Multi-class, multi-residue analysis of trace veterinary drugs in milk by rapid screening and quantification using ultra-performance liquid chromatography–quadrupole time-of-flight mass spectrometry

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ABSTRACT

A simple and rapid multi-class multi-residue analytical method was developed for the screening and quantification of veterinary drugs in milk by ultra-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS). A total of 90 veterinary drugs investigated belonged to almost 20 classes including lincomycins, macrolides, sulfonamides, quinolones, tetracyclines, β -agonists, β -lactams, sedatives, β -receptor antagonists, sex hormones, glucocorticoids, nitroimidazoles, benzimidazoles, nitrofurans, and some others. A modified quick, easy, cheap, effective, rugged, and safe (QuEChERS) procedure was developed for the sample preparation without the solid-phase extraction step. The linearity, sensitivity, accuracy, repeatability, and reproducibility of the method were fully validated. The response of the detector was linear for each target compound in a wide concentration range with a correlation coefficient (R^2) of 0.9973 to 0.9999 (among them $R^2 > 0.999$ for 73 of 90 analytes). The range of the limit of quantification for these compounds in the milk ranged from 0.10 to 17.30 $\mu\text{g}/\text{kg}$. The repeatability and reproducibility were in the range of 2.11 to 9.62% and 2.76 to 13.9%, respectively. The average recoveries ranged from 72.62 to 122.2% with the RSD ($n = 6$) of 1.30 to 9.61% at 3 concentration levels. For the screening method, the data of the precursor and product ions of the target analytes were simultaneously acquired under the all ions MS/MS mode in a single run. An accurate mass database for the confirmation and identification of the target compounds was established. The applicability of the screening method was verified by applying to real milk samples. The proposed analytical method allows the identification and confirmation of the target vet-

erinary drugs at trace levels employing quick analysis time. Certain veterinary drugs were detected in some cases.

Key words: veterinary drug, milk, QuEChERS, ultra-performance liquid chromatography, quadrupole time-of-flight mass spectrometry

INTRODUCTION

Veterinary drugs have been widely used in medical and veterinary practice to treat and prevent diseases and to enhance growth rate and feed efficiency. If they are not used correctly, the practice could lead to the presence of veterinary drug residues in foods of animal origin. Their control is highly important for the agricultural environment and food industry. Bovine milk is one of the most important components of the human diet. The risk of veterinary drug residues in milk is significant. To ensure human food safety, the European Union has set a maximum residue limit (MRL) of 4 to 30 $\mu\text{g}/\text{kg}$ for penicillins, 20 to 100 $\mu\text{g}/\text{kg}$ for cephalosporins, 30 to 100 $\mu\text{g}/\text{kg}$ for quinolones, and 100 $\mu\text{g}/\text{kg}$ for sulfonamides (SA) in milk (European Union, 2010). These low values require the development of analytical methods that are sensitive enough to monitor and determine these drugs in bovine milk.

Recently, Garrido Frenich et al. (2014) provided an overview of current methods based on ultra-high-performance liquid chromatography coupled to mass spectrometry analyzers (UPLC-MS), for the determination of organic contaminants and residues in food. Currently, UPLC-MS has become a very common tool to analyze drug residues in various food matrices. Several papers are available on the analysis of veterinary drugs in milk, but most focus on only few compounds or one class of antibiotics, such as quinolones (Hermo et al., 2008), penicillins (Martínez-Huélamo et al., 2009), β -lactams (Kantiani et al., 2009), and ionophores (Thompson et al., 2011). These single-class methods are relatively easy for the optimization of both extrac-

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tion and instrumental parameters due to the similar physical and chemical properties of targeted drugs from the same family. For the high number of veterinary drugs that need to be controlled, it is highly important to develop multi-class, multi-residue methods that permit analysis for a variety of drugs with a single procedure. Several UPLC-MS/MS methods concerning milk have been reported for the multi-residue determination of β -lactams and veterinary drugs (Aguilera-Luiz et al., 2008; Turnipseed et al., 2008), pesticides and mycotoxins (Aguilera-Luiz et al., 2011), some anthelmintic drugs and phenylbutazone (Kaufmann et al., 2011), and 18 residues of antibiotics, coccidiostats, and corticosteroids (Nebot et al., 2012). Recently, the developed generic and rapid method was able to analyze 8 classes of veterinary drug, but with average relative recoveries from 63 to 141%, the method accuracy needs to be improved further (Zhan et al., 2012). The UHPLC-Orbitrap-MS can offer efficient performance for screening purposes, and it can also effectively provide adequate quantification/confirmation values of veterinary drugs in the positive milk samples (Romero-González et al., 2011).

Ultra-performance liquid chromatography coupled to time-of-flight mass spectrometry (**UPLC-TOF-MS**) also showed very good performances for quantification and allowed the determination of majority of compounds below MRL in milk (Ortelli et al., 2009). Liquid chromatography–quadrupole–time-of-flight–mass spectrometry (**LC-Q-TOF-MS**) was used for the analysis of sulfamethazine, flunixin, cephalirin, or enrofloxacin and metabolites in milk (Turnipseed et al., 2011). The UPLC-QTOF-MS method was able to quantify 34 or screen up to 59 drugs in milk (Wang and Leung, 2012), and to investigate the veterinary drugs belonging to 9 families (Kang et al., 2014). The screening levels of detection for more than 150 veterinary drug residues in milk were determined by the LC-Q-TOF-MS method, and more than half of those tested can be detected at concentrations of 10 ng/mL or less; 72% can be found in milk when present at 100 ng/mL (Turnipseed et al., 2014). Recently, UPLC-Q-TOF-MS was applied for qualitative multi-residue screening of 143 veterinary drugs and pharmaceuticals in milk and fish tissue (Dasenaki et al., 2015), and satisfactory results were obtained at concentration levels of 15 ng/mL in milk and 20 μ g/kg in fish (>60% of the compounds detected and identified). However, the sensitivity of these methods needs to be improved further for trace residue analysis.

