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The challenges of developing a generic extraction procedure to analyze multi-class veterinary drug residues in milk and honey using ultra-high pressure liquid chromatography quadrupole time-of-flight mass spectrometry

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This paper discusses the analytical challenges to develop a generic extraction procedure to analyze or screen multi-class veterinary drugs in milk and honey using ultra-high pressure liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC QqTOF MS). The veterinary drugs in this study included aminoglycosides, endectocides, fluoroquinolones, ionophores, β-lactams or penicillins, macrolides, NSAIDs, phenicols, sulfonamides and tetracyclines. Veterinary drugs were extracted using a QuEChERS (quick, easy, cheap, effective, rugged, and safe) method, which entailed the use of acetonitrile containing 1% acetic acid, sodium acetate, ethylenediaminetetra acetic acid disodium (EDTA) and magnesium sulfate, and no clean-up was performed. Chromatographic separation was achieved on a reversed-phase Acquity UPLC BEH C_{18}, 100 × 2.1 mm, 1.7 μm column with 0.1% formic acid and 10 mM ammonium formate in water, and acetonitrile as mobile phases. Due to poor chromatographic retention, aminoglycosides were first dropped from the list, and because of poor extractability, β-lactams and tetracyclines were also excluded from the method. The method was able to quantify 31 or screen up to 54 drugs (unbound) in honey, and to quantify 34 or screen up to 59 drugs in milk. UHPLC QqTOF data were acquired in TOF MS full-scan mode that allowed both quantification and confirmation of veterinary drugs and identification of their degradation products in samples. The method could achieve detection limits as low as 1 μg/kg with analytical range from 1 to 100 μg/kg. The developed method was intended to be used for screening of as many analytes as possible in one single analysis, or unequivocal confirmation of positive findings and degradation product identification based on accurate mass measurement and isotopic patterns.

Keywords: UHPLC QqTOF MS; QuEChERs; veterinary drugs; honey; milk

Introduction

Veterinary drugs have been widely utilized in medical and veterinary practice to treat and prevent diseases and to enhance growth rate and feed efficiency. Consequently, if the withdrawal time after treatment is not respected, or if veterinary drugs are not used correctly, the practice could lead to the presence of veterinary drug residues in foods of animal origin. The residues in turn may induce allergic reactions in some hypersensitive individuals or cause the problem of drug-resistant pathogenic bacterial strains.[1–3] In order to address these problems, to increase food safety, and to facilitate international trade, the US Food and Drug Administration (FDA),[4] the European Union,[5] Health Canada,[6] and other international bodies[7] have established maximum residue limits (MRLs) for veterinary drug residues to monitor the level of approved veterinary drugs present in food.

In general, veterinary drug residues in food can be determined through (1) biological screening methods such as microbial inhibition tests, rapid test kits, and (2) quantitative and confirmatory methods including gas chromatography (GC) with electron capture, flame ionization or MS detection, as well as liquid chromatography (LC) with ultraviolet (UV), fluorometric or electrochemical detection or MS. Currently, LC-MS has become a very common tool to analyze for drug residues in various food matrices.

Historically, veterinary drug residues in food are grouped and analyzed based on a single class or related families with the number of compounds being no more than 20. A single-class method is relatively easy to optimize for both extraction and instrumental parameters due to the similar physical and chemical properties of veterinary drugs from the same group. However, in the last few years, there have been an increased number of publications on multi-class methods for analysis of veterinary drugs in food using either LC-MS/MS or LC-TOF instruments. The extraction techniques that have been reported included a molecular weight cut-off filter (3 kD) for milk (150 veterinary drugs),[8] liquid-liquid extraction (LLE) for honey (42 antibiotics),[9]

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solid-phase extraction (SPE) using StrataX for egg, milk, animal tissues (100 veterinary drugs),[10,11] and QuEChERS for animal tissues (41 veterinary drugs),[12] and milk (21 veterinary drugs)[13] for different classes of veterinary drugs at various concentrations.

In this study, we explored the applicability of UHPLC QqTOF MS and QuEChERS for quantification and confirmation of veterinary drugs in honey and milk at low μg/kg (parts per billion) levels. The study included 111 veterinary drugs in its initial experiment. However, aminoglycosides, β-Lactams, penicillins, and tetracyclines were not analyzed by the method because of chromatographic retention issue and sample extraction challenges. The developed method was able to analyze up to 59 veterinary drugs of 7 groups that included endectocides, fluoroquinolones, ionophores, macrolides, NSAIDS, phenicols, and sulfonamides, in honey and milk for screening, quantification, confirmation, and identification of their degradation products.

