APPLICATION NOTE

QSight™ LC-MS/MS

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Determination of Sulfonamides in milk by Ultra-Performance Liquid Chromatography coupled with Tandem Mass Spectrometry (QSight™)

Introduction

Sulfonamides are commonly known as "sulfa drug" which are derivatives of sulfanilamide (p-amino benzene sulfonamide), commonly used as antibacterial veterinary drug for treatment. Due to the excellent antibacterial property, low toxicity and low cost, it is opular against common bacterial diseases[2]. However, uncontrolled use of veterinary drugs and noncompliance within the withdrawal period, pave the way for drug residues to remain in animal tissues and pass into their milk[3–4].

Use of various antibiotics in dairy cattle for different treatments is a common practice but eventually it leads to accumulation of these residues in milk. Presence of such residues in human food can be responsible for allergic reactions, toxic effects and develops resistant strains of bacteria[5-6]. To protect consumers, regulatory agencies in the European Union published several official documents to regulate the control of veterinary drugs. Council Directive 96/23/EC[7] establishes the veterinary residue control in food producing animals. In India, as per FSSAI (gazette notification dated 20th July 2018) and EIC (RMP for Aquaculture, Egg, Honey, Milk Poultry etc.) it is a mandate to do the analysis of antibiotics. EIC and FSSAI both have maximum residue limits (MRLs) for various antibiotic residues for food matrices from animals. It is essential to test milk for residues of sulfonamide considering safety and prevent exposure of consumers to veterinary drugs. In this work, we present a fast and sensitive LC-MS/MS method for the quantitative analysis of sulfonamide antibiotics in milk.

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Experimental

Hardware/software:

The chromatographic separation was conducted by a PerkinElmer LX-50 UHPLC System and detection was achieved using a PerkinElmer QSight™ 220 triple quadrupole mass spectrometer, equipped with ESI and APCI ionization sources. All instrument control, data acquisition and data processing were performed using the Simplicity 3Q™ software in a single window. LC parameters including Column and mobile phase gradient program are given in the table 1.

Materials

Reagents & Chemicals

All CRM, reagents, chemicals were used NIST traceable and LC-MS/MS grade. Type 1 water used for this study.

Method

Stock solution and calibration standard Preparation:

Stock solution of 10 mg/L of 10 mL standard mixture of all analytes were prepared by adding appropriate volume from mother stock to 10 mL volumetric flask and finally made up with solvent in which antibiotic analyte is soluble. The standard solutions were stored at -20°C. The calibration standards were prepared from working solutions by serial dilution (5, 10, 25, 50, 100 and 200 ng/ml) of the stock solution with water–acetonitrile (80:20, v/v).

Sample extraction procedure:

- Weighed 2 g ± 0.1 g of sample in 50 mL PTFE centrifuge tube.
- Added 4 mL of water, shook in a vortex 30 second and left for 30 minutes.
- Added 10 mL of ethyl acetate and shook/vortexed properly for 2-3 min for proper interaction of analytes and solvent.
- Centrifuged for 10 min at 10,000 rpm.
- Took 5 mL of supernatant in evaporating tube and evaporated it up to dryness under nitrogen evaporator at 40° C.
- Reconstituted with 1 mL of Acetonitrile: water (80:20) and vortexed it properly.
- Filtered the sample through 0.2 µm filter paper.

Result and discussion

The linearity study covers from 5 µg/L to 200 µg/L concentration level with 6 calibration points. The developed method showed excellent linearity with r^2 >0.99 linearity for all antibiotics studied in milk matrix. The limit of quantification (LOQ) for all compounds achieved 10 µg/L for all antibiotics. The LC method & MS source parameters are shown in Table 1. The MRM mode transitions of the studied antibiotics are shown in Table 3.

