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Simultaneous Determination of Sulfonamides, Trimethoprim, Ormethoprim and Dapsone in Livestock Products by LC-MS/MS with QuEChERS

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Abstract : In this study, we developed simultaneous method for twenty-four compounds (sulfonamides, trimethoprim, ormethoprim and dapsone) in variety of livestock products (beef, pork, chicken, egg, and milk) using LC-MS/MS with multiple reaction monitoring. To validate the method, five representative livestock products were evaluated at the three spiked levels considering the maximum residual limit (MRL) and limit of quantification (LOQ), 0.5 ×MRL, 1 × MRL, 2 × MRL, or 1 × LOQ, 2 × LOQ, 10 × LOQ. The samples are extracted with acetonitrile, dispersive solid phase extraction using $MgSO_4$ and sodium acetate with C_{18} for effective clean up. As a result, recoveries ranged from 68.5 to 118.0%, coefficient variation was below 22%. Linearity was evaluated by correlation coefficients greater than 0.98. LOQ were ranged from 1 to 50 μg kg^{-1}. The results indicate that the proposed method is suitable for determining the sulfonamides, trimethoprim, ormethoprim and dapson in livestock products. This analytical method can be used for sulfonamide class routine monitoring in livestock products.

Keywords : antibiotics, sulfonamides, multi-residue, livestock, LC-MS/MS

Introduction

Sulfonamides are widely used in veterinary medicine to prevent and treat microbial diseases in farmed livestock and fishery products.[1] As synthetic antimicrobials derived from sulfanilic acid, they have been employed since the 1930s. Beyond their therapeutic benefits, sulfonamides are also used as a feed additive to promote growth.[2] Trimethoprim, ormethoprim, and dapsone that have similar antimicrobial activities and often enhance antimicrobial effects.[3] Dapsone, a synthetic sulfone with antimicrobial and antiprotozoal activities, is used as an antibiotic for disease prevention and treatment.

However, dapson can induce hemolytic anemia and methemoglobinemia, leading to serious health consequences. Although rare, aplastic anemia can also occur, potentially resulting in fatal outcomes. Consequently, the use of dapsone in animal products is prohibited.[4] Additionally, sulfonamides are well-absorbed after oral administration, leading to their potential residue in food and posing risk to human health. Trimethoprim, ormethoprim, and dapsone have been associated with various adverse effects in humans including allergies, thyroid dysfunction, renal disorders, and suppression of immune system formation. Furthermore, the overuse of antibiotics contributes to the development and spread of antibiotic resistance.[5,6]

To mitigate these risks, maximum residue limit (MRL) have been established in many countries for sulfonamides. Korean Ministry of Food and Drug Safety (MFDS) has also implemented MRL for sulfonamide compounds. In fish, the MRL is set at 100 μg kg^{-1}, while in eggs, it is managed as undetectable at 10 μg kg^{-1}. Furthermore, trimethoprim is regulated at 20 μg kg^{-1} in eggs and up to 50 μg kg^{-1} in bovine, porcine, chicken, and milk. As non-detection limit of 10 μg kg^{-1} is also enforced.[7] From January 1, 2024, the positive limit system (PLS) will be implemented for all livestock and fishery products. Therefore, it is necessary to develop a testing method at the quantification limit of 10 μg kg^{-1} or lower levels.

The advances analytical method is needed to enhance for efficiency of analytical procedures for food safety control in Korea.[7] According to previous research, testing methods for simultaneous determination of various classes of veterinary drugs, including the sulfonamide, are being developed.[8-10] However, new sulfonamide class is steadily increased as

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veterinary drugs. Therefore, our study developed an analytical method including most frequently used sulfonamide compounds.[11] This study aimed to develop a reliable and quantifiable testing method for the simultaneous determination of 24 sulfonamide compounds, trimethoprim, ormethoprim, and dapsone, applicable for routine monitoring in both livestock and marine products.

Materials and Methods

Standards and reagents

Sulfonamides, dapsone, ormethoprim, and trimethoprim used for the validation of each test method were purchased from Sigma-Aldrich (St. Louis, MO, USA), Dr. Ehrenstorfer (Augsburg, Germany), and US Pharmacopeia (MD, USA). HPLC grade of acetonitrile, methanol, and water were obtained from Merck Inc. (Darmstadt, Germany). $MgSO_4$ and sodium acetate were purchased from Sigma-Aldrich, while C_{18} (Octadecylsilane) was procured from Waters (Milford, MA, USA). Formic acid (≥ 95%) was acquired from Sigma-Aldrich (St. Louis, MO, USA). Polytetrafluoroethylene (PTFE) syringe filter (0.2 μm) was employed from Teknokroma (Barcelona, Spain). The samples of beef, pork, chicken, eggs, and milk were collected from online market. The edible tissues (muscle) were thoroughly homogenized, egg and milk were sufficiently homogenized. The samples were stored in a freezer (-20°C) until analysis. After confirming that no residue of target compounds, the samples were used for validation process.