Despite the advances in separation and detection techniques, sample extraction is still a cornerstone of the analytical process, and effective sample prepara-

tion is essential to achieve reliable results and maintain instrument performance. Because milk is a complex matrix due to its high protein and fat content, which often interfere in analytical procedures, special focus has been placed on sample preparation (extraction and cleanup). The previous sample pretreatments focused on a few compound or a single class of veterinary drugs. For multi-class, multi-residue analysis, it is difficult to develop a common extraction procedure and chromatographic conditions. In general, after protein precipitation and centrifugation, solid-phase extraction can be applied to determine fluoroquinolones in milk (Zheng et al., 2014), veterinary drugs in milk (Stolker et al., 2008), and mycotoxins and pesticides in milk (Aguilera-Luiz et al., 2011); however, these methods are time consuming and require large volumes of organic solvent. Recently solid-phase microextraction has been reported for monitoring estrogens in milk powder, low solvent consumption, and ease of coupling with chromatographic instruments (Lan et al., 2014). A dispersive liquid–liquid microextraction method was used for the determination of quinolones and β -lactams in raw cow milk (Junza et al., 2014). To simplify the sample pretreatment procedure, the quick, easy, cheap, effective, rugged, and safe (**QuEChERS**) method is a streamlined approach that makes it easier and less expensive for analytical chemists to examine pesticide residues in food (Schenck and Hobbs, 2004). However, it is necessary to modify QuEChERS procedure for different target analytes and matrices. A new sample preparation procedure loosely based on QuEChERS methodology was used for the extraction of nitroimidazoles, sulfonamides, fluoroquinolones, quinolones, ionophores, and dinitrocarbanilide in animal tissues (Stubbings and Bigwood, 2009). A modified QuEChERS procedure for the preparation of milk sample was reported to determine 18 veterinary drugs, but with limits of detection (**LOD**) ranging from 1 to 4 μ g/kg (Aguilera-Luiz et al., 2008). A total of 21 veterinary drugs were extracted from milk samples using a modified QuEChERS method, and no further cleanup steps were necessary (Martínez Vidal et al., 2010). It should be pointed out that the original and reported relative QuEChERS methods need to be modified further, including the type and amount of extraction solvent, the appropriate acid, salting-out procedure, and the type and amount of sorbent, for the analysis of multi-residue, multi-class veterinary drugs with physical and chemical performances in complex matrices.

A total of 90 veterinary drugs selected for the study belong to almost 20 classes of veterinary drugs including lincomycins, macrolides, sulfonamides, quinolones, tetracyclines, β -agonists, β -lactams, sedatives, β -receptor

antagonists, sex hormones, glucocorticoids, nitroimidazoles, benzimidazoles, nitrofurans, and some others (Table 1). The purpose of this study is to develop a modified QuEChERS method coupled with UPLC-QTOF-MS for the identification and quantification of the 90 residues in milk. The parameters for QuEChERS were optimized in this study. The effectiveness of the proposed method was confirmed by detecting trace residues in real milk samples.

MATERIALS AND METHODS

Chemicals and Solutions

Ninety veterinary standards (purity $\geq 95\%$) listed in Table 1 were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Individual stock solutions (1,000 mg/L) were prepared by dissolving 10 mg of each compound in 10 mL of methanol, and stored at -18°C . Mixed standard solution at concentrations of 1 mg/L of each standard was prepared by additive mixing 10 μL of each stock solution and diluting to 10 mL with methanol. The mixed standard solutions were stored in amber bottles at -18°C . The penicillins are not very stable drugs, and these compounds were therefore added to the mixed standard solution just before analysis.

The HPLC-grade methanol, acetonitrile, ethyl acetate, dichloromethane, and formic acid were purchased from Dikma Technologies Inc. (Tianjin, China). Guaranteed reagent-grade acetic acid (36%), magnesium sulfate, and sodium acetate, and analytical reagent-grade sodium chloride, sodium sulfate, sodium citrate, disodium citrate, and disodium EDTA were purchased from Guangfu Research Institute (Tianjin, China). ProElut QuEChERS kit including 4 sorbents (150 mg of PSA/900 mg of MgSO_4 , 400 mg of PSA/1,200 mg of MgSO_4 , 400 mg of PSA/400 mg of C18/1,200 mg of MgSO_4 , 150 mg of PSA/150 mg of C18/900 mg of MgSO_4) were purchased from Dikma Technologies Inc. (Tianjin, China), and 5 sorbents (C18, PAX, PSA, NH_2 , and Alumina-N) were purchased from Tianjin Bonna-Agela Technologies Ltd. (Tianjin, China).

Instrumentation

The LC-30A HPLC (Shimadzu Co., Kyoto, Japan) and QTOF5600 mass spectrometer (Applied Biosystems, Waltham, MA) with an electrospray ionization (ESI) source were used. A 3K-30 high-speed refrigerated centrifuge (Sigma Co., St. Louis, MO), TurboVap LV Cyclone concentrator (Caliper Co., USA), G560E Vortex mixer (SI Vortex-Genie 2 Co., USA), and SA300 horizontal oscillator (Yamato Co., Japan) were used for sample preparation.

UPLC-QTOF-MS Analysis Conditions

Separation of the analytes was achieved on an Acquity UPLC BEH C18 column (100 mm $\times$ 2.1 mm i.d., 1.7 μm ; Waters, Milford, MA) at 40°C with the mobile phase consisting of (A) 0.1% formic acid and (B) acetonitrile. Gradient elution was conducted as follows: 97% $\rightarrow$ 95% A (0–3 min), 95% $\rightarrow$ 10% A (3–15 min), 10% A (15–19 min), 10% $\rightarrow$ 97% A (19–19.1 min), and 97% A (19.1–26 min). Sample injection volume was 3 μL , and flow rate was 0.3 mL/min.

In QTOF-MS experiments with full scan mode, ESI⁺ mode was used for the simultaneous determination of the 90 analytes. The MS parameters were as follows: ESI⁺ electrospray ionization: temperature, 550°C ; ionization source spray voltage 5.4 kV; the atomization gas pressure and temperature: 0.345 MPa and 550°C ; auxiliary gas, 0.414 MPa, 550°C ; air curtain air pressure, 0.207 MPa; declustering potential, 80.0 V; collision energy, 20 V, 35 V, 50 V; mass scan range, 50 to 1,000 m/z . The TOF score needs to be $\geq 70\%$, which was calculated using the deviation of the accurate mass, retention time, and the distribution and proportion of isotopic clusters between the measured and theoretical values. Total ion current chromatogram was observed and the extracted ion chromatograms of the 90 veterinary drugs were obtained using PeakView software (AB Sciex Co., Foster City, CA). The data of the precursor and product ions of the target analytes were simultaneously acquired under the All Ions MS/MS mode in a single run. For the method validation, various parameters such as linearity, recovery, repeatability, reproducibility, LOD, and limits of quantification (LOQ) were evaluated. The peak area of the most intense transition versus the concentration was used to establish the linear regression equation. The linearity of the method was evaluated based on matrix-matched calibration.

