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Multiresidue Screening of Veterinary Drugs in Meat, Milk, Egg, and Fish Using Liquid Chromatography Coupled with Ion Trap Time-of-Flight Mass Spectrometry

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Abstract New approaches to veterinary drug screening based on liquid chromatography-mass spectrometry (LC-MS/MS) and time-of-flight mass spectrometry (ToF/MS) are rapid and have high selectivity and sensitivity. In this study, we developed a multiresidue method for screening over 100 veterinary drug residues using ion trap (IT)-ToF/MS. The screened compounds comprised major drug classes used in veterinary practice, representing the following: amphenicols, anthelmintics, benzimidazoles, β-lactams, coccidiostats, ionophores, macrolides, non-steroidal anti-inflammatory drugs, quinolones, sulfonamides, tetracyclines, and tranquilizers. The method was developed based on chromatographic retention time, specific accurate mass, isotope distribution, and fragment data. Each compound was validated at three levels, and the mass accuracy, accuracy, and repeatability were calculated. All parameters showed acceptable values and conformed to the Commission Decision 2002/657/EC criteria. This screening method can simultaneously analyze over 100 veterinary drugs in meat, milk, eggs, and fish in a single analytical run.

JeongWoo Kang and Su-Jeong Park contributed equally to this study.

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Introduction

A maximum residue limit (MRL) established in Korea for drugs in food covers over 140 types of veterinary drugs including β-lactams, tetracyclines, macrolides, aminoglycosides, polyethers, peptides, sulfonamides, quinolones, and nitrofurans [1]. The framework of the Korean National Residue Program (NRP), the system currently employed to detect antimicrobial substances including antibiotics and synthetic antibacterial compounds, mostly involves rapid screening methods such as microbiological testing via the European Community (EC) four-plate method. In case of positive results, precision analysis is carried out using such techniques as high-performance liquid chromatography (HPLC) or liquid chromatography-mass spectrometry (LC-MS/MS) with or without a confirmation test using the Charm II receptor assay [2]. The microbiological and simple chemical screening tests used for domestic and imported foods of animal origin are advantageous because they are inexpensive and allow broad-spectrum screening of antimicrobial residues [3]. However, except for some antimicrobial agents such as penicillins, tetracyclines, macrolides, and quinolones, these techniques cannot ensure the detection of drugs at concentrations below the class-specific MRLs or control standards for which non-detection standards apply [4]. Such drugs include aminoglycosides, polyethers, peptide antibiotics, sulfonamides, chloramphenicol, and nitrofurans. With the rapid development of analytical techniques over recent years, LC-MS/MS-based class-specific quantification methods have been widely used for individual antibiotics [5]. Broader spectrum screening techniques are being developed to enable multiresidue screening of veterinary drugs using Q-ToF-based or IT-ToF-based analysis methods for simultaneous screening of multiclass antimicrobial substances [6, 7]. The ToF-based screening technique allows rapid, accurate, and simultaneous multiclass detection of over 100 individual antimicrobial agents found in microbiological screening tests, thus replacing class-specific screening test methods for identifying presumptive positive substances using the Charm II receptor assay of LC-MS. This technique is expected to replace microbiological, immunological, and chemical screening tests in the near future. Against this background, it is considered necessary to develop a ToF-MS-based method for simultaneous multiclass screening of veterinary drug residues and to introduce it to the national residue monitoring system. This would provide an NRP with an advanced and efficient residue screening system for domestic and imported food of animal origin. Samples found positive in rapid microbiological screening should be subjected to rapid, accurate, and simultaneous multiclass screening without the need for class-specific confirmatory tests such as the Charm II receptor assay. Additionally, a high-precision screening method is essential to yield accurate monitoring results for the precise evaluation of health hazards from substances that cannot be detected below their MRLs by microbiological screening methods. Given that a ToF-based screening method can perform the multiclass residue screening of over 100 different substances, once introduced, it could reduce screening time and cost. It could also enhance the efficacy and reliability of screening with a broader range of detection compared with conventional immunochemical class-specific or substance-specific test kits [8].

This study was conducted to develop a method for the simultaneous screening of multiclass veterinary drug residues in foods of animal origin, such as meat, eggs, and milk, using an LC-IT-ToF/MS analyzer. Another objective was to improve the efficacy of residue screening tests

of domestic and imported foods of animal and fish origin for the simultaneous detection of multiclass veterinary drug residues for the NRP.

Materials and Methods

Chemicals and Reagents

All veterinary standard drugs including penicillins, tetracyclines, macrolides, aminoglycosides, ionophores, amphenicols, quinolones, sulfonamides, nitroimidazoles, benzimidazoles, tranquilizers, and non-steroidal anti-inflammatory drugs (NSAIDs) were purchased from Sigma-Aldrich (St. Louis, MO, USA), Dr. Ehrenstorfer GmbH (Augsburg, Germany), Riedel-de Haën (Buchs, Switzerland), and Fluka (Buchs, Switzerland). Most standard reagents were purchased as 1 mL solutions at a concentration of 100 μg/mL in methanol. Stock solutions were kept in a dark, dry storage environment at −70 °C until required. Acetonitrile and methanol were purchased from Burdick & Jackson (Morristown, NJ, USA); ethyl acetate was purchased from Fisher (Fair Lawn, NJ, USA); acetone was purchased from J.T. Baker (Phillipsburg, NJ, USA), and formic acid was purchased from Fluka (Darmstadt, Germany).

Instrumentation

Experiments were performed using liquid chromatography coupled with ion trap time-of-flight mass spectrometry (Shimadzu Corp., Kyoto, Japan). Chromatographic separation of veterinary drugs was conducted using a YMC-Triart C18 column (3.0 × 100 mm, 3 μm). The column temperature was 40 °C, and the injection volume was 15 μL. Several combinations of mobile phases were tested. Formic acid (0.1%) in water for mobile phase A and formic acid (0.1%) in acetonitrile for mobile phase B gave the best performance in terms of selectivity and peak shapes. The eluent gradient increased from 10% B to 95% B in 11 min, stayed at 95% for 15 min, and then returned to the initial composition in 15.1 min for 20 min. The flow rate was 0.4 mL/min, and the injection volume was 15 μL. IT-ToF/MS was performed in positive/negative electrospray ionization (ESI) switching mode. The external mass calibration of ToF was conducted using sodium trifluoroacetate once per week.