Preparation of standard solutions

Each standard (10 mg) was dissolved in methanol in a 100 mL volumetric flask to prepare a standard stock solution with a concentration of 100 mg L^{-1}. The prepared standard stock solution was then diluted with methanol to an appropriate concentration, and individual standard solutions were prepared. To mitigate the interference effect, matrix-matched calibration was employed with adding 200 μL of the standard solution to sample pretreatment process. The standard stock solution and standard solutions were stored in a brown glass bottle in a freezer at -4°C and diluted immediately before the experiment. The concentration ranges for the recovery experiments were as follows: trimethoprim (bovine, porcine, chicken, and milk), 12.5, 25, 50, 100, 200, and 400 μg kg^{-1}; trimethoprim (eggs), 2.5, 5, 10, 20, 40, and 80 μg kg^{-1}; and ormethoprim (bovine, porcine, chicken, eggs, and milk), 1.25, 2.5, 5, 10, 20, and 40 μg kg^{-1}; sulfonamides (bovine, porcine, chicken, and milk), 12.5, 25, 50, 100, 200, and 400 μg kg^{-1}. The dapson and sulfonamides (egg) were validated at concentrations of 2.5, 5, 10, 20, 100, and 200 μg kg^{-1}.

Sample preparation

Each tissue sample was thoroughly homogenized using a food blender, and then 2 g was accurately weighed and transferred into a 50 mL polypropylene tube. After adding 10 mL of acetonitrile for dispersion, 2 g of $MgSO_4$ and 1 g of sodium acetate were added. The mixture was shaken for 10 minutes and then centrifuged at 4500 × *G* and 4°C for 10 minutes. The resulting supernatant was collected, and 300 mg of C_{18} was added. The mixture was dispersed and shaken for 1 minute. After centrifugation at 4700 × *G* and 4°C for 10 minutes, 5 mL of the extract was taken and concentrated in a water bath below 40°C using nitrogen. After, nitrogen concentration, 1 mL of methanol/water (1:1, *v*/*v*) was added for dissolution. The final solution was then filtered through a 0.2 μm PTFE membrane filter and injected into LC-MS/MS.[11]

LC-MS/MS analysis

A Liquid Chromatograph-Tandem Mass Spectrometer (LC-MS/MS, XEVO TQ-S) from Waters (Milford, MA, USA) was used. An X SELECT-C_{18} (2.1 mm × 150 mm, 3.5 μm) column was selected. The column temperature was maintained at 40°C. The flow rate and injection volume were set to 0.3 mL/min and 5 μL, respectively. The mobile phase was optimized for gradient elution using acetonitrile/water (5:95, *v*/*v*) containing 0.1% formic acid and acetonitrile/water (95:5, *v*/*v*) containing 0.1% formic acid. For ionization of analytes, a precursor ion was selected using the positive ion mode of electrospray ionization (ESI), and product ions were generated by adjusting the collision energy. Subsequently, quantification ions and qualification ions were determined under multiple reaction monitoring (MRM) conditions. The instrumental conditions of LC-MS/MS are presented in Table 1.

Method validation

In accordance with CODEX guideline CAC/GL-71 (2009), this analytical method was validated for linearity, accuracy, precision, selectivity, limit of detection (LOD), and limit of quantification (LOQ).[12] Selectivity was confirmed by comparing chromatograms of blank livestock samples with addition of standard solutions. The LOD and LOQ were determined based on the signal-to-noise ratio (S/N) of the detected peak in the chromatogram with three times higher value for LOD and ten times higher value for LOQ. Precision and accuracy were assessed by adding standard solutions at concentrations of 0.5 ×, 1 ×, and 2 × MRL (1 ×, 2 ×, and 10 × LOQ for prohibited substances), and each experiment was performed in five replicates to determine recovery and coefficient of variation (CV). Six points calibration curves were constructed. Linearity was confirmed by calculating the coefficient of determination (R^2) for each calibration curve using the peak area of the quantitative ion obtained during the analysis.