Sample Preparation

Submitted milk samples were obtained from different places in China, and stored at -18°C until analysis. Samples were fortified with the targeted compounds during the optimization and validation of the developed procedure. The targeted residues were extracted from milk using an extraction procedure based on the modified QuEChERS methodology. The procedure was as follows: 2.0 g of milk or milk powder samples was weighed in a 50-mL polypropylene tube followed by addition of 20 mL of 1% acetic acid–acetonitrile. Subsequently, the mixture was stirred in a shaker for 30 s; then, 1.0 g of NaCl and 2.0 g of Na_2SO_4 were added and the tubes were shaken for 5 min. After centrifugation at $10,733 \times g$ and 10°C for 5 min, 10 mL of

Table 1. Accurate mass, found mass, error, retention time, purity score, and purity project

No.	Veterinary drug	Accurate mass (Da)	Found mass (Da)	Error (ppm)	Retention time (min)			Purity score (%)	Purity project ¹
					Solvent	Sample	Deviation		
	Lincomycin series antibiotics (2)								
1	Clindamycin	425.1872	425.1872	0	7.39	7.14	0.25	77.1	++++
2	Lincomycin	407.2210	407.2208	−0.5	4.84	3.63	1.21	85.4	+ #+++
	Macrolide antibiotics (7)								
3	Erythromycin	734.4685	734.4650	−4.8	8.47	8.48	0.01	81.9	++++
4	Josamycin	828.4740	828.4739	−0.1	9.84	9.53	0.31	92.8	++++
5	Kitasamycin	786.4634	786.4679	5.7	9.55	8.75	0.8	86.8	##+++
6	Roxithromycin	837.5319	837.5326	0.9	9.53	9.22	0.31	92.5	++++
7	Spiramycin	843.5213	843.5161	−3.6	7.00	6.99	0.01	86.0	++++
8	Tilmicosin	869.5733	869.5698	−4.1	7.86	7.54	0.32	84.5	++++
9	Tylosin	916.5264	916.5286	2.4	8.74	8.47	0.27	92.3	++++
	Sulfonamides (19)								
10	Sulfabenzamide	277.0641	277.0656	5.4	7.71	7.56	0.15	85.3	#+++
11	Sulfachloropyridazine	285.0208	285.0206	−0.6	7.83	7.68	0.15	80.7	++++
12	Sulfadiazine	251.0597	251.0597	−0.2	2.91	2.57	0.34	75.4	++++
13	Sulfadimethoxine	311.0809	311.081	0.3	7.99	7.85	0.14	91.4	++++
14	Sulfadoxine	311.0809	311.0816	2.3	7.0	6.77	0.23	81.9	++++
15	Sulfamethoxazole	254.0594	254.0594	0	6.97	6.8	0.17	89.3	++++
16	Sulfamethoxydiazine	281.0703	281.0708	2	6.5	6.26	0.24	70.9	++++
17	Sulfamethoxypyridazine	281.0703	281.0702	−0.4	5.96	5.64	0.32	91.3	++++
18	Sulfamonomethoxine	281.0703	281.0703	0	5.80	5.58	0.22	76.3	++++
19	Sulfanitran	336.0649	336.0653	1.3	9.09	9.00	0.09	89.2	++++
20	Sulfaphenazole	315.0910	315.0910	0	8.08	7.95	0.13	97.2	++++
21	Sulfapyridine	250.0645	250.0643	−0.9	3.99	3.44	0.55	75.4	+ #+++
22	Sulfaquinoxaline	301.0754	301.0755	0.5	8.04	7.89	0.15	90.1	++++
23	Sulfathiazole	256.0209	256.0206	−1.3	3.73	3.22	0.51	82.6	+ #+++
24	Sulfisoxazole	268.0750	268.0766	5.8	7.33	7.16	0.17	94.2	#+++
25	Sulfaclozine sodium monohydrate	285.0208	285.0206	−0.6	7.83	7.68	0.15	82.9	++++
26	Sulfsomidin	279.0910	279.0912	0.7	5.7	5.26	0.44	80.4	++++
27	Sulfamoxol	268.0750	268.0750	−0.1	5.61	5.16	0.45	96.5	++++
28	Trimethoprim	291.1452	291.1451	−0.2	5.47	4.82	0.65	81.8	+ #+++
	Quinolones (18)								
29	Ciprofloxacin	332.1405	332.1404	−0.3	5.85	5.51	0.34	73.3	++++
30	Danofloxacin	358.1562	358.1560	−0.4	6.07	5.79	0.28	77.2	++++
31	Difloxacin	400.1467	400.1480	3.3	6.64	6.39	0.25	86.5	++++
32	Enrofloxacin	360.1718	360.1718	0	6.2	5.92	0.28	65.3	++++#
33	Fleroxacin	370.1373	370.1375	0.5	5.69	5.27	0.42	87.1	++++
34	Flumequine	262.0874	262.0871	−1	8.97	8.85	0.12	93.4	++++
35	Lomefloxacin	352.1467	352.1467	−0.1	6.05	5.74	0.31	77.5	++++
36	Marbofloxacin	363.1463	363.1465	0.6	5.48	4.92	0.56	94.2	+ #+++
37	Nalidixic acid	233.0921	233.0921	0.3	8.72	8.57	0.15	88.4	++++
38	Norfloxacin	320.1405	320.1392	−4.2	5.72	6.67	0.95	78.6	+ #+++
39	Ofloxacin	362.1511	362.1510	−0.2	5.75	5.38	0.37	94.5	++++
40	Orbifloxacin	396.1530	396.1529	−0.1	6.32	6.03	0.29	86.3	++++
41	Oxolinic acid	262.0710	262.0710	−0.2	7.56	7.38	0.18	89.5	++++
42	Piromidic acid	289.1295	289.1294	−0.5	9.7	9.58	0.12	94.0	++++
43	Sarafloxacin	386.1311	386.1307	−0.9	6.58	6.32	0.26	82.7	++++
44	Sparfloxacin	393.1733	393.1733	0	6.67	6.42	0.25	80.7	++++
45	Cinoxacin	263.0663	263.0663	0.2	7.08	6.88	0.2	92.2	++++
46	Pefloxacin	334.1562	334.1562	0.1	5.8	5.45	0.35	78.2	++++
	Tetracycline antibiotics (4)								
47	Chlortetracycline	479.1216	479.1171	−9.3	6.5	6.88	0.38	91.6	#+++
48	Doxycycline	445.1605	445.1604	−0.2	7.42	5.73	1.69	76.9	+ #+++
49	Oxytetracycline	461.1555	461.1554	−0.1	5.75	5.33	0.42	77.1	++++
50	Tetracycline	445.1605	445.1604	−0.2	6.08	5.73	0.35	82.5	++++
	β-agonist class (6)								
51	Brombuterol	366.9839	366.9835	−0.9	7	6.72	0.28	96.7	++++
52	Cimaterol	220.1444	220.1335	−8.1	1.9	1.54	0.36	84.1	#+++
53	Clenbuterol	277.0869	277.0869	−0.1	6.57	6.25	0.32	90.9	++++
54	Mabuterol hydrochloride	311.1133	311.1133	0.3	7.28	7.00	0.28	88.5	++++
55	Penbutolol	292.2271	292.2274	0.9	9.47	9.18	0.29	93.7	++++
56	Ractopamine	302.1751	302.1749	−0.6	6.01	5.67	0.34	90.8	++++