Experimental

Chemicals and reagents

[Glu[1]]-fibrinopeptide B, leucine enkephalin, and a list of veterinary drugs shown in Table 1 were purchased from Sigma-Aldrich Corp (St Louis, MO, USA) or US Pharmacopeia (Rockville, MD, USA).

Table 1. UHPLC/ESI QqTOF MS Method Performance

	Honey										Milk					
Class	Analytes[a]	Elemental composition	Ionization	Exact mass	Retention time, min	LCL S/N:PtP[b] (μg/kg)	Mass error (ppm)	Overall Recovery[c] (%)	Intermediate Precision[c] (%)	Measurement Uncertainty (%)	Analytes[a]	LCL S/N:PtP[b] (μg/kg)	Mass error (ppm)	Overall Recovery[c] (%)	Intermediate Precision[c] (%)	Measurement Uncertainty (%)
Column	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Endectocides	Abamectin B_{1a}	$C_{48}H_{72}O_{14}$	$[M+H]^+$	873.5000	8.72	54(20)	3.7	91.9	28.9	69.2	**Abamectin B_{1a}**	24(20)	-0.7	—	—	—
	Doramectin	$C_{50}H_{74}O_{14}$	$[M+H]^+$	899.5157	9.13	33(20)	-2.0	91.3	29.4	77.6	**Doramectin**	28(20)	-4.3	—	—	—
	Emamectin B_{1a}	$C_{49}H_{75}NO_{13}$	$[M+H]^+$	886.5317	7.81	154(1)	1.5	100.7	20.6	41.6	**Emamectin B_{1a}**	37(1)	4.6	—	—	—
	Eprinomectin B_{1a}	$C_{50}H_{75}NO_{14}$	$[M+H]^+$	914.5266	8.24	32(5)	0.3	97.4	21.3	54.0	**Eprinomectin B_{1a}**	17(5)	-0.1	—	—	—
	Ivermectin	$C_{48}H_{74}O_{14}$	$[M+NH_4]^+$	892.5422	9.70	14(1)	-2.4	96.2	**35.5**	79.2	**Ivermectin**	14(5)	-1.3	—	—	—
	Moxidectin	$C_{37}H_{53}NO_8$	$[M+H]^+$	640.3849	9.34	12(5)	-2.7	87.7	**60.8**	166.7	**Moxidectin**	13(5)	-3.3	—	—	—
	Selamectin	$C_{43}H_{63}NO_{11}$	$[M+H]^+$	770.4479	9.70	111(1)	0.6	90.1	**47.9**	121.6	**Selamectin**	68(5)	-4.8	—	—	—
Fluoroquinolones	**Cinoxacin**	$C_{12}H_{10}N_2O_5$	$[M+H]^+$	263.0668	2.89	46(5)	-2.3	**16.1**	**34.1**	70.0	**Cinoxacin**	40(1)	-0.8	**22.9**	10.3	41.9
	Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	$[M+H]^+$	332.1410	2.19	NA	NA	NA	NA	NA	**Ciprofloxacin**	15(1)	-3.6	**3.2**	21.0	114.7
	Danofloxacin	$C_{19}H_{20}FN_3O_3$	$[M+H]^+$	358.1567	2.29	23(5)	-2.8	**4.9**	**58.6**	119.6	**Danofloxacin**	49(1)	-1.1	**10.5**	16.6	36.1
	Difloxacin	$C_{21}H_{19}F_2N_3O_3$	$[M+H]^+$	400.1473	2.79	24(5)	-3.7	**15.7**	**30.2**	63.8	**Difloxacin**	44(1)	1.0	**46.2**	10.6	24.0
	Enoxacin	$C_{15}H_{17}FN_4O_3$	$[M+H]^+$	321.1363	2.10	NA	NA	NA	NA	NA	**Enoxacin**	16(1)	-3.4	**2.0**	**38.6**	99.7
	Enrofloxacin	$C_{19}H_{22}FN_3O_3$	$[M+H]^+$	360.1723	2.43	38(5)	1.1	**8.6**	**46.0**	95.3	**Enrofloxacin**	39(1)	1.4	**23.0**	10.1	21.8
	Flumequine	$C_{14}H_{12}FNO_3$	$[M+H]^+$	262.0879	4.18	28(1)	1.5	68.8	20.4	41.4	Flumequine	66(1)	-2.3	68.5	10.6	24.4
	Marbofloxacin	$C_{17}H_{19}FN_4O_4$	$[M+H]^+$	363.1469	2.03	NA	NA	NA	NA	NA	**Marbofloxacin**	17(1)	-5.5	**4.7**	22.3	59.3
	Nalidixic Acid	$C_{12}H_{12}N_2O_3$	$[M+H]^+$	233.0926	4.03	58(5)	2.6	56.7	16.6	35.5	Nalidixic Acid	22(1)	-0.9	59.1	9.4	19.2