Results and Discussion

Optimization of LC-MS/MS parameter

An LC-MS/MS was adopted to achieve quantitative and

sensitive analysis on multiple veterinary drug residues. The addition of formic acid to mobile phase was found to enhance ionization efficiency and increase analyte sensitivity.[13] Consequently, mobile phase A consisted of acetonitrile/water (5:95, *v*/*v*) containing 0.1% formic acid, while mobile phase B consisted of acetonitrile/water (95:5, *v*/*v*) containing 0.1% formic acid. Due to the sensitivity of target compounds, formic acid was able to determine target compounds for LC-MS/MS analysis.[14,15] A C_{18} column was suitable for separating compounds of varying polarity.[16] MRM conditions were optimized by directly injecting standard solutions (100 ng mL^{-1}) into the mass detector at a flow rate of 5 μL/min. The intensity of the precursor and product ions scans were carried out by collision energies ranging from 10 to 42 eV. Full scan mode was used to identify precursor and product ions with most sensitive product ion as the quantification ion. Two additional product ions showing high sensitivity were chosen as qualification ions. The selected ions with their retention times are presented in Table 1.

Optimization of extraction and purification

The sample extraction process was optimized based on

Table 1. Optimized multiple reaction monitoring parameters of all target compounds.

Compounds	ESI	Retention time (min.)	Molecular weight (g/mol)	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)
Sulfabenzamide	+	6.1	276.31	277.2	92.1	26
					108.1	24
					156.1	12
Sulfacetamide	+	1.1	214.24	215.1	92.2	22
					108.1	18
					156.1	10
Sulfacholrpyridazine	+	5.3	284.72	285.1	92.1	28
					108.1	24
					156.1	18
Sulfaclozine	+	6.1	284.72	285.1	92.1	32
					108.1	24
					156.1	18
Sulfadiazine	+	2.3	250.28	251.2	92.1	28
					108.1	18
					156.1	14
Sulfadimethoxine	+	6.1	310.33	311.2	92.1	32
					108.1	32
					156.1	20
Sulfadoxine	+	5.5	310.33	311.2	92.1	28
					108.1	26
					156.1	18
Sulfaguanidine	+	2.2	214.25	215.1	92.1	24
					108.1	22
					156.1	14

Table 1. Continued.

Compounds	ESI	Retention time (min.)	Molecular weight (g/mol)	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)
Sulfamerazine	+	3.1	264.31	265.2	92.1	26
					108.1	24
					156.1	16
Sulfamethazine	+	3.9	278.33	279.2	92.1	32
					108.1	28
					186.1	16
Sulfamethoxazole	+	5.6	253.28	254.1	92.2	28
					108.1	22
					156.1	14
Sulfamethoxypyridazine	+	4.1	280.31	281.2	92.4	30
					108.2	25
					156.3	20
Sulfamonomethoxine	+	5.1	280.31	281.1	92.1	30
					108.1	26
					156.1	18
Sulfamoxol	+	3.4	267.31	268.2	92.1	26
					113.1	16
					156.1	16
Sulfaphenazole	+	6.2	314.40	315.2	92.1	38
					108.1	28
					158.2	32
Sulfapyridine	+	2.6	249.29	250.1	92.2	28
					108.1	24
					156.1	16
Sulfaquinoxaline	+	6.1	300.34	301.1	92.1	28
					108.1	26
					156.1	16
Sulfathiazole	+	2.4	255.30	256.1	92.1	26
					108.1	24
					156.1	14
Sulfisomidine	+	1.7	278.33	279.2	92.1	32
					108.1	28
					186.1	16
Sulfisoxazole	+	5.8	267.31	268.1	92.1	26
					113.2	14
					156.1	14
Dapsone	+	5.1	248.30	249.1	92.2	26
					108.1	26
					156.1	26
Monoacetyldapsone	+	5.5	290.34	291.2	92.2	28
					108.1	20
					156.1	16
Ormethoprim	+	2.7	274.32	275.3	81.1	42
					123.2	28
					259.3	20
Trimethoprim	+	2.3	290.32	291.2	123.2	36
					230.2	24
					261.2	24

*The underlined bold letters expressed as quantification ion.