Continued

Table 1 (Continued). Accurate mass, found mass, error, retention time, purity score, and purity project

No.	Veterinary drug	Accurate mass (Da)	Found mass (Da)	Error (ppm)	Retention time (min)			Purity score (%)	Purity project ¹
					Solvent	Sample	Deviation		
β-lactam antibiotics (6)									
57	Cloxacillin	436.0729	436.0725	−0.8	10.06	9.88	0.18	91.6	++++
58	Dicloxacillin	470.0339	470.0333	−1.1	10.69	10.54	0.15	83.2	++++
59	Nafcillin	415.1322	415.1324	0.4	10.3	10.14	0.16	96.4	++++
60	Oxacillin	402.1118	402.1121	0.7	9.6	9.42	0.18	87.5	++++
61	Penicillin V	351.1009	351.1012	0.8	9.27	9.08	0.19	93.2	++++
62	Piperacillin	518.1704	518.1700	−0.7	8.46	8.23	0.23	91.8	++++
Sedatives and β-receptor antagonists (2)									
63	Carazolol	299.1754	299.1752	−0.8	7.36	7.10	0.26	95.7	++++
64	Chlorpromazine	319.1030	319.1032	0.6	9.56	9.27	0.29	92.1	++++
Sex hormones (5)									
65	Methyltestosterone	303.2319	303.2319	0.1	10.6	10.49	0.11	86.7	++++
66	Testosterone	289.2162	289.2160	−0.6	10.18	10.07	0.11	80.5	++++
67	Progesterone	315.2319	315.2318	−0.1	12.21	12.15	0.06	93.2	++++
68	Testosterone propionate	345.2424	345.2422	−0.6	14	13.98	0.02	82.6	++++
69	Methandienone	301.2162	301.2162	−0.2	9.93	9.82	0.11	78.0	++++
Glucocorticoid (6)									
70	Beclomethasone	409.1776	409.1772	−1	9.2	9.09	0.11	81.1	++++
71	Betamethasone	393.2072	393.2067	−1.3	9.01	8.90	0.11	73.2	++++
72	Dexamethasone	393.2072	393.2067	−1.3	9.01	8.90	0.11	79.4	++++
73	Hydrocortisone	363.2166	363.2171	1.4	8.32	8.17	0.15	64.2	+++ [#]
74	Prednisolone	361.2010	361.2022	3.4	8.25	8.09	0.16	58.3	+++ [#]
75	Trenbolone	271.1693	271.1690	−0.9	9.63	9.51	0.12	90.4	++++
Nitroimidazoles (3)									
76	Dimetridazole	142.0611	142.0610	−1	2.64	2.39	0.25	86.9	++++
77	Metronidazole	172.0717	172.0714	−1.3	2.16	1.9	0.26	76.2	++++
78	Ronidazole	201.0618	201.0607	−5.8	2.83	2.52	0.31	88.3	[#] ++++
Benzimidazoles (2)									
79	Mebendazole	296.1030	296.1028	−0.5	8.48	8.33	0.15	99.7	++++
80	Oxibendazole	250.1186	250.1189	1	7.41	7.15	0.26	99.7	++++
Nitrofurans (4)									
81	Furazolidone	226.0459	226.0457	−0.8	5.91	5.59	0.32	94.1	++++
82	Nitrofurazone	199.0462	199.0449	−6.2	5.03	4.48	0.55	67.2	[#] +++ [#]
83	Furaltadone	325.1143	325.1145	0.7	2.72	2.29	0.43	76.5	++++
84	Nitrofurantoin	239.0411	239.0414	1.2	5.44	5.04	0.4	74.4	++++
Others (6)									
85	Tiamulin	494.3299	494.3296	−0.5	9.11	8.85	0.26	96.8	++++
86	Valnemulin	565.3670	565.3670	0.1	9.63	9.34	0.29	78.4	++++
87	Praziquantel	313.1911	313.1908	−0.7	10.32	10.21	0.11	98.4	++++
88	Toltrazuil	426.0730	426.0736	1.5	12.48	12.41	0.07	83.1	++++
89	Xylazine	221.1107	221.1103	−1.9	6.47	6.13	0.34	99.3	++++
90	Colchicine	400.1755	400.1766	2.8	7.94	7.76	0.18	87.1	++++