Sample Preparation

Samples for analysis were selected from muscle tissue (beef, pork, and chicken), kidney, liver, milk, eggs, and fish. The preparation method for muscle tissue, kidney, and liver samples for method validation was as follows: A 2 g tissue (muscle, liver, or kidney) was taken in conical tube, and 10 mL mixture of acetonitrile and water (4:1, *v*/*v*) containing 2 mM ammonium formate was added. Six concentrations (0.0, 0.1, 0.5, 1.0, 1.5, and 2.0 times of validation level as mentioned in Table 1) of the standard solutions of analytes were spiked with tissues to establish the calibration curve. For the determination of other validation parameters except calibration curve, aliquots of working standard solutions containing all of the analytes were spiked with those tissues in tubes so that the concentrations of those analytes would be 0.5, 1.0, and 2.0 times of their respective validation levels. The tissue in solvent mixture was homogenized, shaken for 5 min, and later centrifuged (Beckman Coulter Avanti J-E, 5000×*g*, 4 °C, 10 min) to extract the drug

Table 1 Veterinary compounds and performance characteristics as determined in this study

Compound	Chemical formula	Calculated (*m/z*)	RT[a] (min)	VL[b] (μg/kg)	MRL (μg/kg)	ΔMass[c] (ppm)	Accu[d] (%)	Rept[e] (%)	Repr[f] (%)	CCβ[g] (μg/kg)
Sulfisomidine	$C_{12}H_{14}N_4O_2S$	279.0910	4.089	100	–	1.4	89	4	9	113
Sulfadiazine	$C_{10}H_{10}N_4O_2S$	251.0597	4.631	10	100	2.4	109	26	21	19
Sulfathiazole	$C_9H_9N_3O_2S_2$	256.0209	4.850	10	100	3.1	66	26	28	16
Sulfamerazine	$C_{11}H_{12}N_4O_2S$	265.0754	5.368	10	100	0.0	94	12	14	14
Sulfamethazine	$C_{12}H_{14}N_4O_2S$	279.0910	5.873	10	100	2.9	90	9	10	13
Sulfamethoxy-pyridazine	$C_{11}H_{12}N_4O_3S$	281.0703	5.891	10	100	3.6	77	5	12	11
Sulfamonomethoxine	$C_{11}H_{12}N_4O_3S$	281.0703	6.387	10	100	0.7	59	8	11	12
Sulfachloropyridazine	$C_{10}H_9ClN_4O_2S$	285.0208	6.656	10	100	2.1	92	23	15	17
Sulfadoxine	$C_{12}H_{14}N_4O_4S$	311.0809	6.978	10	100	1.3	96	8	10	12
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	254.0594	7.002	10	100	1.2	67	9	9	12
Sulfisoxazole	$C_{11}H_{13}N_3O_3S$	268.0750	7.255	10	100	0.0	71	7	11	12
Sulfachloropyrazine	$C_{10}H_9ClN_4O_2S$	285.0208	7.768	10	100	3.5	88	13	28	14
Sulfaquinoxaline	$C_{14}H_{12}N_4O_2S$	301.0754	7.830	10	100	1.7	68	7	24	12
Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$	311.0809	7.847	10	100	0.0	60	6	13	11
Sulfaphenazole	$C_{15}H_{14}N_4O_2S$	315.0910	7.979	10	100	1.6	94	5	8	12
Dapsone	$C_{12}H_{12}N_2O_2S$	249.0692	6.596	10	100	1.3	110	19	11	17
Trimethoprim	$C_{14}H_{18}N_4O_3$	291.1452	4.975	50	50	2.7	167	3	10	59
Minocycline	$C_{23}H_{27}N_3O_7$	229.5997	4.738	100	–	4.8	67	24	24	153
Oxytetracycline	$C_{22}H_{24}N_2O_9$	461.1555	5.154	100	200	3.5	69	30	21	168
Tetracycline	$C_{22}H_{24}N_2O_8$	445.1605	5.490	100	200	4.9	120	16	22	164
Demeclocycline	$C_{21}H_{21}ClN_2O_8$	465.1059	5.959	100	200	1.7	87	33	14	194
Chlortetracycline	$C_{22}H_{23}ClN_2O_8$	479.1216	6.462	100	200	4.2	85	31	8	185
Doxycycline	$C_{22}H_{24}N_2O_8$	445.1605	6.617	100	100	4.7	73	12	35	130
Marbofloxacin	$C_{17}H_{19}FN_4O_4$	363.1463	4.963	150	150	2.8	76	4	15	165

Table 1 (continued)

Compound	Chemical formula	Calculated (*m/z*)	RT[a] (min)	VL[b] (μg/kg)	MRL (μg/kg)	ΔMass[c] (ppm)	Accu[d] (%)	Rept[e] (%)	Repr[f] (%)	CCβ[g] (μg/kg)
Norfloxacin	$C_{16}H_{18}FN_3O_3$	320.1405	5.115	50	50	3.4	106	8	13	63
Ofloxacin	$C_{18}H_{20}FN_3O_4$	362.1511	5.143	50	50	5.2	87	5	13	57
Pefloxacin	$C_{17}H_{20}FN_3O_3$	334.1561	5.182	50	50	4.8	84	6	10	58
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	332.1405	5.227	50	100	5.4	73	5	8	56
Danofloxacin	$C_{19}H_{20}FN_3O_3$	358.1561	5.361	100	100	5.0	76	5	13	113
Enrofloxacin	$C_{19}H_{22}FN_3O_3$	360.1718	5.557	50	100	0.8	75	4	9	55
Orbifloxacin	$C_{19}H_{20}F_3N_3O_3$	396.1530	5.731	200	20	3.5	68	3	13	215
Difloxacin	$C_{21}H_{19}F_2N_3O_3$	400.1467	6.114	300	400	4.5	57	7	15	341
Oxolinic acid	$C_{13}H_{11}NO_5$	262.0710	7.435	50	50	5.0	77	10	12	62
Nalidixic acid	$C_{12}H_{12}N_2O_3$	233.0921	8.696	30	30	3.0	63	7	16	35
Flumequine	$C_{14}H_{12}FNO_3$	262.0874	8.826	200	200	5.3	54	7	11	223
Sarafloxacin	$C_{20}H_{17}F_2N_3O_3$	386.1311	6.018	10	10	2.9	93	15	19	15
Piromidic acid	$C_{14}H_{16}N_4O_3$	289.1295	9.541	100	–	4.8	65	6	12	114
Ampicillin	$C_{16}H_{19}N_3O_4S$	350.1169	4.794	50	400	5.4	87	20	25	79
Penicillin G	$C_{16}H_{18}N_2O_4S$	335.1060	5.083	50	50	5.7	76	22	9	78
Penicillin V	$C_{16}H_{18}N_2O_5S$	349.0864	8.700	300	–	0.3	117	24	9	576
Oxacillin	$C_{19}H_{19}N_3O_5S$	400.0973	9.094	300	–	5.7	106	14	33	443
Cloxacillin	$C_{19}H_{18}ClN_3O_5S$	434.0583	9.482	50	300	5.5	110	6	25	60
Nafcillin	$C_{21}H_{22}N_2O_5S$	415.1322	9.700	300	300	6.5	72	25	29	476
Dicloxacillin	$C_{19}H_{17}Cl_2N_3O_5S$	468.0193	9.983	50	300	6.4	98	6	20	60
Piperacillin	$C_{23}H_{27}N_5O_7S$	516.1558	7.687	100	–	7.0	105	8	17	128
Ceftiofur	$C_{19}H_{17}N_5O_7S_3$	524.0363	7.215	1000	1000	1.7	68	23	11	1526
Desfuroylceftiofur	$C_{19}H_{17}N_5O_7S_3$	430.0308	5.592	1000	–	2.3	82	33	26	1896
Cefquinome	$C_{23}H_{24}N_6O_5S_2$	265.0698	4.320	100	50	1.5	120	23	63	190