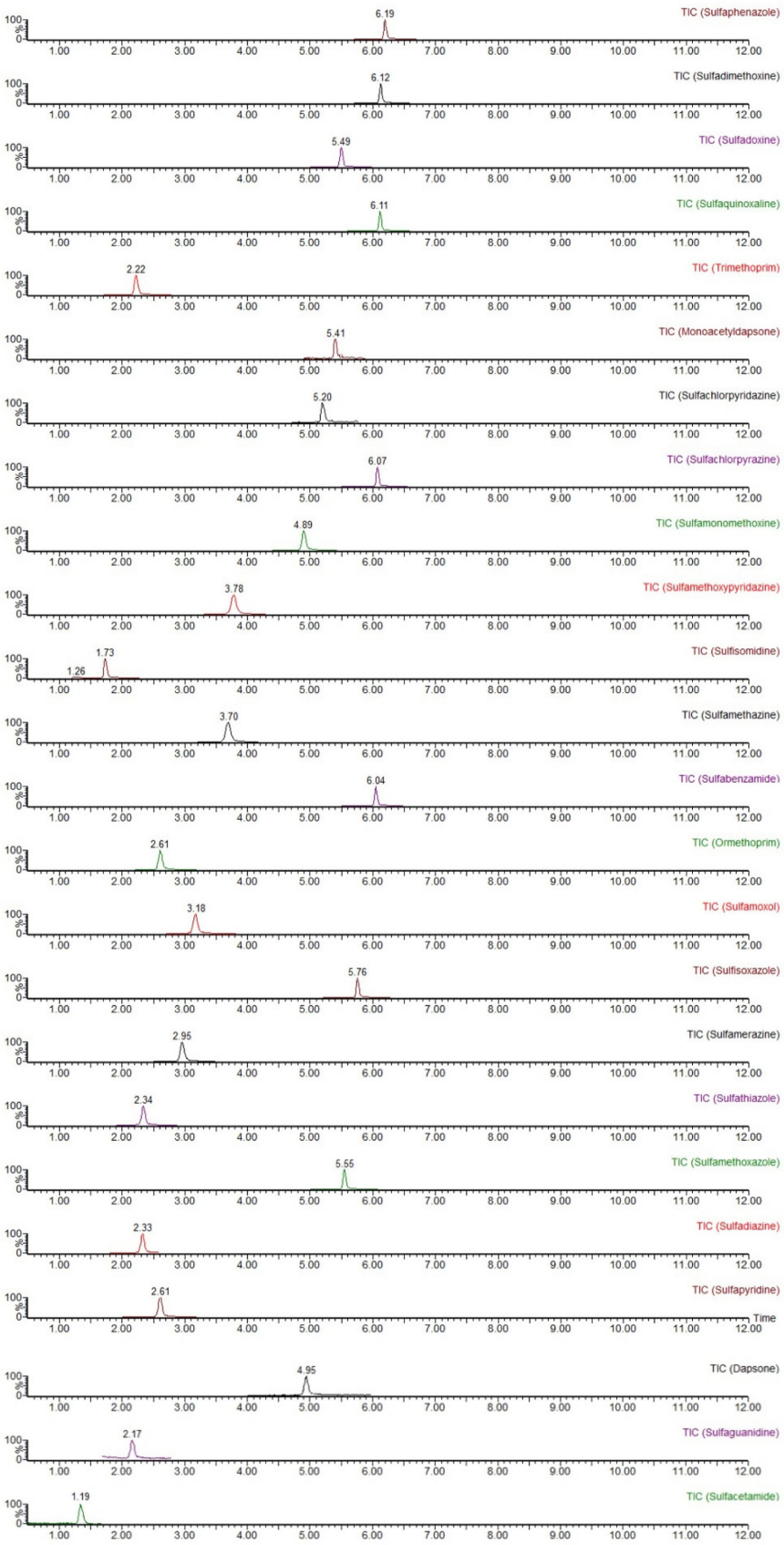

Figure 1. Total ion chromatograms of target compounds in beef (10–50 μg kg^{-1} for sulfonamides, 10 μg kg^{-1} for ormethoprim, and 20 μg kg^{-1} for trimethoprim; 2 μg kg^{-1} for dapsone and monoacetyldapsone).

Table 2. Recovery and coefficient variation at target testing levels for beef, pork and chicken.