Table 1. UHPLC parameters

LC Column	Univ C18AQ 100 mm X 3 mm X 2.1µm (P/N N9304784)
Mobile Phase A	0.1% Formic acid in water
Mobile Phase B	0.1% Formic acid in Acetonitrile
Mobile Phase Gradient	Sr. No. / Time / %A / %B 1 / 0.00 / 98 / 2 2 / 1.50 / 98 / 2 3 / 6.00 / 4 / 96 4 / 6.50 / 4 / 96 5 / 7.00 / 98 / 2 6 / 7.50 / 98 / 2
Column Oven Temperature	40°C
Auto sampler Temperature	15°C
Injection Volume	10 µL
Flow	0.5 mL/Min
Run Time	7.5 minutes
ESI Voltage (+Ve)	5300
Drying Gas	110
Nebulizer Gas	300
Source Temperature	300
HSID Temperature	220

Matrix Effect

The responses of the matrix-matched standards (peak area of pre-extraction spike) were compared with the corresponding peak areas of standards in solvent in six replicates. The matrix effect (ME) was quantified as the average percent suppression or enhancement in the peak area using. For this, the area of Matrix standard and area of solvent standard were used, which is shown in table 2. Negative values of ME signify matrix induced signal suppressions, whereas positive values signify enhancement in the signal.

Table 2. Matrix effect

Analyte	Solvent std Area.	Matrix-matched Area.	% ME
Sulfamethoxypyridine	50655	80960	60
Sulfathiazole	38731.8	43709	13
Sulfadiazin	10873.6	43955.7	304
Sulfapyridine	18719.4	56815	204
Sulfamethizole	76718.8	122715	60
Sulfachloropyridazine	72406.6	111414.3	54
Sulfaisoxazole	94119.2	154994.2	65
Sulfaquinoxazole	30548.2	37611.5	23
Sulfaquinoxaline	29353	37376	27

Recovery study

In this, recovery for all compounds were determined by spiking at 10, 25 and 50 µg/kg level in milk sample in six replicates. The recovery was between 80 to 120 % and percentage RSD for all compounds found below 20 % which is well accepted and as per the regulatory requirements. Table 4-6 represent the all three-level recovery with recovery percent and RSD. All data were analysed in six replicates after calculating final dilution factor.

Table 3. MRM Transitions and Retention time of analytes

Analyte	Precursor	Product 1	CE 1	Product 2	CE 2	RT
Sulfamethoxypyridine	281	156	-23	65	-78	3.68
Sulfathiazole	256	156	-20	65	-73	6.07
Sulfadiazin	251	156	-22	108	-34	3.08
Sulfapyridine	250	156	-22	184	-23	3.24
Sulfamethizole	271	156	-19	92	-41	3.50
Sulfachloropyridazine	285	156	-20	108	-38	3.76
Sulfaisoxazole	268	156	-19	113	-20	3.95
Sulfaquinoxazole	301	156	-23	92	-48	4.15
Sulfaquinoxaline	301	156	-23	208	-25	2.15

Table 4. Recovery at 10 µg/kg spike with 5 times dilution at actual spike

Analyte	Rec 1	Rec 2	Rec 3	Rec 4	Rec 5	Rec 6	Avg	Stdev	%RSD	% Recovery
Sulfamethoxypyridine	10.73	10.61	10.61	10.00	10.60	10.89	10.57	0.30	2.88	105.72
Sulfathiazole	10.60	9.39	8.98	7.92	6.88	7.73	8.58	1.34	15.58	85.83
Sulfadiazin	9.51	8.84	9.38	10.97	10.10	9.16	9.66	0.76	7.92	96.60
Sulfapyridine	10.05	8.33	9.54	10.55	8.86	9.56	9.48	0.80	8.42	94.81
Sulfamethizole	10.25	9.88	9.95	9.28	9.10	8.94	9.57	0.53	5.52	95.65
Sulfachloropyridazine	10.86	10.27	10.75	10.06	10.48	10.54	10.49	0.30	2.83	104.94
Sulfaisoxazole	10.29	10.39	10.08	9.81	9.81	9.93	10.05	0.25	2.45	100.53
Sulfaquinoxazole	10.42	10.31	10.39	9.27	9.19	9.92	9.92	0.56	5.64	99.16
Sulfaquinoxaline	10.41	10.35	10.71	9.67	9.81	10.63	10.26	0.43	4.19	102.64

