

Research Article



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## LC-MS/MS를 이용한 퇴비 및 액비 중 항생제 동시 분석법 개발

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### Development of Simultaneous Analytical Method of Veterinary Antibiotics in Manure using Liquid Chromatography Coupled with Tandem Mass Spectrometry

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#### Abstract

**BACKGROUND:** The current study was to monitor of 9 veterinary antibiotics (ceftiofur, clopidol, florfenicol, sulfamethazine, sulfamethoxazole, sulfathiazole, tetracycline, tiamulin, and tylosin) in manure using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in positive and negative electrospray ionization mode.

**METHODS AND RESULTS:** Sample preparation was carried out using McIlvaine buffer and citrate salts to adjust the pH of the sample followed by purification with dispersive solid phase extraction (d-SPE). Separation of analytes during LC-MS/MS analysis was conducted using an Eclipse Plus C<sub>18</sub> column and the mobile phase was in gradient mode with, 0.1% formic acid and 5 mM ammonium formate in methanol (A) and 0.1% formic acid and 5 mM ammonium formate in distilled water (B). The linearity of the matrix-matched calibrations of all tested antibiotics was good, with R<sup>2</sup> determination coefficients ≥

0.9920. The limit of detection (LOD) and quantifications (LOQ) were 0.1-67.0 µg/kg and 0.4-200.0 µg/kg, respectively. Analysis of 13 solid and liquid manure samples taken from the Republic of Korea revealed concentrations less than 0.7 µg/kg for tiamulin, 1497.6 µg/kg for sulfamethazine.

**CONCLUSION:** To monitor 9 veterinary antibiotics from manure samples in 13 provincial areas throughout the Republic of Korea, an analytical method was developed. The developed method was fully validated and successfully applied for monitoring various veterinary antibiotics in manure samples.

**Key words:** LC-MS/MS, Liquid, Monitoring, Solid manure, Veterinary antibiotics

#### 서론

가

(Arikan *et al.*,

2009).

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Table 1) pH 6 McIlvaine buffer Tokyo, Japan) . C<sub>18</sub> PSA (Primary  
 QuEChERS LC-MS/MS 가 Secondary Amine) Agilent Technologies (Palo Alto,  
 가 . California, USA)  
 HPLC

### 재료 및 방법

#### 시약 및 재료

ceftiofur (purity, 97.0%), clopidol (purity, 99.7%), florfenicol (purity, 99.0%), sulfamethazine (purity, 99.0%), sulfamethoxazole (purity, 99.9%), sulfathiazole (purity, 99.9%), tetracycline (purity, 98.0%), tiamulin (purity, 98.3%), tylosin tetrade (purity, 95.5%)  
 Sigma-Aldrich (St. Louis, Missouri, USA)  
 (MeOH) , (MeCN), (H<sub>2</sub>O)  
 SK Chemical ( , , ) ,  
 (Formic acid), (Ammonium formate),  
 sodium citrate dibasic sesquihydrate (Na<sub>2</sub>Cit. 5H<sub>2</sub>O, purity, 99.0%) Sigma-Aldrich (St. Louis, Missouri, USA)  
 sodium citrate tribasic dihydrate (Na<sub>3</sub>Cit. 2H<sub>2</sub>O, purity, 99.0%) Junsei Chemical Co. Ltd. (Chuo-ku,

#### 표준용액

Ceftifor clopidol (Florfenicol, sulfamethazine, sulfamethoxazole, sulfathiazole, tetracycline, tiamulin tylosin) 1000 µg/mL