Compounds	Beef (n = 5)					Pork (n = 5)					Chicken (n = 5)				
	Linearity	LOQ (ng kg^{-1})	Target testing levels (μg kg^{-1})	Recovery (%)	CV (%)	Linearity	LOQ (ng kg^{-1})	Target testing levels (μg kg^{-1})	Recovery (%)	CV (%)	Linearity	LOQ (ng kg^{-1})	Target testing levels (μg kg^{-1})	Recovery (%)	CV (%)
Sulfabenzamide	0.9996	30	50	98.3	19.0	0.9982	50	50	96.2	19.0	0.9992	30	50	104	6.20
			100	113	9.60			100	90.4	4.60			100	88.0	2.90
			200	109	15.5			200	92.3	4.90			200	82.8	3.90
Sulfacetamide	0.9964	50	50	101	4.50	0.9949	50	50	97.6	18.7	0.9950	50	50	94.7	2.40
			100	108	7.30			100	90.1	5.90			100	94.9	1.80
			200	112	3.30			200	91.2	6.40			200	88.6	4.10
Sulfacholrpyridazine	0.9983	30	50	74.3	18.3	0.9991	50	50	98.0	16.8	0.9979	30	50	103	7.40
			100	91.5	9.10			100	98.8	6.30			100	89.5	3.80
			200	82.6	17.0			200	95.5	8.50			200	81.7	4.00
Sulfaclozine	0.9983	40	50	98.4	13.6	0.9989	50	50	93.3	19.5	0.9993	40	50	99.6	7.10
			100	96.4	9.20			100	91.9	5.00			100	85.8	2.50
			200	97.4	4.10			200	92.8	7.30			200	79.5	5.20
Sulfadiazine	0.9991	50	50	100	19.0	0.9965	20	50	102	19.7	0.9987	30	50	99.1	9.00
			100	116	7.50			100	88.7	4.40			100	87.4	4.30
			200	118	15.5			200	87.7	7.50			200	81.1	2.50
Sulfadimethoxine	0.9981	40	50	85.0	14.3	0.9979	50	50	98.1	17.4	0.9985	30	50	102	7.10
			100	91.1	7.90			100	89.8	7.00			100	92.1	2.90
			200	90.7	5.30			200	97.3	6.80			200	87.9	4.00
Sulfadoxine	0.9985	40	50	90.6	15.1	0.9995	20	50	98.0	21.1	0.9997	20	50	103	7.50
			100	88.2	9.60			100	94.2	6.10			100	89.5	1.50
			200	90.4	4.30			200	95.2	6.80			200	84.6	2.10
Sulfaguanidine	0.9986	30	50	114	17.0	0.9993	50	50	85.0	19.0	0.9990	40	50	100	9.10
			100	105	2.90			100	84.2	7.60			100	89.0	5.10
			200	109	16.1			200	86.8	5.20			200	83.9	4.20
Sulfamerazine	0.9996	30	50	99.3	20.0	0.9991	40	50	98.4	16.5	0.9992	20	50	100	8.80
			100	115	9.90			100	97.6	5.30			100	90.5	2.70
			200	111	17.7			200	97.5	4.80			200	83.5	2.80
Sulfamethazine	0.9975	40	50	83.6	16.2	0.9997	30	50	96.9	21.6	0.9996	30	50	102	6.80
			100	91.9	11.6			100	91.9	4.60			100	87.9	2.50
			200	84.0	16.3			200	94.0	6.10			200	82.6	3.10

Table 2. Continued.

Compounds	Beef (n = 5)					Pork (n = 5)					Chicken (n = 5)				
	Linearity	LOQ (ng kg^{-1})	Target testing levels (μg kg^{-1})	Recovery (%)	CV (%)	Linearity	LOQ (ng kg^{-1})	Target testing levels (μg kg^{-1})	Recovery (%)	CV (%)	Linearity	LOQ (ng kg^{-1})	Target testing levels (μg kg^{-1})	Recovery (%)	CV (%)
Sulfamethoxazole	0.9996	30	50	109	17.4	0.9990	20	50	102	16.2	0.9995	30	50	102	7.90
			100	115	11.1			100	95.4	5.90			100	86.6	2.90
			200	118	4.20			200	92.3	8.70			200	80.9	3.40
Sulfamethoxypyridazine	0.9980	40	50	81.7	16.6	0.9992	20	50	98.9	20.0	0.9990	20	50	102	5.90
			100	89.8	10.3			100	91.5	4.20			100	88.0	2.70
			200	82.9	16.8			200	93.1	6.70			200	82.0	2.90
Sulfamonomethoxine	0.9979	40	50	84.7	15.4	0.9990	40	50	97.9	18.7	0.9991	20	50	102	6.20
			100	91.8	8.90			100	91.5	3.80			100	89.1	2.80
			200	90.6	5.90			200	94.7	6.50			200	82.1	3.50
Sulfamoxol	0.9991	50	50	89.7	14.3	0.9990	40	50	88.0	16.5	0.9988	30	50	104	5.80
			100	101	9.80			100	85.0	4.40			100	90.6	3.20
			200	99.9	4.50			200	86.8	7.70			200	84.5	2.90
Sulfaphenazole	0.9990	40	50	98.3	15.1	0.9996	30	50	98.2	17.7	0.9995	30	50	103	6.90
			100	104	8.00			100	94.8	7.20			100	87.9	4.00
			200	94.3	4.40			200	95.0	5.40			200	81.2	3.20
Sulfapyridine	0.9984	40	50	84.6	16.3	0.9990	50	50	98.5	19.7	0.9996	30	50	101	8.10
			100	93.6	9.50			100	90.2	3.3			100	91.0	2.30
			200	84.5	15.9			200	92.5	5.8			200	84.0	2.50
Sulfaquinoxaline	0.9993	40	50	86.5	16.9	0.9925	50	50	97.8	20.6	0.9932	20	50	103	5.90
			100	92.8	8.10			100	93.6	3.70			100	79.1	2.80
			200	90.3	10.8			200	95.1	13.3			200	70.6	9.70
Sulfathiazole	0.9996	30	50	109	16.3	0.9988	20	50	99.7	17.2	0.9984	20	50	99.7	9.10
			100	117	8.80			100	89.6	3.30			100	90.4	2.60
			200	115	16.1			200	90.0	4.70			200	74.8	2.20
Sulfisomidin	0.9989	40	50	90.6	15.0	0.9996	30	50	98.5	21.6	0.9997	20	50	103	8.80
			100	92.7	13.2			100	94.5	11.4			100	90.6	3.30
			200	96.0	7.30			200	95.1	8.10			200	85.1	2.20
Sulfisoxazole	0.9979	40	50	88.5	16.9	0.9992	40	50	96.3	19.3	0.9984	30	50	104	6.80
			100	98.3	10.5			100	92.6	5.50			100	87.2	5.60
			200	86.8	16.1			200	94.6	6.0			200	81.5	5.60