¹Score standards: ++++: mass deviation ≤ 0.5 ppm, retention time deviation ≤ 0.5 min, isotope-ratio deviation ≤ 0.20 , and purity score $> 70.0\%$; +++: mass deviation > 0.5 ppm, retention time deviation ≤ 0.5 min, isotope-ratio deviation ≤ 0.20 , and purity score $> 70.0\%$; ++: mass deviation ≤ 0.5 ppm, retention time deviation > 0.5 min, isotope-ratio deviation ≤ 0.20 , and purity score $> 70.0\%$; #: mass deviation > 0.5 ppm, retention time deviation > 0.5 min, isotope-ratio deviation ≤ 0.20 , and purity score $> 70.0\%$; ++#: mass deviation ≤ 0.5 ppm, retention time deviation ≤ 0.5 min, isotope-ratio deviation ≤ 0.20 , and purity score 50 to 70.0%; +++#: mass deviation > 0.5 ppm, retention time deviation ≤ 0.5 min, isotope-ratio deviation ≤ 0.20 , and purity score 50 to 70.0%.

the acetonitrile layer was transferred to an Eppendorf tube containing 100 mg of C18 sorbents followed by oscillation for 5 min and centrifuged again under the same conditions described above. Five milliliters of the supernatant was evaporated to dryness by a nitrogen blowing concentrator in a water bath at 50°C. The residue was re-dissolved with 1 mL of 25% acetonitrile aqueous solution. The sample extract was filtered through a 0.22- μ m PTFE filter into an auto-sampler vial for the UPLC-QTOF-MS analysis.

RESULTS AND DISCUSSION

Optimization of Extraction and Cleanup

A wide range of analytes with different physicochemical properties was used in this study. For this reason, a more generic treatment should be applied when developing multi-class residue methods (Mol et al., 2008). Because the tested milk and milk powder matrix contains interferences such as proteins, fat, lactose, and

Figure 1. Effect of different solvents (a) and sorbents (b) on the extraction of the analytes. Solvent serial number: 1: dichloromethane, 2: methanol, 3: ethyl acetate, 4: acetonitrile, 5: 1% acetic acid in acetonitrile, 6: 3% acetic acid in acetonitrile, 7: 5% acetic acid in acetonitrile, 8: EDTA-acetonitrile. Sorbent serial number: 1: 150 mg of PSA/900 mg of MgSO_4 , 2: 400 mg of PSA/1200 mg of MgSO_4 , 3: 400 mg of PSA/400 mg of C18/1,200 mg of MgSO_4 , 4: 150 mg of PSA/150 mg of C18/900 mg of MgSO_4 , 5: 200 mg of C18, 6: 200 mg of NH_2 , 7: 200 mg of PAX, 8: 200 mg of PSA, 9: Alumin-N200 mg. Color version available online.

AA, the success of the extraction procedure depended on the effective degreasing and deproteinization. The effect of several solvents and their combinations, as well as salting-out agents on recovery was investigated, including methanol, acetonitrile, ethyl acetate, dichloromethane, Na_2EDTA -acetonitrile, and 1, 3, and 5% acetic acid in acetonitrile (Figure 1a).

Better recoveries were obtained for most of the compounds when acetonitrile acidified with acetic acid was used. It should be noted that the elimination of acetic acid could only extract free sulfonamides present in the samples, and did not help to regain β -lactams and penicillins and led to the low recovery for quinolones (Gómez-Pérez et al., 2012). The pH of extraction solvent influences the ionization for acidic-alkaline amphoteric substances such as sulfonamides macrolides, quinolones, tetracyclines, and nitroimidazoles. The result indicated that 20 mL of acetonitrile acidified with 1% acetic acid was better; a mean recovery of 68.5 to 120% was obtained for 90 analytes, and among them, recovery of 80 to 120% was obtained for 73 analytes.

In this work, 4 groups of salt were used as a salting-out agent to partition drug residues into the acetonitrile layer, including 1 g of sodium acetate/4 g of sodium sulfate, 1 g of sodium chlorate/4 g of sodium sulfate, 1

g of sodium chlorate/4 g of magnesium sulfate, 1 g of sodium chlorate/4 g of magnesium sulfate/1 g of sodium citrate/0.5 g of disodium citrate. The result showed that the addition of sodium acetate or magnesium sulfate decreased recovery of quinolones, sulfonamides, tetracyclines, and macrolides, among them, markedly decreasing for tetracyclines because the formation of complex precipitation between Mg^{2+} and tetracyclines. We found that 1 g of sodium chloride/4 g of sodium sulfate instead of sodium acetate-magnesium sulfate resulted in recovery of 62.52 to 129.1%. Further testing showed that when using 1 g of sodium chloride/2 g of sodium sulfate as a salting-out agent, a recovery of 67.34 to 124.7% was achieved, which was used in the next work.

In this work, the cleanup for 9 sorbents was compared and recovery of 90 analytes was determined (Figure 1b). Two hundred milligrams of C18 sorbent made 69 analytes achieving the recovery of 80 to 120%. The effect of different C18 dosage (100, 200, 400, and 600 mg) on recovery was investigated further. The result showed using 400 and 600 mg of C18 sorbent could not improve the recovery, whereas using 100 mg of C18 sorbent obtained the same recovery with 200 mg of C18 sorbent, which was used in next work.

The effect of sample quality (1, 2, 3, and 4 g) on recovery was also investigated. The result showed that when 2 g of sample was extracted with 20 mL of acetonitrile followed by cleanup with 100 mg of C18 sorbent, the recovery of 68.54 to 119.8% was achieved for 90 analytes.

Optimization of UPLC-QTOF-MS Parameters

An 100-mm-long Acquity BEH C_{18} column showed the best elution and separation of the analytes compared with a 50-mm-long Acquity BEH C_{18} column. Regarding UPLC separation, the mobile phase composition was investigated to maximize the method sensitivity. The result showed that the mobile phase consisted of 0.1% formic acid aqueous solution and acetonitrile was optimal. The gradient was optimized to render a rough separation between the 90 selected target compounds in the complex matrix. The elution gradient was also optimized by varying the percentage of the selected mobile phase (A) throughout the run. Finally, linear mobile phase gradient is described as above. The optimal separation of 90 detected compounds was achieved using a 100-mm-long Acquity BEH C_{18} column (Waters) with a 1.7- μm particle and elution gradient under 0.3 mL/min of mobile phase.