Table 1 (continued)

Compound	Chemical formula	Calculated (*m/z*)	RT[a] (min)	VL[b] (μg/kg)	MRL (μg/kg)	ΔMass[c] (ppm)	Accu[d] (%)	Rept[e] (%)	Repr[f] (%)	CCβ[g] (μg/kg)
Cephalexin	$C_{16}H_{17}N_3O_4S$	348.1013	4.825	200	–	6.0	80	22	25	317
Cefadroxil	$C_{16}H_{17}N_3O_5S$	396.1224	5.735	50	–	3.9	68	7	11	58
Erythromycin	$C_{37}H_{67}NO_{13}$	734.4685	7.610	50	50	1.4	77	50	33	113
Oleandomycin	$C_{35}H_{61}NO_{12}$	688.4267	7.245	150	150	2.5	74	22	16	229
Tilmicosin	$C_{46}H_{80}N_2O_{13}$	435.2903	6.843	100	100	3.9	51	50	8	183
Roxithromycin	$C_{41}H_{76}N_2O_{15}$	419.2696	8.885	40	–	2.1	66	12	9	51
Spiramycin	$C_{43}H_{74}N_2O_{14}$	422.2643	6.041	100	200	0.9	125	16	9	165
Josamycin	$C_{42}H_{69}NO_{15}$	828.4740	9.300	200	40	6.3	112	22	22	358
Tiamulin	$C_{41}H_{79}N_3O_{12}$	494.3299	8.494	100	100	5.1	58	26	16	149
Valnemulin	$C_{31}H_{52}N_2O_5S$	565.3670	8.957	10	–	0.7	49	42	38	17
Lincomycin	$C_{18}H_{34}N_2O_6S$	407.2210	4.411	100	100	7.1	106	7	12	125
Tulathromycin	$C_{41}H_{79}N_3O_{12}$	269.5294	5.033	50	–	9.3	70	33	13	88
Thiabendazole	$C_{10}H_7N_3S$	202.0433	4.785	100	100	5.9	82	8	17	121
5-Hydroxy-thiabendazole	$C_{10}H_7N_3OS$	218.0383	4.170	100	100	6.4	100	6	11	119
Oxibendazole	$C_{12}H_{15}N_3O_3$	250.1186	6.918	100	100	2.8	87	9	32	127
Triclabendazole	$C_{14}H_9Cl_3N_2OS$	358.9574	11.574	100	200	5.6	82	18	19	148
Albendazole	$C_{12}H_{15}N_3O_2S$	266.0958	8.217	200	100	1.1	82	15	58	281
Amino albendazole sulfone	$C_{10}H_{13}N_3O_2S$	240.0801	4.577	100	–	8.3	110	10	9	136
Mebendazole	$C_{16}H_{13}N_3O_3$	296.1030	8.187	100	–	5.1	62	12	44	124
Flubendazole	$C_{16}H_{12}FN_3O_3$	314.0935	8.544	100	10	2.2	117	10	49	138
2-Amino flubendazole	$C_{14}H_{10}FN_3O$	256.0881	6.529	100	–	2.7	93	9	53	126
Febantel	$C_{20}H_{22}N_4O_6S$	447.1333	11.113	50	100	0.3	77	6	42	58
Fenbendazole	$C_{15}H_{13}N_3O_2S$	300.0801	9.349	50	–	1.2	78	19	59	75
Fenbendazole sulfone	$C_{15}H_{13}N_3O_4S$	332.0700	8.043	50	–	4.1	73	5	14	56

Table 1 (continued)

Compound	Chemical formula	Calculated (*m/z*)	RT[a] (min)	VL[b] (μg/kg)	MRL (μg/kg)	ΔMass[c] (ppm)	Accu[d] (%)	Rept[e] (%)	Repr[f] (%)	CCβ[g] (μg/kg)
Oxfendazole	$C_{15}H_{13}N_3O_3S$	316.0750	6.775	50	–	0.8	72	5	18	55
Oxfendazole sulfone	$C_{15}H_{13}N_3O_4S$	332.0700	8.043	50	–	4.1	73	5	8	56
Lasalocid	$C_{34}H_{54}O_8$	613.3711	13.974	100	20	1	69	26	83	159
Monensin	$C_{36}H_{62}O_{11}$	693.4184	15.453	10	50	5.8	89	26	94	18
Narasin	$C_{43}H_{72}O_{11}$	787.4967	15.977	10	–	9.8	67	26	92	16
Salinomycin	$C_{42}H_{70}O_{11}$	773.4810	15.081	1000	20	2.3	68	19	24	1422
Semduramicin	$C_{45}H_{76}O_{16}$	895.5026	14.384	100	–	6.6	74	18	128	144
Clopidol	$C_7H_7Cl_2NO$	191.9977	4.397	20	200	10.4	118	12	7	30
Decoquinate	$C_{24}H_{35}NO_5$	418.2588	12.639	100	2	6.5	75	28	52	168
Diaveridine	$C_{13}H_{16}N_4O_2$	261.1346	4.691	50	–	0.3	102	11	11	68
Dicyclanil	$C_8H_{10}N_6$	191.1055	2.419	20	–	5.2	88	22	36	32
Diclazuril	$C_{17}H_9Cl_3N_4O_2$	404.9757	11.004	100	500	1	104	22	22	175
Ethopabate	$C_{12}H_{15}NO_4$	238.1074	7.730	2000	500	0.8	112	10	9	2724
Imidocarb	$C_{19}H_{20}N_{6O}$	349.1769	3.815	500	300	4.9	109	20	14	858
Nicarbazin	$C_{19}H_{18}N_6O_6$	301.0578	10.559	200	200	6.6	115	20	22	354
Robenidine	$C_{15}H_{13}Cl_2N_5$	334.0603	9.787	200	–	12.6	99	39	52	455
Toltrazuril	$C_{18}H_{14}F_3N_3O_4$	424.0626	11.266	200	–	0.5	115	17	27	330
Toltrazuril sulfone	$C_{18}H_{14}F_3N_3O_6S$	456.0513	10.579	500	–	9.4	119	13	78	755
Dinitolmide	$C_8H_7N_3O_5$	224.0313	6.936	200	–	3.1	130	13	18	307
Roxarsone	$C_6AsNH_6O_6$	263.9484	3.257	200	–	–	70	11	5	249
Toltrazuril sulfoxide	$C_{18}H_{14}F_3N_3O_5S$	440.0533	9.806	500	–	–	118	6	5	616
Niclosamide	$C_{13}H_8Cl_2N_2O_4$	324.9788	11.682	200	–	6.8	91	22	8	330
Morantel	$C_{12}H_{16}N_2S$	221.1107	6.312	300	–	1.4	70	12	21	380
Pyrantel	$c_{11}H_{12}N_2S$	207.0950	5.386	200	–	2.4	110	12	28	287