ceftiofur clopidol 100 µg/mL

(the lowest calibrated level, LCL) 100

LCL 1, 2, 5, 10, 20, 40 50

matrix-matched

-26°C

4°C

#### 대상 항생제 및 시료 채취(퇴·액비)

flofenicol, sulfamethazine, sulfamethoxazole, sulfathiazole, tiamulin tylosin

Table 2. Sampling points of the manure in Republic of Korea

Liquid manure			
No.	Province	City/District	Name of Establishment
1	Gangwon	Chulwon	Chulwon Chukhyup
2	Kyunggi	Pocheon	Pocheon Chukhyup
3	Kyunggi	Icheon	Icheon pigsties
4	Chungnam	Hongseong	Hongseong Chukhyup
5	Chungnam	Gongju	Gyeryongsan Hanwoo Youngnong
6	Chungbuk	Chungju	Chungju Yangdon Youngnong
7	Kyungnam	Tongyeong	Tongyeong Chukhyup
8	Kyungbuk	Goryeong	Goryeong Woogoek Yangdon
9	Kyungbuk	Gunwi	Gunwi Chukhyup
10	Jeonnam	Gurye	Gurye liquid manure Yangdon Youngnong
11	Jeonnam	Muan	Yangdon muan Yongnong
12	Jeonbuk	Buan	Nambuan liquid manure
13	Jeonbuk	Namwon	Ecobio Youngnong
Solid manure			
No.	Province	City/District	Name of Nonghyup
1	Gangwon	Chulwon	Chulwon
2	Kyunggi	Anseong	Iljook
3	Kyunggi	Icheon	Daewall
4	Chungnam	Hongseong	Hongdong
5	Chungnam	Cheonan	Ipzang
6	Kyungnam	Sancheong	Sancheong Chukhyup
7	Kyungnam	Hadong	Yokjong
8	Kyungbuk	Seongju	Beokjin
9	Kyungbuk	Gunwi	Gunwi Chukhyup
10	Jeonnam	Jangheung	Jangheung Chukhyup
11	Jeonnam	Muan	Mokpo-Muan-Shinan Chukhyup
12	Jeonbuk	Buan	Nambuan
13	Jeonbuk	Namwon	Jerisan Nakhyup

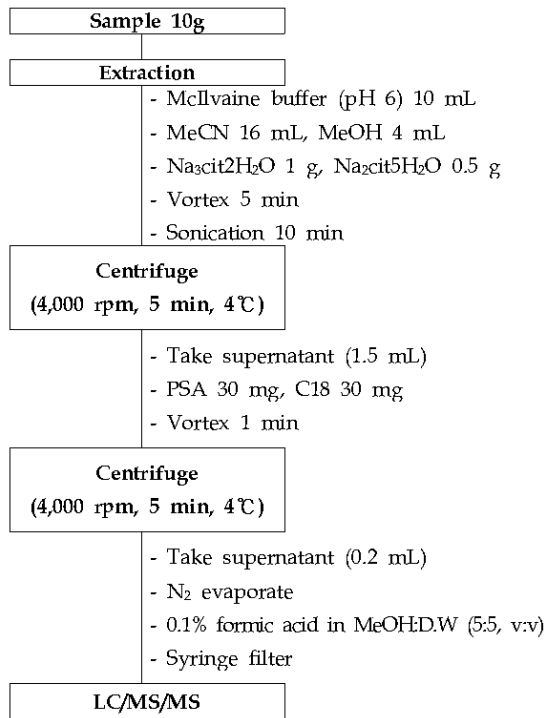


Fig. 2. Flow chart of analytical method of antibiotics in manure.

ceftiofur, clopidol, sulfamethazine, sulfamethoxazole, sulfathiazole tetracycline  
 13 (Lee *et al.*, 2016).  
 (Table 2).

시료추출 및 정제

10 g 50 mL  
 pH 6 McIlvaine buffer  
 16 mL 4 mL 가  
 1 g Na<sub>2</sub>Cit.5H<sub>2</sub>O, 0.5 g Na<sub>3</sub>Cit.2H<sub>2</sub>O 5  
 10 Branson 8510 (St.  
 Louis, Missouri, USA)  
 (4,000 rpm, 5 min, 4°C, Combi514R, Hanil Biomed  
 Inc. Incheon, Republic of Korea)  
 1.5 mL C<sub>18</sub> PSA 0.03 g 1  
 (4,000 rpm, 5 min, 4°C)  
 0.2 mL  
 (N<sub>2</sub> evaporate) 0.1%  
 / (50/50, v/v) 1 mL  
 syringe filter (PTFE-hydrophilic, 0.2 μm, ADVANTEC  
 Co., Ltd. Ehime, Japan)

Fig. 2

Table 3. Analytical HPLC conditions for the veterinary antibiotics

HPLC Condition			
Column	Eclipse C <sub>18</sub> (3.0 mm i.d.×150 mm, 3.5 μm, Agilent Technologies, Palo Alto, CA, USA)		
Mobile phase	A: 0.1% formic acid+5 mM ammonium formate in MeOH B: 0.1% formic acid+5 mM ammonium formate in H <sub>2</sub> O		
Flow rate	0.3 mL/min		
Injection volume	5 μL		
Gradient	Time	A (%)	B (%)
	0	5	95
	1	5	95
	4	50	50
	9	100	0
	18	100	0
	21	5	95
MS/MS Condition			
Mode	LC/MS/MS ESI, positive/negative mode		
Capillary voltage	3.25 (positive), 3.50 (negative) kV		
Source temp.	150°C		
Desolvation temp.	350°C		
Software	MassLynx (Ver 4.1)		