The MS experiments were performed in TOF full scan mode. Total ion chromatograms for matrix-matched

multi-compound standard solutions, an acceptable baseline chromatographic separation was achieved in 26 min for 90 target compounds monitored simultaneously using the elution program.

For the screening method, the data of the precursor and product ions of the target analytes were simultaneously acquired under the all ions MS/MS mode in a single run. The better sensitivity for 90 analytes was obtained at ESI⁺ capillary 3000 V and cone voltage of 35 V. In QTOF experiments, we selected the protonated molecule [M+H]⁺ ion as precursor ion. Using PeakView software, the defined search criteria were accurate mass and retention time tolerances. The compound database established with Analyst TF1.6 software (AB Sciex Co.) in this work is shown in Table 1. In addition, the database attached with the 4 MS/MS spectra for each target analyte. The most intense product ion was used for quantification with MultiQuant 2.1 software (AB Sciex Co.).

Screening and Confirmation by UPLC-QTOF-MS

The accurate-mass database for the 90 analytes includes data not only on the accurate masses, retention time, and isotopic pattern data of the target ions but also on the characteristic in-source fragment ions. It has to be mentioned that the confirmatory analysis of the suspected samples has to be done by the use of an MS/MS technique because criteria for confirmation of the identity of drugs by QTOF-MS are not included yet in the European Union guidelines (Stolker et al., 2008). Figure 2 shows the 4 MS/MS spectra of the most representative compounds (ciprofloxacin, sulfadiazine, and tetracycline) at different collision energies.

Qualitative screening using UPLC-QTOF-MS with PeakView 1.2.0.3 software is an indispensable step to search and identify the presence of target compounds in the samples by comparing the compound list with an accurate mass database. The defined search criteria was accurate mass, retention time tolerances, isotope-ratio deviation, and purity score. The list of identified compounds also includes a score value, which is calculated taking into account the accurate masses and the isotopic distribution. The higher the value, the more plausible is the elemental composition. To evaluate the database, blank extracts spiked with 110 µg/L for kitasamycin, spiramycin, chlortetracycline, ciprofloxacin, ronidazole, nitrofurazone, and nitrofurantoin, and 10 µg/L for another 83 analytes were analyzed using UPLC-QTOF-MS instruction with the MultiQuant 2.1 software. The obtained mass error, retention time error, and purity score are listed in Table 1.

The screening result showed that 73 analytes could be correctly identified with low mass errors (≤ 5.0 ppm), retention time (≤ 0.5 min), and isotope-ratio deviation (≤ 0.20), and high degrees of the purity score ($> 70.0\%$). In the light of these results, it is possible to estimate the efficiency of the screening method for identify the selected compounds according to the matrix tested. The identification capabilities were assessed based on the criterion of the identification points (IP) established by the Commission Decision (2002/657/EEC). An ion measured with high resolution QTOF-MS can provide high IP (Meng et al., 2015). Because some compounds only presented one important product ion, only 2.5 IP are earned and ion ratio could not be measured using the triple quadrupole (QqQ) instrument; in contrast, using QTOF instrument, the number of IP obtained is the minimum of 3 required and the ion ratio of precursor/product can also be measured. An ion measured with high resolution QTOF-MS can provide high IP. Because some compounds only presented one important product ion, only 2.5 IP were earned and ion ratio could not be measured using the QqQ instrument (Meng et al., 2015); in contrast, using QTOF instrument, the number of IP obtained is the minimum of 3 required and the ion ratio of precursor/product can also be measured. The developed method was further applied for the analysis of real milk samples, and some positive samples were detected.

Matrix Effect

Matrix effects in LC-MS with electrospray ionization source can severely compromise quantitative analysis of the compounds at trace levels, as well as it can greatly affect the method reproducibility and accuracy. To evaluate this possible effect, 2 types of test solutions (A: standard solution in solvent, B: standard solution prepared with the extract from a blank milk sample with solvent) were used to measure the matrix effect (B/A). The results showed that suppression effect was major, showing the matrix effect of 0.51 to 0.8 for 25 analytes and 0.8 to 1.0 for 47 analytes, among them 0.51 for sulfadiazine; and enhancement effect of 1.0 to 1.2 for another 15 analytes, except ciprofloxacin (1.39), tetracycline (1.65), and oxytetracycline (1.81). Although the matrix effect for 62 analytes was in the range of 0.8 to 1.2, standard calibration curve could not be used for the quantification. Consequently, quantification was performed with the external calibration using matrix-matched standards. The results demonstrated that the quantification of these analytes using a matrix-matched standard calibration curve is the most

Figure 2. The MS/MS spectra of ciprofloxacin, sulfadiazine, and tetracycline at different collision energies: (a) 20 V, (b) 35 V, (c) 50 V, (d) >35 V ~ <50 V. Color version available online.

effective method for reducing indirect matrix effects present in this method. Therefore, matrix-matched standard curves were required in the multi-component analysis for accurate quantification.

Method Validation

Linearity of analytical method was validated using the matrix-matched calibration curves for each com-

pound at 7 different concentration levels. Table 2 shows matrix-matched calibration parameters including the regression equation (based on peak areas) and the correlation coefficients (R^2). The response of the detector was linear for each target compounds in the wide range with correlation coefficient (R^2) of 0.9981 to 0.9999 (among them $R^2 > 0.999$ for 73 of 90 analytes), except for sulfamethoxypyridazine (0.9973). Quantification was performed using MultiQuant 2.1 software based on

matrix-matched calibration plots using the peak area of the most intense ion versus the concentration of the analyte for all real samples.

Method sensitivity was evaluated by measuring the LOD and the LOQ of examined analytes. The LOD and LOQ were based on the chromatogram peak with a signal-to-noise ratio of 3 and a peak 10 times the background chromatographic noise, respectively. Estimated LOD and LOQ values for all of the matrices were obtained based on analyses of spiked blank milk samples. The data in Table 2 show that LOD are in the range of 0.03 to 5.20 $\mu\text{g}/\text{kg}$, and LOQ ranged from 0.10 $\mu\text{g}/\text{kg}$ for nafcillin to 17.30 $\mu\text{g}/\text{kg}$ for spiramycin, which is lower than the MRL.