	Norfloxacin	$C_{16}H_{18}FN_3O_3$	$[M+H]^+$	320.1410	2.10	NA	NA	NA	NA	NA	**Norfloxacin**	11(1)	2.5	**2.1**	28.9	65.1
	Ofloxacin	$C_{18}H_{20}FN_3O_4$	$[M+H]^+$	362.1516	2.14	34(5)	0.8	**3.1**	**61.8**	126.1	**Ofloxacin**	21(1)	1.9	**6.6**	21.8	46.4
	Orbifloxacin	$C_{19}H_{20}F_3N_3O_3$	$[M+H]^+$	396.1535	2.53	20(5)	-2.5	**18.3**	**43.2**	87.3	**Orbifloxacin**	58(1)	-3.8	**39.9**	9.8	19.8
	Oxolinic Acid	$C_{13}H_{11}NO_5$	$[M+H]^+$	262.0715	3.21	25(1)	1.9	62.9	17.2	35.8	Oxolinic Acid	47(1)	-0.8	63.4	7.0	16.8
	Sarafloxacin	$C_{20}H_{17}F_2N_3O_3$	$[M+H]^+$	386.1316	2.72	NA	NA	NA	NA	NA	**Sarafloxacin**	30(1)	2.1	**15.3**	14.3	33.7
	Sparfloxacin	$C_{19}H_{22}F_2N_4O_3$	$[M+H]^+$	393.1738	2.77	19(5)	-1.0	**15.1**	**37.2**	77.5	**Sparfloxacin**	38(1)	-0.8	**29.5**	11.3	28.7
Ionophores	**Lasalocid**	$C_{34}H_{53}O_8$	$[M+NH_4]^+$	608.4162	10.46	131(1)	-2.1	84.5	**46.2**	128.2	**Lasalocid**	46(1)	-1.5	—	—	—
	Monensin	$C_{36}H_{62}O_{11}$	$[M+NH_4]^+$	688.4636	9.65	121(1)	-0.6	80.3	29.5	91.8	**Monensin**	43(1)	1.2	—	—	—
	Narasin	$C_{43}H_{72}O_{11}$	$[M+NH_4]^+$	782.5418	10.11	145(1)	0.4	54.8	**37.1**	104.0	**Narasin**	53(20)	5.5	—	—	—
	Salinomycin	$C_{42}H_{70}O_{11}$	$[M+NH_4]^+$	768.5262	9.73	224(1)	1.2	**49.4**	**32.4**	88.6	**Salinomycin**	45(1)	-0.3	—	—	—
Macrolides	**Erythromycin**	$C_{37}H_{67}NO_{13}$	$[M+H]^+$	734.4691	4.23	87(1)	1.5	**42.0**	**39.4**	79.6	**Erythromycin**	36(1)	1.2	71.1	**34.6**	105.3
	Neospiramycin I	$C_{36}H_{62}N_2O_{11}$	$[M+H]^+$	699.4432	2.93	50(1)	4.0	66.2	24.9	63.1	Neospiramycin I	88(5)	-0.2	80.4	8.7	19.6
	Oleandomycin	$C_{35}H_{61}NO_{12}$	$[M+H]^+$	688.4272	3.97	28(1)	2.2	101.7	11.8	24.9	Oleandomycin	35(1)	-1.0	88.0	8.6	17.9
	Roxithromycin	$C_{41}H_{76}N_2O_{15}$	$[M+H]^+$	837.5324	5.17	126(1)	3.0	98.8	11.0	25.4	Roxithromycin	65(1)	3.2	90.6	9.2	23.0
	Spiramycin I	$C_{43}H_{74}N_2O_{14}$	$[M+H]^+$	843.5218	3.24	63(1)	-2.4	92.7	18.5	43.2	Spiramycin I	40(5)	3.8	82.3	8.6	17.5
	Tilmicosin	$C_{46}H_{80}N_2O_{13}$	$[M+H]^+$	869.5739	3.94	90(1)	2.1	99.5	17.5	35.4	Tilmicosin	61(5)	1.4	90.3	11.4	25.7
	Tylosin A	$C_{46}H_{77}NO_{17}$	$[M+H]^+$	916.5270	4.44	82(1)	2.9	102.9	12.4	25.2	Tylosin A	35(1)	-5.1	83.3	15.3	30.9
NSAIDS	Flunixin	$C_{14}H_{11}F_3N_2O_2$	$[M+H]^+$	297.0851	5.19	17(1)	2.4	95.8	10.5	22.7	Flunixin	29(1)	-0.7	92.1	19.0	55.8
	Phenylbutazone	$C_{19}H_{20}N_2O_2$	$[M+H]^+$	309.1603	6.38	15(1)	1.3	63.7	23.2	103.9	**Phenylbutazone**	76(20)	-1.3	72.5	**69.1**	165.6
Phenicols	Florfenicol	$C_{12}H_{15}Cl_2FNO_4S$	$[M+NH_4]^+$	375.0348	2.96	22(5)	-4.5	99.1	12.3	25.2	Florfenicol	44(5)	-3.2	89.4	7.4	17.0
	Thiamphenicol	$C_{12}H_{15}Cl_2NO_5S$	$[M+NH_4]^+$	373.0392	2.16	13(5)	-5.1	108.9	24.8	50.1	Thiamphenicol	38(5)	-2.7	103.9	7.9	19.0
Sulfonamides	**Dapsone**	$C_{12}H_{12}N_2O_2S$	$[M+H]^+$	249.0698	2.60	23(5)	-2.0	57.9	**39.0**	78.8	Dapsone	53(1)	-0.4	83.1	8.7	17.5