LC-MS/MS conditions의 분석조건 설정

Ceftiofur, clopidol, florfenicol, sulfamethazine, sulfamethoxazole, sulfathiazole, tetracycline, tiamulin tylosin 9 Waters Alliance 2695 Separations Module LC Micromass Quattro Microtripole tandem mass (Waters Crop., Milford, Ohio, USA) MS/MS Eclipse C<sub>18</sub> (3.0 mm i.d. 150 mm, 3.5 μm, Agilent Technologies, Palo Alto, CA, USA) peak intensity (Mobile Phase A : 0.1% formic acid+5 mM ammonium formate in MeOH, Mobile phas B : 0.1% formic acid+5 mM ammonium formate in H<sub>2</sub>O) gradient mode 0.3 mL/min, 5 μL ammonium formate (5 mM) 가 formic acid ammonium formate 가 가 (Darryl *et al.*, 2013). HPLC MS/MS Table 3

분석법의 검증

(Linearity, R<sup>2</sup>). (limit of detection, LOD), (limit of quantitation, LOQ), (accuracy) (precision) peak



**Table 5. Molecular weight (MW), precursor and product ion masses, cone voltage (CV), collision energy (CE) of the tested veterinary antibiotics in LC-(ESI)-MS/MS**

No.	Veterinary antibiotics	Electrospray ionication	Precursor ion ( <i>m/z</i> )	Product ion ( <i>m/z</i> )		CV (V)
				Quantitation (CE)	Confirmation (CE)	
1	Ceftiofur	Positive	523.97	241.06 (16)	94.99 (38)	30
2	Clopidol	Positive	193.93	102.97 (24)	103.42 (28)	44
3	Florfenicol	Negative	357.93	185.02 (21)	337.99 (13)	-26
4	Sulfamethazine	Positive	279.03	186.01 (17)	155.97 (19)	30
5	Sulfamethoxazole	Positive	253.97	155.99 (15)	107.99 (29)	26
6	Sulfathiazole	Positive	255.97	155.98 (15)	107.99 (23)	26
7	Tetracycline	Positive	445.03	154.01 (29)	410.08 (17)	26
8	Tiamulin	Positive	494.22	192.10 (21)	119.04 (41)	30
9	Tylosin	Positive	916.46	174.10 (41)	100.97 (47)	54

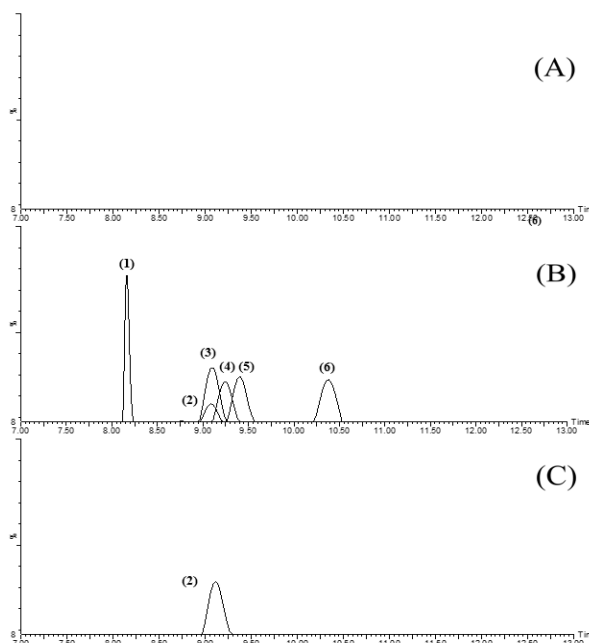
가 precursor/  
product ion pair  
product ion  
(qualification ion)  
Peak 13 (Fig. 3,  
4). MS/MS precursor ion product ion mass,  
collision energy (CE) cone voltage (CV), capillary  
voltage (CP) Table 5

**분석법검증**

가  
가  
peak  
(Fig. 3, 4).  
( 가 )  
matrix-matched  
( $R^2$ )  $\geq 0.9925$   
(Table 6).  
가  
( 가 ) LOQ 5  
LOQ 10  
90.2-120.4% 71.5-106.2%  
29.8% 25.7%  
가  
(LOD) (LOQ)  
가 0.13-67, 0.7-  
13.3  $\mu\text{g/kg}$  (LOD) 0.4-200  $\mu\text{g/kg}$ , 2-40  $\mu\text{g/kg}$  (LOQ)