Table 1 (continued)

Compound	Chemical formula	Calculated (*m/z*)	RT[a] (min)	VL[b] (μg/kg)	MRL (μg/kg)	ΔMass[c] (ppm)	Accu[d] (%)	Rept[e] (%)	Repr[f] (%)	CCβ[g] (μg/kg)
Florfenicol	$C_{12}H_{14}Cl_2FNO_4S$	355.9932	7.036	200	200	9.8	112	7	0.3	254
Thiamphenicol	$C_{12}H_{15}Cl_2NO_5S$	353.9975	5.500	500	500	4.8	79	6	19	579
Azaperone	$C_{19}H_{22}FN_3O$	328.1820	5.760	60	60	8.5	72	9	2	73
Closantel	$C_{22}H_{14}Cl_2I_2N_2O_2$	660.8449	13.465	3000	–	1.6	72	56	67	6990
Clorsulon	$C_8H_8Cl_3N_3O_4S_2$	377.8949	7.604	500	–	2.7	105	40	27	1191
Flunixin	$C_{14}H_{11}F_3N_2O_2$	297.0745	10.258	200	–	8.8	74	10	6	248
Meloxicam	$C_{14}H_{13}N_3O_4S_2$	352.0420	10.093	200	–	5.1	91	11	13	263
Tolfenamic acid	$C_{14}H_{12}ClNO_2$	262.0629	11.735	10	–	7.3	82	12	4	13
Phenylbutazone	$C_{19}H_{20}N_2O_2$	307.1452	11.319	10	10	2.9	60	25	13	15
Oxyphenbutazone	$C_{19}H_{20}N_2O_3$	323.1401	9.854	10	–	3.9	67	23	10	15
Diclofenac	$C_{14}H_{11}Cl_2NO_2$	294.0049	9.487	200	–	4.1	70	13	6	261
Paracetamol	$C_8H_9NO_2$	152.0706	3.661	20	–	0.8	79	33	18	37
Ketoprofen	$C_{16}H_{14}O_3$	253.0870	9.767	20	–	6.7	95	24	15	35

With the exception of validation level (VL) and detection capability (CC_β), the parameters are expressed as the range of the average parameters for the four tested matrices (muscle, milk, eggs, and fish) at the three concentrations (0.5, 1.0, and 2 times VL)

MRL maximum residue limit

[a] RT: retention time in minutes as determined in chromatograms of analyte standard solutions

[b] VL: validation level is the concentration spiked to samples (muscle, kidney, and liver) in g/kg; validation was carried out 0.5, 1.0, and 2 times VL

[c] ΔMass: mass measurement error relative to the calculated exact mass of the analyte

[d] Accur: the recovery of the spiked analyte concentration expressed as a percentage

[e] Rept: repeatability expressed as the relative standard deviation in 20 analyses performed on 1 day

[f] Repr: reproducibility expressed as the relative standard deviation in 20 analyses performed at different days

[g] CCβ: detection capability calculated from the within-lab reproducibility at the validation level 1.0 times VL

compounds in solution. Ten milliliters of hexane was supplemented to the extracted solution, and then, 250 mg octadecyl carbon chain (C_{18})-bonded silica powder was added to that for more purification. The extracted drug solution, silica powder, and hexane were mixed by vortexing for 30 s. The mixture was centrifuged for 5 min at 10,000 rpm to separate the supernatant, and later, hexane was aspirated from that supernatant. The solvents of the purified extract were evaporated under nitrogen till near dryness. The extracted drug compounds were re-dissolved in water and made the volume 1 mL. The resulting liquid was filtered through a 0.2-μm polyvinylidene difluoride (PVDF) syringeless filter prior to analysis.

One gram of milk sample and 2 mL of acetonitrile were taken in conical tube, and standard drug solution was supplemented as mentioned above. The resulting mixture was put into an ultracentrifugal filter (cutoff membrane at 3 kDa, 4 mL) and purified by centrifugal separation (Beckman Coulter Avanti J-E, 5000×*g*, 4 °C, 60 min). The solution was placed in a nitrogen evaporator to be concentrated to 1 mL and filtered with a 0.2-μm PVDF syringeless filter prior to analysis. The egg and fish samples were prepared by taking 2 g of egg or fish in tubes. Standard drug solution was supplemented as mentioned above with the addition of 3 mL of acetonitrile and 1 mL of water that contains 2 mM ammonium formate. The supernatant was obtained by centrifuging for 5 min with 5000×*g* at 4 °C. The supernatant (2 mL) was purified by centrifugation (5000×*g*, 4 °C, 60 min) in an ultracentrifugal filter (cutoff membrane at 3 kDa). Two milliliters of hexane was added to the filtrate, vortexed, and re-centrifuged for further purification. The resulting supernatant (2 mL) was concentrated to 0.5 mL in a nitrogen evaporator and then filtered using a 0.2-μm PVDF syringeless filter prior to analysis (Fig. 1).

All samples were purchased from a local market or collected by the provincial veterinary services from two areas in Korea (Gyeonggi, Gyeongnam) to apply the method for residue determination.