	Sulfabenzamide	$C_{13}H_{12}N_2O_3S$	$[M+H]^+$	277.0647	3.30	75(20)	-1.8	73.5	12.9	26.5	Sulfabenzamide	17(1)	-2.2	83.3	8.2	25.5
	Sulfacetamide	$C_8H_{10}N_2O_3S$	$[M+H]^+$	215.0490	1.52	10(20)	7.9	88.5	**41.2**	84.1	Sulfacetamide	12(5)	-0.9	83.4	10.9	22.1
	Sulfachloropyridazine	$C_{10}H_9ClN_4O_2S$	$[M+H]^+$	285.0213	2.72	29(5)	-2.8	75.3	17.8	36.6	Sulfachloropyridazine	78(5)	-2.8	83.5	9.2	28.2
	Sulfadiazine	$C_{10}H_{10}N_4O_2S$	$[M+H]^+$	251.0603	1.68	35(5)	-8.0	79.1	**35.5**	71.8	Sulfadiazine	27(1)	-6.4	77.5	10.6	25.4
	Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$	$[M+H]^+$	311.0814	3.50	18(1)	0.6	78.8	16.7	33.8	Sulfadimethoxine	56(1)	2.3	87.8	7.9	19.7
	Sulfadoxine	$C_{12}H_{14}N_4O_4S$	$[M+H]^+$	311.0814	2.94	14(1)	1.3	78.7	18.0	37.0	Sulfadoxine	60(1)	-2.6	81.4	10.5	23.0
	Sulfaethoxypyridazine	$C_{12}H_{14}N_4O_3S$	$[M+H]^+$	295.0865	3.04	64(5)	-1.7	71.0	24.2	49.0	Sulfaethoxypyridazine	35(1)	1.4	78.4	7.2	14.6
	Sulfamerazine	$C_{11}H_{12}N_4O_2S$	$[M+H]^+$	265.0759	2.05	25(5)	0.4	78.1	**40.9**	83.3	Sulfamerazine	16(1)	-2.6	97.1	11.7	31.1
	Sulfamethazine	$C_{12}H_{14}N_4O_2S$	$[M+H]^+$	279.0916	2.37	70(5)	-0.7	64.7	20.9	42.3	Sulfamethazine	29(1)	1.1	82.3	10.3	21.6
	Sulfamethizole	$C_9H_{10}N_4O_2S_2$	$[M+H]^+$	271.0323	2.29	12(5)	0.7	65.9	**31.4**	63.6	Sulfamethizole	17(1)	-7.4	86.5	11.3	28.0
	Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	$[M+H]^+$	254.0599	2.91	35(5)	-0.4	83.6	16.6	33.6	Sulfamethoxazole	36(1)	-2.0	95.4	11.3	23.4
	Sulfamethoxypyridazine	$C_{11}H_{12}N_4O_3S$	$[M+H]^+$	281.0708	2.34	15(1)	-5.0	79.7	28.1	57.3	Sulfamethoxypyridazine	34(1)	-3.2	91.3	11.1	24.4
	Sulfamonomethoxine	$C_{11}H_{12}N_4O_3S$	$[M+H]^+$	281.0708	2.61	14(1)	-2.5	74.0	18.4	37.2	Sulfamonomethoxine	24(1)	1.1	78.6	11.0	25.1
	Sulfamoxole	$C_{11}H_{13}N_3O_3S$	$[M+H]^+$	268.0756	2.22	23(1)	5.6	76.9	**30.7**	62.5	Sulfamoxole	26(1)	-0.4	71.4	17.4	37.4
	Sulfaphenazole	$C_{15}H_{14}N_4O_2S$	$[M+H]^+$	315.0916	3.56	10(1)	6.0	79.2	18.2	36.8	Sulfaphenazole	40(1)	-0.3	84.1	7.5	19.2
	Sulfapyridine	$C_{11}H_{11}N_3O_2S$	$[M+H]^+$	250.0650	1.93	11(1)	3.2	75.3	**50.0**	101.1	Sulfapyridine	11(1)	5.2	85.3	9.9	20.1
	Sulfaquinoxaline	$C_{14}H_{12}N_4O_2S$	$[M+H]^+$	301.0759	3.51	14(5)	-1.3	75.2	18.4	37.3	Sulfaquinoxaline	24(1)	0.7	84.1	10.2	28.1
	Sulfathiazole	$C_9H_9N_3O_2S_2$	$[M+H]^+$	256.0214	1.80	12(1)	5.1	68.9	**45.3**	91.5	Sulfathiazole	27(5)	-1.6	77.6	11.1	22.5
	Sulfisomidine	$C_{12}H_{14}N_4O_2S$	$[M+H]^+$	279.0916	1.55	11(5)	-2.9	66.2	**70.5**	142.4	Sulfisomidine	12(1)	1.8	73.8	10.0	20.5
	Sulfisoxazole	$C_{11}H_{13}N_3O_3S$	$[M+H]^+$	268.0756	3.08	11(1)	6.5	77.9	18.7	39.6	Sulfisoxazole	11(1)	2.2	85.1	7.2	16.3
	Trimethoprim	$C_{14}H_{18}N_4O_3$	$[M+H]^+$	291.1457	2.16	44(1)	-2.1	69.6	19.9	42.7	Trimethoprim	32(1)	1.7	88.7	7.6	22.9