**퇴비 및 액비 중 대상 항생제 잔류실태 조사**

13  
Table 7  
. European Commission Decision 2002/657/EC



**Fig. 4. LC-MS/MS chromatograms of the tested veterinary antibiotics in a blank solid manure sample (A); fortified with appropriate concentration of the tested analytes (B); and field-incurred samples collected from Kyunggi (Anseong) (C). (1) : sulfathiazole, (2) : clopidol, (3) : sulfamethazine, (4) : tetracycline, (5) : sulfamethoxazole, (6) : ceftiofur.**

matrix-matched  
peak  
6 florfenicol sulfamethoxazole  
sulfathiazole, sulfamethazine, tiamulin, tylosin  
4 가 Tiamulin  
0.7-6.7  $\mu\text{g/kg}$   
46%,  
6.7  $\mu\text{g/kg}$  . Michael (2003)  
tiamulin 43.0  $\mu\text{g/kg}$

**Table 6. Linear range (LR,  $\mu\text{g}/\text{kg}$ ), coefficient of determination ( $R^2$ ), recovery (%), relative standard deviation (RSD, %), limit of detection (LOD,  $\mu\text{g}/\text{kg}$ ), and limit of quantitation (LOQ,  $\mu\text{g}/\text{kg}$ ) of the tested veterinary antibiotics in manure using LC-MS/MS ( $n=3$ )**

Liquid manure							
No.	Veterinary antibiotics	LR	$R^2$	Recovery (Mean $\pm$ RSD)%		LOD	LOQ
				5 LOQ	10 LOQ		
1	Florfenicol	100 - 5000	0.9985	94.9 $\pm$ 24.4	103.8 $\pm$ 14.2	67.0	200.0
2	Sulfamethazine	2 - 100	0.9981	99.9 $\pm$ 15.4	117.6 $\pm$ 29.8	1.3	4.0
3	Sulfamethoxazole	2 - 100	0.9984	120.4 $\pm$ 10.7	106.0 $\pm$ 19.8	1.3	4.0
4	Sulfathiazole	2 - 100	0.9995	99.9 $\pm$ 9.0	90.2 $\pm$ 9.1	1.3	4.0
5	Tylosin	2 - 100	0.9991	96.6 $\pm$ 4.9	100.2 $\pm$ 11.3	1.3	4.0
6	Tiamulin	0.2 - 10	0.9987	100.0 $\pm$ 5.8	97.8 $\pm$ 1.1	0.1	0.4
Solid manure							
No.	Veterinary antibiotics	LR	$R^2$	Recovery (Mean $\pm$ RSD)%		LOD	LOQ
				5 LOQ	10 LOQ		
1	Ceftiofur	20 - 1000	0.9999	72.6 $\pm$ 19.4	85.1 $\pm$ 25.7	13.3	40.0
2	Clopidol	5 - 250	0.9925	78.3 $\pm$ 11.1	73.7 $\pm$ 17.2	3.3	10.0
3	Sulfamethazine	1 - 50	0.9942	86.7 $\pm$ 3.2	106.2 $\pm$ 8.8	0.7	2.0
4	Sulfamethoxazole	2 - 100	0.9996	71.5 $\pm$ 5.4	76.6 $\pm$ 19.3	1.3	4.0
5	Sulfathiazole	4 - 200	0.9998	75.3 $\pm$ 1.9	79.1 $\pm$ 7.9	2.7	8.0
6	Tetracycline	10 - 500	0.9999	71.7 $\pm$ 6.4	98.1 $\pm$ 6.9	6.7	20.0

**Table 7. Monitoring results of the veterinary antibiotics in liquid and manure samples ( $n=3$ ,  $\mu\text{g}/\text{kg}$ )**

