxine	2410911	4085787	4191986	4143308	3893969	3436776	3693789.50	686415.86	18.58

3.2.2. Specificity :

Specificity was performed by injecting 20 representative blank samples and sample spiked at MRL, observed that there was no interferences at the retention of analyte. The results have been tabulated in table 4.

3.2.3. Calibration Curve (Linearity) :

Three sets Linearity was established using matrix-matched calibration curves. Calibration curves were prepared at levels of matrix blank, 50ppb, 100ppb, 150ppb, 200ppb, 250ppb and 300ppb in Shrimp and analyzed with each batch. The correlation coefficient for all the analytes were >0.99. The results have been tabulated in Table-5.

Table 4. Specificity

S.No.	Analyte Name	Average area of analyte in blank samples	Average of LOQ area
1	Sulfadiazine	57	39903
2	Sulfathiazole	76	30366
3	Sulfapyridine	84	55864
4	Sulfamerazine	74	59493
5	Sulfamethazine	273	186534
6	Sulfamethoxy pyridazine	115	100888
7	Sulfamethizole	47	38871
8	Sulfachloropyridazine	52	47501
9	Sulfadimethoxazole	24	17622
10	Sulfadimethoxine	261	196647

Table 5: Linearity Results

S.No.	Compound	Slope (m)	Coefficient of determination (R^2)
1	Sulfathiazole	535.691	0.9982
2	Sulfadiazine	701.859	0.9974
3	Sufapyridine	1038.709	0.9985
4	Sulfamerazine	1066.684	0.9983
5	Sulfamethazine	3282.815	0.9976
6	Sulfamethoxy pyridazine	1757.475	0.9964
7	Sulfamethizole	722.452	0.9991
8	Sulfamethoxazole	326.049	0.9982
9	Sulfadimethoxine	3470.274	0.9970
10	Sulfachloropyridazine	938.557	0.9987

3.2.4. Limit of Detection and Limit of Quantification:

Calculated the Limit of Detection (LOD) and Limit of Quantification (LOQ) by using standard

deviation (STEYX) of the response and the slope of the linearity and the obtained LOD and LOQ values are given in the table 6.

Table 6: Limit of Detection and Limit of Quantification

S.No.	Compound	SD(STEYX)	Slope	LOD	LOQ
1	Sulfathiazole	550.5	1689.4	10.1	30.7
2	Sulfadiazine	728.0	1579.0	7.2	21.7
3	Sufapyridine	1061.4	3754.4	11.7	35.4
4	Sulfamerazine	1096.1	2923.2	8.8	26.7
5	Sulfamethazine	3394.9	9684.8	9.4	28.5
6	Sulfamethoxy pyridazine	1825.9	7612.1	13.8	41.7
7	Sulfamethizole	735.7	1827.0	8.2	24.8
8	Sulfamethoxazole	328.9	1760.4	17.7	53.5
9	Sulfadimethoxine	3601.8	11273.5	10.3	31.3
10	Sulfachloropyridazine	942.7	4402.0	15.4	46.7

3.2.5. Recovery/Accuracy (Trueness):

Recovery was performed by injecting seven spiked samples at 50ppb, 100ppb and 150ppb and calculated the mean, the standard deviation and the coefficient of variance for these concentrations,

calculate the recovery as accuracy (trueness) by dividing the detected mean concentration by the fortified value and multiply by 100. The results have been tabulated in table 7.

Table 7 Recovery/Accuracy
Table 7.1 Recovery/Accuracy at 50 ppb level

S. No.	Compound	Mean (Concentration in ppb)			%Recovery/Accuracy (Concentration in ppb)		
		Batch -1	Batch -2	Batch -3	Batch -1	Batch -2	Batch -3
1	Sulfathiazole	49.5608	50.1048	44.7392	99.12	100.21	89.48
2	Sulfadiazine	49.5834	50.3630	43.6973	99.17	100.73	87.39
3	Sufapyridine	50.2312	50.1538	45.8167	98.73	97.17	88.37
4	Sulfamerazine	48.8099	48.9302	43.8541	97.62	97.86	87.71
5	Sulfamethazine	48.7339	48.7735	44.8896	97.47	97.55	89.78
6	Sulfamethoxyridazine	48.6084	49.5731	44.5162	97.22	99.15	89.03
7	Sulfamethizole	48.7611	47.6648	44.1774	97.52	95.33	88.35
8	Sulfamethoxazole	50.5765	50.9328	44.5278	101.15	101.87	89.06
9	Sulfadimethoxine	47.7134	49.2290	44.6724	95.43	98.46	89.34
10	Sulfachloropyridazine	49.5735	50.0315	46.6694	99.15	100.06	93.34