[a] Analytes in bold font are qualitatively determined for confirmative purpose only.
[b] Signal-to-noise ratio from peak-to-peak at the lowest concentration level of a matrix-matched standard calibration curve.
[c] Data with overall recovery not between 50 and 120%, and intermediate precision > 30% are bolded and underlined.

Tilmicosin (90.7%) was a gift from Eli Lilly and Company (Indianapolis, IN, USA). Neospiramycin I (97.6%) was obtained from Wako Chemicals USA, Inc. (Richmond, VA, USA). Ammonium acetate (reagent grade), LC-MS water (Chromasolv, 1 L) and LC-MS acetonitrile (Chromasolv, 2.5 L) were purchased from Sigma-Aldrich Corp (St Louis, MO, USA). Bulk anhydrous magnesium sulfate ($MgSO_4$) was from United Chemical Technologies, Inc. (Bristol, PA, USA). PSA (primary secondary amine) was purchased from Varian Inc (Palo Alto, CA, USA). Acetic acid (glacial acetic acid, reagent grade, 99.7%), acetonitrile (distilled in glass), ammonium acetate, EDTA (ethylenediaminetetra acetic acid disodium) and methanol (distilled in glass) were obtained from Caledon Laboratories Ltd (Georgetown, Ontario, Canada). Sodium acetate anhydrous (ACS reagent) was from Thermo Fisher Scientific Inc. (Ontario, Canada). Formic acid (LC-MS grade) and ammonium formate (reagent grade) were from Sigma-Aldrich Corp (St Louis, MO, USA). Raw milk (veterinary drug free) was obtained from a local farm and was kept under −20 °C. Negative control honey samples (veterinary drug free) were taken from the sample monitoring programme. Water used for extraction was from Barnstead Nanopure System (Thermo Fisher Scientific Inc., Ontario, Canada).