Liquid manure							
No.	Antibiotic Area	Florfenicol	Sulfamethazine	Sulfamethoxazole	Sulfathiazole	Tylosin	Tiamulin
		1	Gangwon (Chulwon)	-	131.5 $\pm$ 21.6	-	-
2	Kyunggi (Pocheon)	-	14.6 $\pm$ 4.7	-	-	-	0.7 $\pm$ 0.1
3	Kyunggi (Icheon)	-	-	-	-	-	-
4	Chungnam (Hongseong)	-	-	-	-	-	-
5	Chungnam (Gongju)	-	-	-	-	-	-
6	Chungbuk (Chungju)	-	1497.6 $\pm$ 97.0	-	516.9 $\pm$ 48.3	10.5 $\pm$ 1.2	6.7 $\pm$ 0.4
7	Kyungnam (Tongyeong)	-	-	-	-	-	-
8	Kyungbuk (Goryeong)	-	-	-	-	-	-
9	Kyungbuk (Gunwi)	-	-	-	-	-	-
10	Jeonnam (Gurye)	-	397.3 $\pm$ 43.3	-	15.5 $\pm$ 1.1	7.3 $\pm$ 0.8	6.3 $\pm$ 0.9
11	Jeonnam (Muan)	-	-	-	-	-	-
12	Jeonbuk (Buan)	-	118.9 $\pm$ 26.4	-	508.1 $\pm$ 23.5	13.3 $\pm$ 1.7	3.1 $\pm$ 0.2
13	Jeonbuk (Namwon)	-	61.2 $\pm$ 13.9	-	23.17 $\pm$ 2.2	-	0.7 $\pm$ 0.1
Solid manure							
No.	Antibiotic Area	Ceftiofur	Clopidol	Sulfamethazine	Sulfamethoxazole	Sulfathiazole	Tetracycline
		1	Gangwon (Chulwon)	-	237.3 $\pm$ 34.7	-	-
2	Kyunggi (Anseong)	-	100.1 $\pm$ 15.4	-	-	-	-
3	Kyunggi (Icheon)	-	-	-	-	-	-
4	Chungnam (Hongseong)	-	-	-	-	-	-
5	Chungnam (Cheonan)	-	18.5 $\pm$ 4.1	-	-	-	-
6	Kyungnam (Sancheong)	-	148.8 $\pm$ 30.6	-	-	-	-
7	Kyungnam (Hadong)	-	194.5 $\pm$ 9.9	-	-	-	-
8	Kyungbuk (Seongju)	-	-	2.3 $\pm$ 0.5	-	-	-
9	Kyungbuk (Gunwi)	-	-	-	-	-	-
10	Jeonnam (Jangheung)	-	22.9 $\pm$ 3.9	-	-	-	-
11	Jeonnam (Muan)	-	-	-	-	-	-
12	Jeonbuk (Buan)	-	574.4 $\pm$ 91.2	-	-	-	-
13	Jeonbuk (Namwon)	-	443.4 $\pm$ 41.1	-	-	-	-

tiamulin  
 7 가 . Tylosin , , ,  
 7.3-15.4 µg/kg 가  
 31%, 15.4 µg/kg  
 . Sulfonamide sulfathiazole  
 , , , 23.2-516.9 µg/kg  
 31%,  
 516.9 µg/kg . Sulfamethazine  
 , , , , 14.6-1497.6 µg/kg  
 46%,  
 1497.6 µg/kg .  
 가  
 Table 4  
 sulfonamide 가  
 Hu (2008) sulfamethoxazole  
 sulfamethoxazole  
 (Perez *et al.*, 2005; Hu *et al.*,  
 2008).  
 6 ceftiofur sulfamethoxazole,  
 sulfathiazole, tetracycline clopidol, sulfamethazine  
 2 가 . Clopidol , , ,  
 , , , , 18.5-574.5 µg/kg  
 61.5%,  
 574.5 µg/kg .  
 Sulfamethazine 2.3 µg/kg  
 8% . tetracycline  
 sulfamethoxazole Hu (2008)  
 tetracycline sulfamethoxazole  
 41500.0 µg/kg 5200.0 µg/kg  
 . Zhao (2010)  
 sulfamethoxazole  
 840.0 µg/kg 2800.0 µg/  
 kg . , Karchl Balcloglu  
 (2009) sulfamethoxazole  
 3760.0 µg/kg (Karci and  
 Balcioglu, 2009). Martinez-Carballo (2007)  
 tetracycline 360.0-23000.0 µg/kg  
 70%  
 sulfathiazole sulfamethoxazole  
 . Lee (2016)  
 (clopidol, sulfamethazine,  
 sulfathiazole, tiamulin tylosin)  
 3.0-4.8 (9.8%), 3.8-6.6 (5.9%), 3.1-7.7 (11.8%),

0.2-10 (56.9%), and 0.7-5.3 µg/kg (7.8%)

**요약**

9 (ceftiofur,  
 clopidol, florfenicol, sulfamethazine, sulfamethoxazole,  
 sulfathiazole, tetracycline, tiamulin, tylosin) pH 6  
 McIlvaine buffer  
 Na<sub>2</sub>Cit.5H<sub>2</sub>O, Na<sub>3</sub>Cit.2H<sub>2</sub>O C<sub>18</sub> PSA  
 LC-MS/MS  
 5 sulfamethazine, sulfathiazole, tylosin,  
 tiamulin clopidol

( )

**Notes**

The author declare no conflict of interest.

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