Table 5. Recovery at 25 µg/kg spike with 5 times dilution at actual spike

Analyte	Rec 1	Rec 2	Rec 3	Rec 4	Rec 5	Rec 6	Avg	Stdev	%RSD	% Recovery
Sulfamethoxypyridine	26.00	26.84	26.07	19.43	18.88	19.35	22.76	3.89	17.11	91.04
Sulfathiazole	21.19	20.56	23.42	22.02	22.80	21.75	21.96	1.04	4.75	87.83
Sulfadiazin	24.78	24.87	23.56	22.85	21.04	21.84	23.16	1.55	6.69	92.63
Sulfapyridine	25.99	27.17	28.12	22.12	21.31	23.61	24.72	2.79	11.27	98.88
Sulfamethizole	24.29	24.83	24.69	17.75	17.86	17.47	21.15	3.79	17.92	84.60
Sulfachloropyridazine	26.18	26.12	26.49	19.90	19.55	19.31	22.93	3.67	15.99	91.70
Sulfaisoxazole	25.94	26.04	26.06	18.64	18.46	18.93	22.35	4.02	18.00	89.39
Sulfaquinoxazole	26.54	26.41	25.50	18.67	18.18	19.83	22.52	4.03	17.88	90.08
Sulfaquinoxaline	24.80	25.24	25.31	18.85	18.99	17.89	21.85	3.61	16.51	87.38

Table 6. Recovery at 50 µg/kg spike with 5 times dilution at actual spike

Analyte	Rec 1	Rec 2	Rec 3	Rec 4	Rec 5	Rec 6	Avg	Stdev	%RSD	% Recovery
Sulfamethoxypyridine	47.45	48.29	48.24	53.66	54.03	54.35	51.00	3.32	6.50	102.00
Sulfathiazole	55.00	58.66	57.69	46.03	42.92	44.31	50.77	7.12	14.03	101.54
Sulfadiazin	50.20	50.65	49.93	52.42	53.82	53.27	51.71	1.67	3.23	103.43
Sulfapyridine	47.09	53.56	48.14	41.95	47.72	49.41	47.98	3.75	7.82	95.95
Sulfamethizole	48.12	49.35	48.48	56.60	56.17	56.57	52.55	4.29	8.17	105.10
Sulfachloropyridazine	48.23	49.33	47.41	55.44	54.52	54.99	51.65	3.71	7.18	103.31
Sulfaisoxazole	49.05	48.30	47.64	56.79	54.55	55.72	52.01	4.11	7.91	104.01
Sulfaquinoxazole	49.91	47.25	47.99	55.20	54.52	54.91	51.63	3.67	7.10	103.26
Sulfaquinoxaline	48.85	48.02	48.46	54.06	54.54	56.22	51.69	3.64	7.04	103.38

Conclusion

The results obtained confirm the capability of QSight™ LC-MS/MS method for antibiotic analysis in milk as efficient for routine analysis. The results showed excellent chromatographic repeatability and sample analyte identities were positively confirmed via their qualifier/quantifier ion ratios. A quick and reliable UHPLC-MS/MS method was developed for the simultaneous estimation of sulfonamides in milk matrix. The LOQ for all the analytes are 10 µg/kg. Linerity range is from 5 to 200 mg/kg with the regression coefficient > 0.99. The LOQs achieved using this method are well below that the permitted level, suggesting that PerkinElmer QSight™ 220 LC-MS/MS System provides a very sensitive and robust platform for the analysis of sulfonamides in milk.

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