Preparation of standard solutions

Individual veterinary drug standard stock solutions were generally prepared in a concentration of 1000.0–4000.0 μg/ml in methanol, except for fluoroquinolones which were prepared in acetonitrile. Oxolinic acid and sarafloxacin required a small amount of NH_4OH (a few drops) to aid dissolution. Intermediate veterinary drug standard mix working solution was prepared as 10.0 μg/ml in methanol from stock solutions. Stock and intermediate solutions were stored at −20 °C. Six-level veterinary drug standard mix working solutions were prepared by transferring 0.02, 0.1, 0.4, 0.8, 1.2, and 2.0 ml of 10.0 μg/ml intermediate working solution into six separate 10-ml volumetric flasks and making up to volume with aceonitrile to prepare 0.02, 0.1, 0.4, 0.8, 1.2, and 2.0 μg/ml six-level standard solutions. They were used for constructing matrix-matched standard calibration curves. Four-level sample spike veterinary drug standard working solutions were prepared by transferring 0.2, 0.5, 1.0, and 1.6 ml of 10.0 μg/ml intermediate working solution into separate 10-ml volumetric flasks and making up to volume with acetonitrile to prepare 0.2, 0.5, 1.0, and 1.6 μg/ml four-level standard solutions for sample spikes. All working solutions were prepared fresh for each fortification experiment.

Preparation of reagent solutions

Acetonitrile/acetic acid (99 + 1, *v/v*) was prepared by mixing 990 ml acetonitrile with 10 ml of acetic acid. Ammonium acetate (0.1 M) was made by weighing 7.7 g of ammonium acetate and dissolving in approximately 800 ml water. After transferring into a 1000-ml volumetric flask, the solution was made up to the volume with water. Solvent buffer was a mixture of 0.1 M ammonium acetate and methanol (50 + 50, *v/v*). Leucine enkephalin solution (4 mg/L) for the lock mass was prepared in 20% acetonitrile in water. [Glu1]-fibrinopeptide B solution (0.32 pmol/μl) for the mass calibration was made by dissolving 0.1 mg [Glu^1]-fibrinopeptide B into 2 ml of water, then a 100 μl aliquot was diluted with 9.9 ml of a mix of methanol and water (50 + 50, *v/v*) with 1% acetic acid. Mobile phase B consisted of 10 mM ammonium formate and 0.1 % formic acid in water.