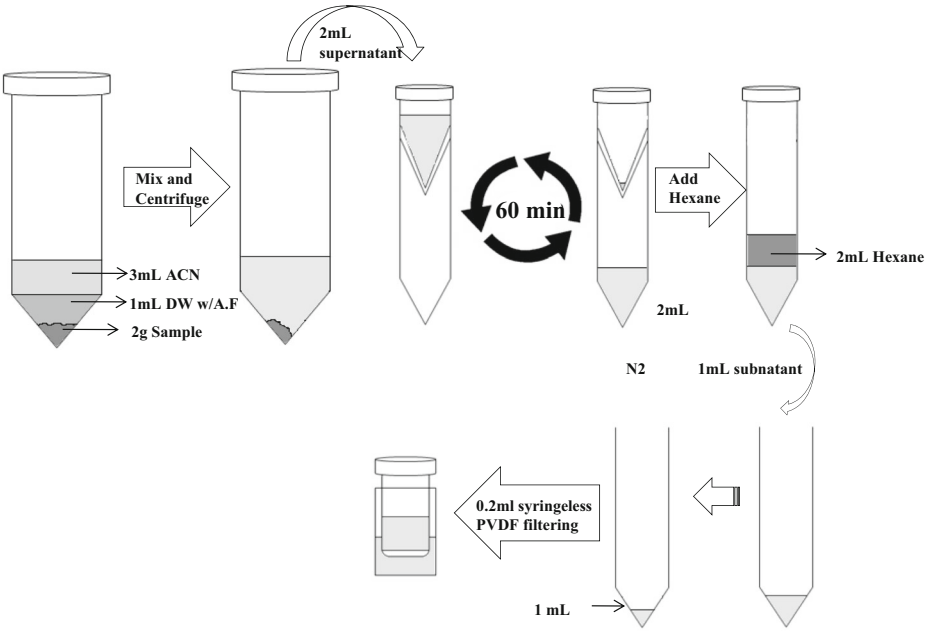

Fig. 1 Sample preparation schematics for egg and fish products

Method Validation

The developed method was validated based on the procedure described in published article [9] which is in accordance with the European Commission Decision 2002/657/EC [10]. All concentration levels used in this validation study are described in Table 1. Validation levels (VLs) regarding muscle, milk, eggs, and fish samples were set below the MRL values of the targeted drugs in the corresponding Korean regulations. Moreover, the VLs of the drugs of interest were chosen below the MRL values to prevent overloading of the analytical column. Six replicates of each sample were injected to the system on each validation day to assess the intraday variability or repeatability. The validation procedure was repeated consecutively in three different days to determine the interday variability or reproducibility of the analytical method.

The differences in mass values detected in the samples were expressed as mass accuracy (△Mass, ppm). Isotope distribution and fragment data were calculated using MetID Solution software (Shimadzu Corp.). The reliability test was conducted via standard material or an international proficiency test. The retention time, repeatability (%), reproducibility (%), accuracy (%), and recovery (%) were calculated. The values of validation parameters at different analyte concentrations were evaluated according to the 2002/657/EC guidelines [10]. The average recovery of the drugs of interest in the sample matrix at the VL is known as accuracy which is acceptable at a range of 70–120% for a multicompound quantitative screening [9]. The linearity of an analytical method is determined by the value of squared regression coefficients (r^2). The calibration curves were constructed in each validation day, and the r^2 values were calculated for each compound. The detection capability (CCβ) at the VL was determined from decision limit (CCα), which, in turn, was calculated from the standard deviation at the VL (SD_{VL}) using the following equations as mentioned in published article [9]:

$CC\beta = CC\alpha + 1.64 \times SD_{VL}$ and $CC\alpha = VL + 1.64 \times SD_{VL.}$

Specificity of the analytical method ensures that the signals measured come from the desired compounds and there is no interference from diluents, endogenous compounds of biological samples, and mobile phase. The specificity of the method was ascertained by analyzing the standard compound in standard and spiked solutions. The chromatograms were monitored for peaks that can potentially interfere with the analytes of interest.

Results and Discussion

The aim of this study was to develop a comprehensive screening method for quantifying and confirming residue of more than 100 veterinary drugs in different matrixes. For regulatory needs, the method must give good results for concentrations below or near the MRL. The objective was thus to validate the method for confirmation and quantification at concentrations near to this limit (<MRL, MRL, >MRL) in spiked blanks obtained from tissues (muscle, kidney, liver), milk, eggs, and fishes. This requires simple and efficient extraction strategies, chromatographic separation, and sensitive detection. The procedure described here uses the very good performances of LC-IT-ToF/MS and a simplified, efficient, and time-saving sample preparation method. A simple, fast, and robust sample preparation procedure based on protein precipitation associated with dispersive solid-phase extraction (SPE) cleanup for tissues (muscle, kidney, liver) and ultrafiltration for milk, egg, and fish was efficient to realize a generic sample preparation for tissues (muscle, kidney, liver), eggs, fishes, and raw milk. The

different polarities needed to be considered for enabling the analysis of a large amount of samples [8]. Extraction methods for the multicompound determination of veterinary drugs in milk, meat, fish, and feed have been described in the previously published articles [9]. Cleanup optimization of multiclass residue can be complex, particularly if a relatively large number of compounds are considered. In most cases, extraction procedures adopted in analytical methodologies for determining drug residues in food are based on solvent extraction followed by cleanup through SPE [8].

Although LC-MS/MS outperformed LC-IT-TOF/MS in terms of qualitative function and utilized in different multiresidue determination methods [11–14], TOF/MS is a perfect instrument for the analysis of unknown compounds, new metabolites, or breakdown products which is not suitable in LC-MS/MS. LC-IT-TOF-MS provides higher sensitivity and accuracy than both TOF and IT-MS. The inherent characteristics of TOF-MS in accurate mass measurements and high resolution make this analyzer attractive in the qualitative analysis of chemical constituents in complex matrixes. While the parent ion of the fragment ions acquired by TOF-MS may not be easier to confirm, the IT-MS should be a good solution due to its abilities of ion isolation and accumulation, as well as multiple stage analysis. The integration of TOF and IT-MS could facilitate the identification and structure elucidation of target and non-target compounds in complex matrices. Furthermore, multiple scans of food products in MSN modes and accurate mass measurements can be performed simultaneously using LC-IT-TOF/MS, which will achieve an unequivocal confirmation of the target analytes by increasing confidence about the origin of the fragmentations. The analysis procedures will also be simplified, and the analysis time will be greatly reduced [15]. We utilize LC-IT-TOF/MS in our current study to analyze multiresidues of veterinary drugs in food products due to these wide advantages of this instrumentation.