Table 2. Continued.

Compounds	Beef (n = 5)					Pork (n = 5)					Chicken (n = 5)				
	Linearity	LOQ (ng kg^{-1})	Target testing levels (μg kg^{-1})	Recovery (%)	CV (%)	Linearity	LOQ (ng kg^{-1})	Target testing levels (μg kg^{-1})	Recovery (%)	CV (%)	Linearity	LOQ (ng kg^{-1})	Target testing levels (μg kg^{-1})	Recovery (%)	CV (%)
Dapsone	0.9990	1	10	79.1	17.7	0.9951	1	10	98.9	16.1	0.9996	1	10	100	6.20
			20	117	9.90			20	92.6	4.10			20	91.0	3.20
			100	89.4	14.5			100	91.6	5.60			100	93.1	2.60
Monoacetyldapsone	0.9989	1	10	68.5	11.9	0.9812	1	10	117	7.70	0.9989	1	10	103	8.70
			20	84.6	11.5			20	94.0	4.40			20	97.1	4.80
			100	84.2	16.4			100	78.0	6.30			100	80.1	3.90
Ormethoprim	0.9989	5	5	88.4	15.7	0.9984	5	5	98.9	19.6	0.9979	5	5	106	11.3
			10	90.6	11.7			10	94.5	7.30			10	91.2	6.20
			20	84.9	17.6			20	94.9	5.80			20	86.7	4.50
Trimethoprim	0.9993	20	25	81.5	17.4	0.9988	20	25	95.8	21.8	0.9974	10	25	103	11.1
			50	89.7	12.6			50	87.6	6.60			50	86.0	6.10
			100	86.0	18.0			100	93.3	10.5			100	78.2	3.20

Table 3. Recovery and coefficient variation at target testing levels for egg and milk.

Compounds	Egg (n = 5)					Milk (n = 5)				
	Linearity	LOQ (μg kg^{-1})	Target testing levels (μg kg^{-1})	Recovery (%)	CV (%)	Linearity	LOQ (μg kg^{-1})	Target testing levels (μg kg^{-1})	Recovery (%)	CV (%)
Sulfabenzamide	0.9987	10	10	94.4	8.80	0.9929	50	50	102	6.60
			20	101	11.8			100	101	4.80
			100	101	8.70			200	103	6.70
Sulfacetamide	0.9997	10	10	109	9.20	0.9990	50	50	105	4.60
			20	102	7.80			100	94.3	13.8
			100	99.8	11.5			200	98.1	8.30
Sulfacholrpyridazine	0.9994	10	10	101	9.50	0.9970	50	50	103	6.60
			20	106	12.3			100	109	6.50
			100	101	10.4			200	107	10.6
Sulfaclozine	0.9981	10	10	88.5	8.60	0.9882	50	50	95.4	6.40
			20	104	7.30			100	101	8.00
			100	99.0	8.30			200	102	8.80

Table 3. Continued.