Repeatability and reproducibility were assessed with intra- and inter-day precisions (relative standard deviation, **RSD**) for the determination of 90 analytes in spiked negative milk sample at 0.5 $\times$, 2 $\times$, and 5 $\times$ MRL levels or at 1 $\times$, 5 $\times$, and 10 $\times$ LOQ levels for the analyte without MRL value. All data concerning intra- and interday RSD are given in Supplemental Table S1 (<http://dx.doi.org/10.3168/jds.2015-9825>). For 6 determinations in a day and 2 determinations in a day within 3 d, the intra- and inter-day RSD were 2.11 to 9.62% and 2.76 to 13.9%, respectively. It was shown that the repeatability and reproducibility of the method are better than the results for repeatability (RSD <20% for 86% of the compounds) and reproducibility (RSD <40% for 96% of the compounds; Stolker et al., 2008).

For the recovery test, the spiked sample was left for at least 10 min at room temperature to ensure the appropriate distribution of the analytes in the matrices. The drug contents were determined by using the corresponding the matrix-matched calibration curves. Average recoveries of all test analytes at 3 spiked levels (0.5 $\times$, 2 $\times$, and 5 $\times$ MRL levels, or at 1 $\times$, 5 $\times$, and 10 $\times$ LOQ for the analyte without MRL value) are given in Supplemental Table S1 (<http://dx.doi.org/10.3168/jds.2015-9825>). They ranged from 72.62% for lincomycin to 122.2% for sulfamethoxypyridazine with an RSD of 1.30 to 9.61%. These results show the accuracy is satisfactory.

Analysis of Real Samples

Submitted samples from different places in China were analyzed to demonstrate the applicability of the method. A total of 120 milk samples included high-calcium milk, breakfast milk, wheat-flavored milk, and pure milk (5 packing boxes for each type and 6 bottles for each packing box). These samples were treated with the modified QuEChERS procedure and screened by UPLC-QTOF-MS.

According to an accurate-mass database, using the information in View 1.2.0.3 software (AB Sciex Co.) discriminated between positive and negative samples. None of the pure milk samples gave a positive result; a packing box of breakfast milk gave a positive result for sulfathiazole; a packing box of wheat-flavored milk gave a positive result for ciprofloxacin; and 3 packing boxes of breakfast milk gave a positive result for tetracycline, sulfadiazine, or both. For these positive samples, a false positive did not occur. The data were evaluated with product ion spectra. Figure 3 shows extracted mass spectra for ciprofloxacin, sulfadiazine, and tetracycline.

Quantification of the confirmed positive sample was carried out by the UPLC-QTOF-MS instrument. The matrix-matched calibration method was applied for the analysis of these positive samples, avoiding significant quantification errors and misinterpretation of results. The average residue concentrations were obtained as follows: 21.98 $\mu\text{g}/\text{kg}$ with RSD of 5.98% for sulfadiazine in 6 breakfast milk samples, 18.76 $\mu\text{g}/\text{kg}$ with RSD of 3.25% for ciprofloxacin in 6 wheat-flavored milk samples, 3.25 $\mu\text{g}/\text{kg}$ with RSD of 6.21% for sulfadiazine alone in 6 high-calcium milk samples, and 13.78 $\mu\text{g}/\text{kg}$ with RSD of 4.29% for tetracycline alone in 6 high-calcium milk samples, 17.35 $\mu\text{g}/\text{kg}$ with RSD of 5.03%, and 9.5 $\mu\text{g}/\text{kg}$ with RSD of 3.64 for both sulfadiazine and tetracycline in 6 high-calcium milk samples, respectively. Results indicated that these residue concentrations are lower than their MRL and other veterinary drug residues below their LOQ.

CONCLUSIONS

This work proposed a generic multi-class analysis to extract and quantify 90 veterinary drug residues in milk. The developed extraction and cleanup steps were easy and rapid, and the modified QuEChERS procedure was quick, easy, cheap, effective, rugged, and safe, and no further cleanup steps were necessary. Furthermore, utilizing the UPLC technology shortened analysis times for all analytes. The UPLC-QTOF-MS offers the best performance for screening purposes and can also effectively provide concentration values. It is accurate enough to differentiate between positive and negative samples or drug concentrations below or above the MRL.

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Table 2. Linear range, limit of detection (LOD), limit of quantification (LOQ), maximum residue limit (MRL), intra-day RSD, and inter-day RSD for the determination of 90 veterinary drugs