Establishment of LC-IT-ToF/MS Screening Method and Validation for Muscle, Kidney, and Liver

A total of 108 substances were targeted for analysis in muscle (beef, pork, and chicken), kidney, and liver tissues, including aminoglycosides, antiprotozoals, benzimidazoles, cephalosporins, ionophores, macrolides, penicillins, quinolones, sulfonamides, and tetracyclines. Multiresidue analysis of the drugs was applied to the various possibilities for sample treatment. Finding suitable extraction conditions for a large range of target analytes displaying different chemical properties (lipophilicity, hydrophilicity, alkaline and acidic characteristics, etc.), along with keeping the procedure as short and simple as possible, is a great challenge [16]. Proper adjustment of sample pH is necessary to avoid deprotonation of acidic compounds and protonation of basic compounds as well as to enhance extraction efficiency [17]. To determine the optimal pH for the sample homogenizing solvent, we tested 1% formic acid (pH 2.3), distilled water (pH 6.5), and 5% ammonium hydroxide (pH 11.3). As shown in Fig. s1a, distilled water (pH 6.5) was identified as the best solvent for homogenizing the analytes. Kaufmann et al. clearly stated that the pH value (5.5) of extraction solvent constitutes a compromise and might not be the optimum for some very acidic and strongly basic analytes [18]. Neutral pH values of the extract loaded in the SPE have been reported in cases when the extraction was held with water, whereas a pH value of 4 was selected by Bohm et al., who used McIlvaine buffer as the extraction solvent [16]. Acidic extraction with trichloroacetic acid was found to be suitable for quinolones, lincomycin, and tetracyclines [16]. Acetonitrile, ethyl acetate, and acetone are the most commonly used for extraction. When we evaluated the most

suitable extraction solvent among acetonitrile, ethyl acetate, acetonitrile/methanol (1:1, *v*/*v*), and acetonitrile/ethyl acetate (1:1, *v*/*v*), acetonitrile produced the best results (Fig. s1b). Having a look at the literature in the field of veterinary drug residue analysis, it becomes apparent that acetonitrile extraction is the most common extraction route used for many veterinary drugs including antibiotics, anthelmintics, and coccidiostats [5, 19, 20].

After extraction and deproteination with acetonitrile, purification using hexane produced better results. Hexane was also used in the final cleanup process in a previous study which ensured the removal of fats from the matrix [16]. As compared with conventional liquid–liquid extraction, SPE exhibits lower cost, reduced processing time, and solvent consumption [21]. In SPE, C_8 or C_{18}-bonded silica, graphitized carbon, ionic exchange resins, or polymeric materials, commercially available in cartridge and disk systems, are the most extensively used SPE sorbents for preconcentration and cleanup of samples [21]. Purification using 250 mg of C_{18}-bonded silica powder yielded the best results compared with the use of other sorbents utilized in purification processes including pressure swing adsorption (PSA) to 125 or 250 mg (Fig. s1c). In a sample filtering test, samples filtered through a 0.2-μm PVDF filter yielded better results than those using 0.2-μm GHP, PTFE, or nylon filters (Fig. s1d). The PVDF was also chosen as the best among different membrane filters in a previously published multiresidue determination method of veterinary drugs [22]. The final sample preparation system chosen comprised acetonitrile/water (4:1, *v*/*v*) containing 2 mM ammonium formate as extraction buffer, 250 mg C_{18}-bonded silica powder and hexane for purification, and a 0.2-μm PVDF syringeless filter.

We validated the designed analysis method for the simultaneous analysis of 110 veterinary drugs analyzed concurrently with substances for which MRLs were established (Fig. 2). Using the standard solvent, qualitative ions were selected by substance, and the differences in mass values detected in the samples were expressed as mass accuracy (ΔMass, ppm). Of the 110 veterinary drugs targeted, 108 (98%) were measured below 10 ppm. The average accuracy of measurement of drug residues in muscle, liver, and kidney tissues at VL concentration ranged from 63 to 122%, with repeatability and reproducibility ranging from 5 to 22% and from 7 to 23%, respectively (Table 1).

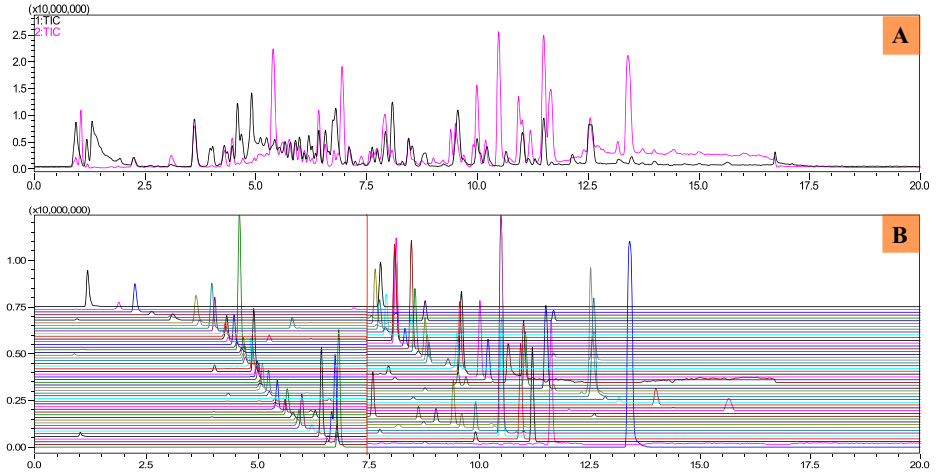

Fig. 2 Total ion chromatograms (**a**) and extracted ion chromatograms (**b**) of 110 veterinary drugs at the validation level in muscle

Establishment of LC-IT-ToF/MS Screening Method and Validation for Milk

The veterinary drugs with MRLs or simultaneous analyzable drugs were targeted for analysis in milk, eggs, and fish. Owing to the very low recovery rate in milk using the preparation method established in meat, the establishment of a second pretreatment process was necessary using an ultracentrifugal filter (cutoff membrane at 3 kDa) for the milk sample [23, 24]. The matrix effects were also tried to reduce in previous study by three different ultrafiltration devices with cutoff membrane at 3, 10, and 30 kDa. Ultrafiltration was achieved faster with 10 and 30 kDa membrane, but the cutoff membrane at 3 kDa was utilized finally for all the sample analysis because the resulting extract by ultrafiltration with 10 and 30 kDa membrane was less clean and a precipitation was observed after the evaporation step. Thus, the ultrafiltration device with cutoff membrane at 3 kDa was used in our study to overcome the matrix effects [23]. This method was faster (time requirement approximately 2 h) and easier to use than the conventional methods. It also reduced the use of organic solvents and increased recovery. The solvent-dependent extraction performance comparison in our study revealed that acetonitrile outperformed methanol and ethyl acetate and showed the best results for the recovery of all drugs except for benzimidazoles. Acetonitrile was normally used in different studies as the preferred extraction solvent due to its excellent hydrophilic and lipophilic extraction performance with the addition of different modifiers such as acetic acid, formic acid, and so forth [8]. As shown in Fig. s2a, extraction using acetonitrile yielded almost 100% recoveries in comparison with the other solvents. The solvent volume-dependent extraction performance comparison revealed that acetonitrile achieved the highest average recovery when the extraction volume was over 2 mL, as compared with 1 and 3 mL (Fig. s2b).