Compounds	Egg (n = 5)					Milk (n = 5)				
	Linearity	LOQ (μg kg^{-1})	Target testing levels (μg kg^{-1})	Recovery (%)	CV (%)	Linearity	LOQ (μg kg^{-1})	Target testing levels (μg kg^{-1})	Recovery (%)	CV (%)
Sulfadiazine	0.9964	10	10	89.8	8.70	0.9992	50	50	108	4.80
			20	104	6.80			100	95.3	13.3
			100	96.6	8.30			200	102	5.30
Sulfadimethoxine	0.9987	10	10	93.7	6.20	0.9874	50	50	95.4	6.40
			20	99.3	13.5			100	98.1	19.6
			100	99.2	7.80			200	103	8.30
Sulfadoxine	0.9991	10	10	100	7.40	0.9892	50	50	99.5	3.10
			20	102	11.3			100	106	10.3
			100	98.1	6.30			200	104	6.40
Sulfaguanidine	0.9996	10	10	96.0	7.20	0.9943	50	50	99.5	5.70
			20	102	8.00			100	102	5.70
			100	93.3	8.70			200	106	7.00
Sulfamerazine	0.9994	10	10	95.3	7.60	0.9915	50	50	99.1	7.40
			20	105	7.20			100	94.6	18.1
			100	96.5	8.20			200	101	8.70
Sulfamethazine	0.9991	10	10	94.8	8.40	0.9985	50	50	101	4.70
			20	105	6.40			100	98.2	7.80
			100	98.7	7.60			200	97.1	5.50
Sulfamethoxazole	0.9990	10	10	91.3	6.50	0.9908	50	50	96.5	5.40
			20	97.4	2.90			100	92.6	16.2
			100	95.6	5.10			200	102	7.90
Sulfamethoxypyridazine	0.9995	10	10	94.4	7.30	0.9988	50	50	106	4.10
			20	101	12.6			100	91.6	13.3
			100	97.1	7.90			200	97.4	5.60
Sulfamonomethoxine	0.9990	10	10	95.8	7.20	0.9964	50	50	103	4.40
			20	99.3	11.5			100	103	6.40
			100	96.8	7.40			200	100	5.50
Sulfamoxol	0.9994	10	10	88.1	7.60	0.9943	50	50	99.3	6.10
			20	102	12.6			100	98.0	11.0
			100	97.2	9.50			200	97.3	10.1

Table 3. Continued.

Compounds	Egg (n = 5)					Milk (n = 5)				
	Linearity	LOQ (μg kg^{-1})	Target testing levels (μg kg^{-1})	Recovery (%)	CV (%)	Linearity	LOQ (μg kg^{-1})	Target testing levels (μg kg^{-1})	Recovery (%)	CV (%)
Sulfaphenazole	0.9995	10	10	89.4	6.50	0.9895	50	50	96.9	6.20
			20	100	12.3			100	102	6.20
			100	99.7	9.70			200	99.6	7.40
Sulfapyridine	0.9994	10	10	99.0	6.70	0.9935	50	50	99.6	6.20
			20	99.9	1.90			100	94.8	17.3
			100	92.9	2.10			200	102	8.00
Sulfaquinoxaline	0.9988	10	10	94.3	7.90	0.9937	50	50	103	13.4
			20	105	12.4			100	91.4	4.20
			100	100	9.00			200	97.3	14.5
Sulfathiazole	0.9992	10	10	92.9	8.20	0.9984	50	50	105	4.30
			20	104	6.40			100	94.1	14.5
			100	97.9	6.30			200	101	5.80
Sulfisomidin	0.9989	10	10	93.3	5.30	0.9988	50	50	111	9.20
			20	102	14.0			100	97.1	15.5
			100	102	6.40			200	102	6.60
Sulfisoxazole	0.9991	10	10	94.7	9.00	0.9965	50	50	104	4.20
			20	100	11.9			100	92.5	7.90
			100	99.2	9.20			200	91.9	6.80
Dapsone	0.9992	10	10	96.1	11.1	0.9995	1	10	115	5.40
			20	100	10.4			20	95.7	14.2
			100	96.1	3.80			100	97.4	9.70
Monoacetyldapsone	0.9994	2	10	97.6	4.70	0.9974	1	10	107	6.30
			20	106	8.90			20	101	9.90
			100	96.5	7.30			100	88.9	9.50
Ormethoprim	0.9953	5	5	92.8	4.10	0.9907	5	5	105	10.5
			10	95.7	17.0			10	115	8.40
			20	96.8	9.40			20	107	8.40
Trimethoprim	0.9940	10	10	87.4	7.40	0.9846	25	25	95.8	7.80
			20	96.0	17.9			50	109	8.00
			40	97.3	6.10			100	102	12.1