Table 7.2 Recovery/Accuracy at 100 ppb level

S. No.	Compound	Mean (Concentration in ppb)			%Recovery/Accuracy (Concentration in ppb)		
		Batch -1	Batch -2	Batch -3	Batch -1	Batch -2	Batch -3
1	Sulfathiazole	89.28	100.32	99.13	89.28	100.32	99.13
2	Sulfadiazine	88.75	101.69	98.34	88.75	101.69	98.34
3	Sufapyridine	87.21	97.62	97.82	87.21	97.62	97.82
4	Sulfamerazine	87.77	98.66	97.78	87.77	98.66	97.78
5	Sulfamethazine	88.87	100.06	101.27	88.87	100.06	101.27
6	Sulfamethoxyridazine	89.74	100.95	101.45	89.74	100.95	101.45
7	Sulfamethizole	88.29	96.51	97.57	88.29	96.51	97.57
8	Sulfamethoxazole	88.54	99.45	96.80	88.54	99.45	96.80
9	Sulfadimethoxine	88.50	95.99	96.36	88.50	95.99	96.36
10	Sulfachloropyridazine	87.14	96.91	97.54	87.14	96.91	97.54

Table 7.3 Recovery/Accuracy at 150 ppb level

S. No.	Compound	Mean (Concentration in ppb)			%Recovery/Accuracy (Concentration in ppb)		
		Batch -1	Batch -2	Batch -3	Batch -1	Batch -2	Batch -3
1	Sulfathiazole	156.86	155.46	150.19	104.57	103.64	100.13
2	Sulfadiazine	159.52	156.02	149.91	106.35	104.02	99.94
3	Sufapyridine	157.02	151.00	149.63	104.68	100.66	99.75
4	Sulfamerazine	157.25	152.81	148.77	104.83	101.87	99.18
5	Sulfamethazine	158.47	154.47	152.03	105.64	102.98	101.35
6	Sulfamethoxyridazine	158.91	156.30	153.65	105.94	104.20	102.43
7	Sulfamethizole	154.92	149.29	147.36	103.28	99.52	98.24
8	Sulfamethoxazole	154.86	151.84	138.91	103.24	101.23	92.60
9	Sulfadimethoxine	159.03	149.43	147.44	106.02	99.62	98.29
10	Sulfachloropyridazine	152.15	149.63	146.12	101.43	99.75	97.41

3.2.6. Decision Limit (CC α) :

Decision limit was performed by injecting seven spiked samples at 50ppb, 100ppb and 150ppb in three batches and calculated the y-intercept and slope. The decision limit (at $\alpha = 5\%$) was calculated at

permitted limit for all the compounds, the concentration at permitted limit (MRL) plus 1.64 times the standard deviation of the within-laboratory reproducibility. The results are tabulated in Table-8.

Table 8: Decision limit (CC α) and Detection capability (CC β)

S.No.	Compound	Decision limit (CC α)	Detection capability (CC β)
1	Sulfathiazole	105.56	114.33
2	Sulfadiazine	105.29	114.98
3	Sufapyridine	105.56	114.52
4	Sulfamerazine	105.17	115.07
5	Sulfamethazine	105.79	114.37
6	Sulfamethoxypyridazine	105.79	116.16
7	Sulfamethizole	105.71	115.30
8	Sulfamethoxazole	106.60	117.64
9	Sulfadimethoxine	104.72	117.00
10	Sulfachloropyridazine	105.47	117.98

3.2.7. Detection Capability (CC β) :

Detection capability ($\beta = 5\%$) was calculated, the concentration at decision limit plus 1.64 times the standard deviation of the 20 samples fortified at decision limit. The results are tabulated in Table-8 and the recoveries of the 20 samples are meeting the criteria.

3.2.8. Ruggedness :

Ruggedness was performed by injecting seven spiked samples with two different analysts and two different lots of extraction solvents at permitted level. Calculated the Mean, standard deviation and % RSD. The results were within the acceptance criteria i.e. % RSD of area ≤ 20 and the results are tabulated in table-9.

Table 9: Ruggedness

S.No.	Compound	Mean	SD	%RSD
1	Sulfathiazole	102.9811	4.6379	4.50
2	Sulfadiazine	101.7489	4.0404	3.97
3	Sufapyridine	101.3539	4.2323	4.18
4	Sulfamerazine	101.4549	4.5273	4.46
5	Sulfamethazine	103.4838	3.1053	3.00
6	Sulfamethoxypyridazine	104.6107	4.1270	3.95
7	Sulfamethizole	102.2208	5.3023	5.19
8	Sulfamethoxazole	99.7507	4.3965	4.41
9	Sulfadimethoxine	97.9155	3.0410	3.11
10	Sulfachloropyridazine	100.2537	3.5235	3.51

Conclusions

A sensitive and selective positive ion LC/M/MS method was developed and validated for the

determination of sulfonamide antibiotic residues in raw shrimp meat. Based on the results obtained, acceptance criteria for all validation parameters such

as System Suitability, Specificity, Calibration curve (Linearity), Repeatability, within laboratory Reproducibility, Recovery, Decision Limit ($CC\alpha$) and Detection Capability ($CC\beta$) have been met for the method used to estimate the sulfonamides in shrimp samples as per protocol and as well as EU guideline council Directive 2002/657/EC. Henceforth considered the method is validated and can be used for further intended purpose. This method can be used for the determination of sulfonamide antibiotic residues in shrimp meat.

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