Efficacy validation of the analysis method was performed on the milk analytes for the simultaneous analysis of 88 veterinary drugs that can be analyzed concurrently with substances for which national MRLs have been established: sulfonamides, antiprotozoals, benzimidazoles, cephalosporins, ionophores, macrolides, penicillins, quinolones, and tetracyclines [25, 26]. An analysis was performed by evenly applying the VL to the 88 targeted drugs at the spike levels of 5, 10, 20, 50, and 100 μg/kg. The final data were expressed in terms of the minimum validation level (mVL). Analysis accuracy was calculated by comparing the mass accuracy (ΔMass, ppm) between actual m/z values of the veterinary drugs to be analyzed with LC-IT-ToF/MS and the m/z values measured with IT-ToF. All 88 veterinary drugs were estimated within the range of 20 ppm, as per the recommended specification of the measuring instrument, LCMS-IT-ToF (Shimadzu Corp., Kyoto, Japan). Analysis accuracy and repeatability were calculated as the recovery (%) and relative standard deviation (%), respectively. Recoveries of the 88 analytes at the VL were as follows: sulfonamides, 65–99%; tetracyclines, 60–116%; antiprotozoals, 56–94%; benzimidazoles, 78–105%; cephalosporins, 62–95%; macrolides, 51–124%; penicillins, 66–98%; quinolones, 76–99%; amphenicols, 78–95%; and NSAIDs and others, 62–92%. The recoveries of 105 veterinary drugs in milk in another study ranged from 52 to 92%, where the extraction solvent was acetonitrile containing 0.1% formic acid [8]. The recoveries of six macrolide antibiotics in milk by HPLC-MS-MS were 30–115%, where the extraction solvent was Tris buffer [11]. Eight quinolones for veterinary use in bovine raw milk were quantified and validated in CZE-MS/MS with a recovery of 81–110% where the drugs were extracted from ammonia solution [27]. In the current study, sulfonamides, quinolones, macrolides, antiprotozoals, and NSAIDs showed excellent recoveries. Although the recoveries of tetracyclines, penicillins, and cephalosporins were slightly low, they were more than the recoveries obtained in previous studies [8]. Repeatability was

expressed as the value of the relative standard deviation (%) of the analytes on the same day, and it was approximately 20% in 85% (74/88) of the analytes.

In the case of analytes for which no regulatory limit has been established, CCβ must be as low as possible. But, CCβ must be less than or equal to the MRL for the analytes having an established regulatory limit [10]. Thus, the detection capability (CCβ) of the 88 analytes was expected to measure at levels below their respective MRLs, except for ofloxacin, pefloxacin, and phenylbutazone, which have undetectable domestic MRLs, and penicillins, which have an extremely low MRL (4 μg/kg). The remaining substances with no MRLs were measured in the range 10–100 μg/kg (Table 2). Unlike LC-MS/MS, which only analyze separate targeted ions, the ion-trapping method of ToF-MS/MS, which simultaneously screens all ions, did not show favorable results for the estimation of aminoglycosides, avermectins, and undetectable substances, which have low analysis sensitivity. Moreover, several vermicides that dissolve in dimethyl sulfoxide (DMSO) solvent could not be measured accurately because DMSO interferes with the ion-trapping method of IT-ToF-MS/MS.

Establishment of LC-IT-ToF/MS Screening Method and Validation for Eggs

The preparation method described for milk samples was applied to eggs and fish. To minimize the matrix effect, samples were re-purified with hexane after passing through an ultrafilter required for eggs and fish. The validation method was performed on egg samples for the simultaneous analysis of 51 veterinary drugs that can be analyzed concurrently with substances for which national MRLs apply: sulfonamides, antiprotozoals, benzimidazoles, cephalosporins, ionophores, macrolides, penicillins, quinolones, and tetracyclines. All 51 veterinary drugs were measured within the range of 20 ppm. Recoveries of the 51 analytes at the mVL were as follows: sulfonamides, 61–92%; doxycycline, 61%; tiamulin, 76%; benzimidazoles, 60–105%; macrolides, 51–124%; penicillins, 60–102%; quinolones, 60–104%; and NSAIDs and others, 60–151%. The recoveries of six macrolide antibiotics in Tris buffer from egg matrix were 44–92% which were quantitated by LC-MS/MS [11]. Twelve sulfonamides were quantified from eggs with the recovery rates of 76–93% by LC-MS [28]. Thus, the recovery rates of most of the samples in this study are higher than the conventional methods reported earlier. Repeatability was measured within the range of 20% for 29 analytes and 33% for the remaining 22 analytes. The mVLs of the 51 analytes were set as the lowest analyzable values. Sulfonamides and enrofloxacin/ciprofloxacin, which have undetectable domestic MRLs, were measured at the level of 2.5 μg/kg, close to the limit of quantification value. All substances with MRLs, except for doxycycline (undetectable) and penicillins (4 μg/kg), could be measured below their respective MRLs. The remaining substances with no MRLs were measured in the range of 2.5–125 μg/kg (Table 1). The screening analysis method applied to egg samples was found to be slightly higher in the matrix effect and lower in mass accuracy and repeatability compared with milk.

Establishment of LC-IT-ToF/MS Screening Method and Validation for Fish

The recovery tests were performed on the samples of mudfish, salmon, and eel (Fig. 3) by applying the method that established for milk samples. The highest recovery was shown for eel, followed by salmon and mudfish. Unlike for eels and salmon, mudfish sampling included the whole of the fish, taking into account the intake characteristics of the entire body being consumed. This may have affected recovery, which was only 20% of that for eels. Therefore,

Table 2 Validation parameters are expressed as the range of the average parameters for the three tested matrices (milk, eggs, and fish) at the three concentrations (0.5, 1.0, and 2 times validation level (VL))