the our previous study for determination of sulfonamides in fishery products.[11] The QuEChERS method (quick, easy, cheap, effective, rugged, and safe) has been widely adopted for simultaneous multi-residue analysis of veterinary drugs.[17,18] The QuEChERS method was based on acetonitrile extraction, followed by the addition of anhydrous magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl) to eliminate moisture and interfering substances with salting-out process. Additionally, pH adjustment using acids or salts can be employed for residue determination of veterinary drugs.[19-21] The extract undergoes purification through the d-SPE (dispersive-solid phase extraction). During the sample cleanup process, $MgSO_4$ is used to reduce the polarity of the extract in the sample, primary secondary amine (PSA) is utilized to remove organic acids or sugars, and octadecyl (C_{18}) is employed to eliminate non-polar interfering substances.[22] Acetonitrile (ACN) is commonly employed for the extraction of various compounds due to its low solubility in the matrix and minimal interference.[23] ACN was also used to precipitate proteins in the sample and facilitate the separation of the aqueous and organic layers through the salting-out principle. Our method was optimized based on the QuEChERS method to effectively extract and purify veterinary drug residues from various matrix interferences.

Method validation

The analytical methods were validated according to the CODEX guideline CAC/GL-71. Selectivity was evaluated by comparing chromatograms of untreated samples, standard solutions, and test solutions with added standard solutions. No interfering substances were detected with the same retention time and mass-to-charge ratio (*m/z*), indicating high resolution and selectivity of the test method (Fig. 1). Our results demonstrate acceptable quantification limits compared to official methods in Korean Food Code. The coefficient of determination (R^2) for all analytes was 0.98 or higher. The results showed that the recovery for livestock and fishery product samples ranged from 68.5% to 118%, and the coefficient of variation was below 22%. While certain compounds (sulfadoxine, sulfamethazine, sulfaquinoxaline, sulfisomidin and trimethoprim) in pork exhibited CV values slightly above the CODEX guidelines (CAC GL-71, 2009), they were still within an acceptable range for multi-compound residue analysis. Further investigation are needed to reduce within-laboratory variation (repeatability) and the between-laboratory variability (reproducibility). The LOQs were ranged from 1 μg kg^{-1} to 50 μg kg^{-1} with a signal-to-noise ratio (S/N) of ≥ 10 (Table 2 and 3). In previous study, Varenina et al., 2016 showed a similar result when using SPE cartridges.[6] However, our study used the QuEChERS method simplifying sample treatment. Moreover, we achieve acceptable results for recovery, linearity and standard deviation without using internal standards. Compared to other previous studies (LOQs for 5–250 μg kg^{-1}), our results show lower levels of LOQs (1–50 μg kg^{-1}).[25,26] Previous studies have developed hybrid linear ion trap-Orbitrap to detect sulfonamides in animal tissue [27] and LC-MS/MS for analyzing residues of sulfonamides and trimethoprim in honey.[3,28,29] However, there are no reported multi-residue methods for sulfonamides combined with dapsone (its metabolite), ormethoprim, and trimethoprim in livestock products. Our study focused on livestock products to verify the testing method in beef, pork, chickens, eggs, and milk.[11,30] Compared to our previous study, the method was also successfully applied to fishery products.[8-10]

This study has several limitations. First, the matrix effect should evaluate to improve the method reliability including decision limit (ccα), and detection capability (ccβ) performance. In particular, matrix effects can have a significant impact on analyte quantification. Second, further investigation is necessary to confirm the method's applicability through residue monitoring studies. Future study is needed to focus on inter-laboratory validation to enhance reliability of analytical method.

Conclusion

This study presents a simultaneous testing method of 24 sulfonamide compounds including three antimicrobials (trimethoprim, ormethoprim, dapsone) commonly used in livestock products. To enhance the extraction efficiency, salt was added to the conventional acetonitrile extraction process, and optimal solvents were selected for dissolution after nitrogen concentration. LC-MS/MS analysis was optimized to achieve high-sensitivity quantification. The developed method was validated in five livestock products. The results demonstrated acceptable accuracy and precision, mostly satisfied with the validation guideline of CAC/GL 71-2009, confirming the reliability of the testing method. This study established a simultaneous multi-residue analytical method of sulfonamides class for livestock products.

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