No.	Linear range ($\mu\text{g/kg}$)	LOD ($\mu\text{g/kg}$)	LOQ ($\mu\text{g/kg}$)	MRL ($\mu\text{g/kg}$)	Intra-day RSD (%)	Inter-day RSD (%)
1	2–1,000	0.56	1.86		2.88–4.86	6.14–7.99
2	2–400	0.36	1.20	150	2.66–4.13	6.54–8.32
3	10–200	0.82	2.75	40	3.20–4.50	6.15–10.1
4	4–400	0.03	0.10		3.37–4.30	6.58–7.98
5	20–1,000	3.67	12.23		3.32–4.63	8.09–9.98
6	2–400	0.59	1.98		3.34–4.32	6.18–8.35
7	20–1,000	5.2	17.30	200	3.61–4.56	8.12–9.45
8	2–400	0.13	0.43	50	3.30–5.14	6.67–7.96
9	2–400	0.41	1.38	50	2.45–4.62	6.14–8.36
10	2–200	0.14	0.47	100	3.13–4.83	6.78–8.81
11	2–500	0.36	1.2	100	3.06–4.98	5.30–8.48
12	5–200	0.11	0.38	100	2.26–3.26	6.58–9.28
13	2–200	0.59	1.98	100	3.15–4.27	5.77–8.25
14	2–200	0.51	1.71	100	2.18–5.97	7.16–9.41
15	2–500	0.58	1.92	100	3.12–3.54	6.14–8.18
16	2–500	0.11	0.37	100	2.98–3.33	7.33–8.78
17	2–200	0.13	0.44	100	3.31–5.25	7.53–10.7
18	2–100	0.10	0.34	100	3.10–4.55	3.06–4.44
19	2–500	0.17	0.56	100	4.14–5.11	7.69–8.84
20	2–500	0.55	1.82	100	3.09–3.64	7.21–8.64
21	2–500	0.36	1.20	100	2.96–4.37	6.85–9.36
22	2–500	0.58	1.93	100	3.05–4.51	7.18–9.01
23	2–500	0.11	0.36	100	3.13–4.28	6.79–8.74
24	2–500	0.52	1.72	100	2.97–5.11	5.77–8.58
25	2–500	0.29	0.98	100	2.66–4.02	6.77–8.56
26	5–200	0.40	1.33	100	2.49–3.69	5.45–10.0
27	2–200	0.16	0.53	100	2.88–4.41	6.17–7.19
28	2–500	0.59	1.98	50	2.91–4.71	5.45–7.77
29	2–200	0.53	1.75	100	3.73–4.83	7.40–9.27
30	5–500	1.29	4.29	30	4.20–6.28	3.97–4.44
31	2–200	0.57	1.91	800	3.80–5.65	2.76–6.24
32	2–500	0.40	1.33	100	3.35–5.74	7.21–12.2
33	2–500	0.10	0.34		2.57–4.09	7.70–9.57
34	2–500	0.41	1.36	50	4.08–5.07	5.44–8.00
35	2–500	0.53	1.77		2.94–5.49	5.03–8.55
36	2–500	0.11	0.38	75	2.13–5.46	8.38–10.3
37	10–500	1.18	3.93		2.14–3.49	6.50–7.71
38	10–500	0.23	0.76		3.41–4.76	7.11–9.79
39	10–500	1.28	4.28		4.03–4.95	6.40–9.18
40	10–500	0.84	2.80		3.08–4.41	6.08–10.2
41	10–500	1.05	3.49		4.94–8.25	8.94–13.9
42	10–500	0.90	3.00		3.72–5.23	7.01–10.8
43	10–500	1.50	4.99		3.67–4.94	5.07–8.58
44	10–500	1.05	3.50		3.62–5.38	7.23–9.30
45	4–500	0.13	0.42		3.83–4.79	7.79–9.30
46	4–500	0.14	0.47		3.32–4.76	7.33–9.08
47	10–1,000	2.91	9.71	100	3.00–4.67	6.94–11.7
48	20–1,000	3.31	11.03		3.49–4.74	5.80–8.60
49	10–1,000	2.78	9.27	100	3.15–4.70	6.22–9.86
50	20–1,000	2.62	8.73	100	3.70–5.26	8.00–9.29
51	1–1,000	1.00	3.34		3.16–4.22	6.88–9.20
52	10–1,000	0.53	1.78		2.70–5.39	5.38–9.39
53	5–1,000	1.46	4.87		2.72–4.61	6.60–9.12
54	2–500	0.49	1.62		3.32–4.65	6.00–8.32
55	2–500	0.46	1.53		3.57–6.06	7.54–11.6
56	10–1,000	1.30	4.32		2.77–3.42	6.05–9.12
57	10–1,000	0.08	0.27	30	3.04–4.09	7.44–9.90
58	5–1,000	0.13	0.42	30	3.59–5.89	8.75–10.3
59	5–1,000	0.06	0.20	30	3.24–6.81	7.08–10.1
60	5–1,000	0.07	0.22	30	3.20–5.83	8.82–11.3
61	5–1,000	0.62	2.06		2.91–4.85	7.92–9.30
62	5–1,000	0.19	0.63		2.79–4.33	5.11–8.21
63	2–200	0.45	1.49		2.79–4.10	5.83–9.62
64	2–500	0.53	1.77		3.37–5.45	6.62–8.96

Continued

Table 2 (Continued). Linear range, limit of detection (LOD), limit of quantification (LOQ), maximum residue limit (MRL), intra-day RSD, and inter-day RSD for the determination of 90 veterinary drugs

No.	Linear range ($\mu\text{g/kg}$)	LOD ($\mu\text{g/kg}$)	LOQ ($\mu\text{g/kg}$)	MRL ($\mu\text{g/kg}$)	Intra-day RSD (%)	Inter-day RSD (%)
65	2–500	0.60	2.00		3.39–3.64	5.92–8.48
66	2–1,000	0.56	1.86		2.88–4.76	5.03–10.7
67	2–1,000	0.05	0.18		3.01–9.62	7.68–13.3
68	2–1,000	0.59	1.97		3.31–5.32	7.83–9.08
69	2–500	0.40	1.34		2.78–5.01	8.39–11.4
70	5–1,000	0.10	0.33		4.04–5.57	5.93–7.14
71	5–1,000	0.16	0.53		3.22–4.49	6.78–9.86
72	5–1,000	0.16	0.53		4.04–5.98	6.15–9.03
73	5–1,000	0.33	1.10		3.38–5.30	7.55–9.75
74	2–1,000	0.10	0.34	6	4.73–11.4	7.96–12.7
75	2–500	0.60	2.00		3.97–5.61	5.47–8.54
76	10–1,000	0.79	2.63		3.15–5.32	8.82–11.3
77	5–1,000	0.58	1.93		3.17–4.74	6.14–9.68
78	2–500	0.53	1.76		3.71–4.95	6.19–9.12
79	2–500	0.56	1.86		3.02–4.59	7.69–9.30
80	2–500	0.51	1.70		4.88–6.62	8.30–11.6
81	5–1,000	0.33	1.10		3.74–5.83	7.70–10.6
82	5–1,000	1.07	3.56		3.30–6.16	6.73–9.97
83	5–1,000	0.44	1.45		3.64–4.85	7.45–9.30
84	5–1,000	1.19	3.98		3.08–6.13	7.85–9.53
85	2–500	0.48	1.61		4.31–5.25	5.02–9.63
86	5–500	0.33	1.10		3.54–4.35	6.70–9.69
87	5–1,000	1.09	3.63		3.26–4.97	5.93–8.60
88	10–1,000	0.24	0.79		2.54–4.91	7.25–9.28
89	2–1,000	0.46	1.52		3.74–5.61	6.08–9.81
90	2–200	0.51	1.70		3.61–4.74	5.74–9.06

Figure 3. Chromatograms of positive milk samples; sulfadiazine: $t_R = 2.59$ min, mass error = -0.42 ppm, 251.049 to 251.069 Da; ciprofloxacin: $t_R = 5.53$ min, mass error = -0.3 ppm, 332.130 to 332.150 Da; tetracycline: $t_R = 5.74$ min, mass error = -0.2 ppm, 445.150 to 445.170 Da. Color version available online.

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