Class	No. of compounds	Calculated (*m/z*)	RT[a] (min)	VL[b] (μg/kg)	MRL (μg/kg)	ΔMass[c](ppm)	Accur[d](%)	Rept[e] (%)	Repr[f] (%)	CCβ[g] (μg/kg)
Sulfonamides	17	215.0597–315.0910	4.0–8.1	2.5–100	0–100	0.7–20.6	60–99	2–35	6–28	3.1–112
Tetracyclines	6	229.5997–479.1216	4.6–6.7	25–100	0–400	3.5–20.4	50–116	7–31	2–19	27–110
Quinolones	14	233.0885–400.1467	4.9–9.5	2.5–125	0–500	0.2–21.7	60–104	5–33	10–23	3.5–132
β-Lactams	13	202.0392–518.1515	2.6–9.9	5–1000	4–100	0.3–20	50–111	4–35	8–41	5.1–1013
Macrolides	7	269.5252–828.4740	4.3–9.3	5–200	50–1000	0.7–20.5	51–124	4–35	5–27	5.1–211
Benzimidazoles	10	202.0433–447.1333	3.8–20.7	2.5–100	30–400	3.8–20.7	74–105	1–18	7–22	2.8–105
Ionophores	3	613.3711–895.5026	13.9–15.4	12.5–100	10–50	10–15.5	64–86	7–16	4–33	13.2–113
Coccidiostats	11	191.1038–456.0513	2.2–11.2	12.5–100	10–50	1.3–20.9	50–217	3–53	1–16	12.8–112
NSAIDs	6	152.0723–354.0005	3.7–11.6	5–100	0	1.7–19.8	61–151	9–28	7–21	5.2–111
Others	11	207.0902–660.8449	2.2–13.4	10–100	200	0.1–22.2	56–105	1–23	5–28	12–114

MRL maximum residue limit

[a] RT: retention time in minutes as determined in chromatograms of analyte standard solutions

[b] VL: validation level is the concentration spiked to samples (muscle, kidney, and liver) in g/kg; validation was carried out 0.5, 1.0, and 2 times VL

[c] ΔMass: mass measurement error relative to the calculated exact mass of the analyte

[d] Accur: the recovery of the spiked analyte concentration expressed as a percentage

[e] Rept: repeatability expressed as the relative standard deviation in 20 analyses performed on 1 day

[f] Repr: reproducibility expressed as the relative standard deviation in 20 analyses performed at different days

[f] CCβ: detection capability calculated from the within-lab reproducibility at the validation level 1.0 times VL

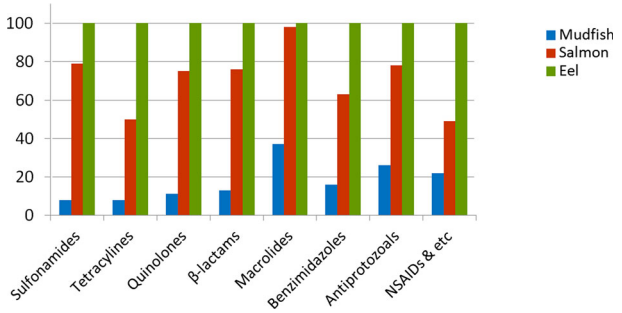

Fig. 3 Comparison of recovery results from mudfish, salmon, and eel using the extraction buffer

eels were used as the validation sample. Efficacy validation of the analysis method was performed on eel samples for the simultaneous analysis of 61 veterinary drugs concurrently analyzable with the substances for which national MRLs apply: sulfonamides, antiprotozoals, benzimidazoles, cephalosporins, ionophores, macrolides, penicillins, quinolones, and tetracyclines. Analysis was performed by evenly applying the VL to the 61 analytes at the spike levels of 12.5, 25, 50, and 100 μg/kg. The final data were expressed as mVLs. All 61 veterinary drugs were measured within the range of 20 ppm, the recommended specification of the measuring instrument, LC-IT-ToF-MS (Shimadzu Corp., Kyoto, Japan) as mass accuracy (ΔMass, ppm). The recoveries of the 61 analytes at the mVL ranged between 60 and 217%. Repeatability was measured within the range of 20% for 58 analytes and 35% for the remaining 3 analytes. The mVLs of the 61 analytes were set as the lowest analyzable values. Norfloxacin, ofloxacin, and pefloxacin, which have zero tolerance domestic MRLs, were measured at the level of 12.5–25 μg/kg. Except for norfloxacin, ofloxacin, and pefloxacin, all substances with MRLs were measured below their respective MRLs. The remaining substances with no MRLs were measured in the range 12.5–100 μg/kg (Table 1).

Reliability Test via Proficiency Test and NRP Sample

The screening method developed in this study passed the screening test administered by the proficiency test of the ISO 17025-accredited laboratory by detecting four substances out of three unknown samples (pig muscle) and five classes of antibiotic and antibacterial substances (quinolones, β-lactams, sulfonamides, macrolides, and tetracyclines). The substances detected using LC-IT-ToF/MS were ceftiofur (β-lactam class), desfuroylceftiofur (metabolite), sulfadiazine (sulfonamide class), and tulathromycin (macrolide class). Using the LC-IT-ToF/MS screening method established for milk, we applied to the NRP and confirmed that the samples screened (raw milk) did not contain any of the 88 veterinary drug residues.

According to the validation strategy mentioned in CD 2002/657/EC, a screening method can be considered as qualitative when detection capability (CCβ), specificity, and applicability were determined [10]. The qualitative screening method is considered as quantitative, only when the precision of the method is determined together with the determination of detection capability (CCβ), specificity, and applicability [10]. Detection capability (CCβ), specificity, applicability, and precision (repeatability and reproducibility) of the currently developed multiresidue method are determined in this study, which demonstrated that the developed method is qualitative as well as quantitative.

Conclusion

Here, we reported a simple and rapid multiresidue screening method for the simultaneous measurement of over 100 veterinary drugs found in the food of animal origin. With the current method, the highest sample recoveries were achieved using distilled water (pH 6.5) for sample pretreatment, acetonitrile for extraction, hexane for protein purification, and C_{18}-bonded silica powder (500 mg) for the solid-state extraction phase. For some samples, using 0.2-μm PVDF and high-density ultracentrifugal filters (cutoff membrane at 3 kDa) further improved the detection accuracy. The simple pretreatment and rapid detection method significantly reduce the time (2–3 h), human resource, and hazardous organic solvent requirements compared with conventional methods. The efficacy validation of the current analytical method was satisfactory in terms of average accuracy, repeatability, reproducibility, VL, mass accuracy, and detection capability (CCβ), with values that were under the MRLs confirmed as measured parameters. Moreover, LC-IT-ToF/MS was far superior in terms of simultaneously detecting a large number of veterinary drug residues in a single run. Moreover, it was capable of screening over 100 substances of different classes, thereby overcoming the disadvantages of conventional immunochemical test kits, which are limited to class-specific or material-specific screening. Once the initial cost of the equipment has been met, the system will greatly reduce screening cost and time in inspection labs and will improve testing efficacy and outcome reliability. The currently established method provides an effective tool for the determination of veterinary drug residues in livestock and food products, and thus, we recommend this validated method for routine analysis in food safety control.

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