Sample extraction

Sample extraction procedures followed the buffered QuEChERS or AOAC Official Method 2007.01[14,15] without dispersive solid-phase extraction clean-up step. A 10 g honey or milk sample was weighed into a 50-ml centrifuge tube (VWR International, Mississauga, Ontario, Canada). Ten millilitres of water was added to a honey sample which was shaken on a mechanical shaker to dissolve the honey. Five hundred μl per four-level sample spike veterinary drug standard working solution was added into four centrifuge tubes to provide 10.0, 25.0, 50.0, and 80.0 μg/kg of veterinary drugs equivalent in samples. After 15 min, 0.4 g EDTA were transferred into the sample, which was capped and shaken on a shaker for 10 min. Then, 10 ml of a mixture of acetonitrile and acetic acid (99 + 1, *v/v*) was added using a bottle-top dispenser. The sample was capped and vortexed to mix for 45 s. For raw milk, a sample was allowed to stand for 10 min for the protein to denature after the mixture of acetonitrile and acetic acid was added. And then, 4 g $MgSO_4$ and 1 g sodium acetate were combined with the sample, which was capped and shaken for 45 s by hand. The sample mixture was centrifuged at 2100 g for 3 min in a centrifuge (Allegra X-15R Centrifuge, Beckman Coulter, Inc. Fullerton, CA, USA). Five millilitres of supernatants (5 g sample/5 ml) was transferred into individual 5-ml PYREX brand centrifuge tubes, pre-calibrated with 1 ml volume accuracy (VWR International, Mississauga, Ontario, Canada). Each of the sample extracts was evaporated to 0.1–0.2 ml, which took approximately 2 h, using an N-EVAP nitrogen evaporator (Organomation Associates Inc., Berlin, MA, USA) at 30 °C under a stream of nitrogen. The extracts were made up to 0.5 ml with acetonitrile, vortexed for 30 s, and then made up to 1.0 ml with 0.1 M ammonium acetate and vortexed again for 30 s. Five hundred μl of each extract was transferred into a Mini-UniPrep vial (Whatman Inc., USA). Sample extracts were analyzed by UHPLC QqTOF system.

Preparation of matrix-matched calibration standards and calculation

Matrix-matched calibration standards were prepared by adding standards to blank sample extracts after sample extraction. Multiple blank honey or milk samples (10.0 g/sample) were weighed into separate 50-ml centrifuge tubes and were processed through the extraction procedure as described above. To each of the six remaining 0.1–0.2 ml sample extracts, 250 μl of each six-level veterinary drug standard mix working solution was added accordingly, providing 1.0, 5.0, 20.0, 40.0, 60.0, and 100.0 μg/kg of standard equivalent in samples. The extracts were made up to 0.5 ml with acetonitrile, vortexed for 30 s, made up volume to 1.0 ml with 0.1 M ammonium acetate and then vortexed again for 30 s. The extracts were analyzed by UHPLC QqTOF system.

UHPLC QqTOF MS

The UPLC Q-TOF system used was Waters Acquity UPLC coupled with a Q-TOF Premier, a quadrupole and orthogonal acceleration TOF-MS/MS utilizing electrospray interface. The system was operated under MassLynx 4.1 software (Waters, Milford, MA, USA). The Q-TOF Premier has two function types, i.e. Tof MS (MS scan only) and TOF-MS/MS (MS/MS scan only). Therefore, the Q-TOF could be utilized as either a straight TOF instrument (TOFMS) or a tandem TOF mass spectrometer (TOF-MS/MS). The former has the advantage of being able to capture all ions from the electrospray ionization (ESI) source, and the latter is somewhat selective because it uses the first quadrupole as a mass filter to select the precursor ion of a target analyte and to record the product ion spectrum by the TOF analyzer after breakdown in the collision cell.

In a regulatory routine practice, although a TOF-MS/MS product ion spectrum allows a further unequivocal confirmation of incurred chemicals, TOFMS is more practical than TOF-MS/MS to screen as many chemical contaminants as possible in one single run. Any incurred chemical contaminants could be further confirmed using the TOF-MS/MS if necessary. In our study, we used the Q-TOF instrument as a single-stage TOF instrument (TOFMS) for all data acquisitions.

UPLC profile

Mobile phase components were acetonitrile (solvent A), 0.1% formic acid and 10 mM ammonium formate in water (solvent B). Gradient profile consisted of 0 min: 8% A; 0–9 min: 8–95% A; 9–11 min: 95–100% A; 11–12 min: 8% A. Flow rate was 0.4 ml/min. Injection volume was 10 µl. The UPLC column utilized was an Acquity UPLC BEH C_{18} column 100 mm × 2.1 mm, 1.7 µm and the pre-column was the Acquity VanGuard BEH C_{18} 5 mm × 2.1 mm, 1.7 µm (Waters, Milford, MA, USA). The column oven was set at 45 °C and auto-sampler temperature was set at 5 °C.

Q-TOF conditions

Ionization mode: electrospray positive ion mode; capillary voltage: 3.20 kV; source temperature: 120 °C; desolvation temperature: 300 °C; nebulizer nitrogen flow rate: 50 L/hr; desolvation nitrogen gas flow rate: 800 L/hr; collision gas argon pressure: 5.3×10^{-3} mbar; collision energy: 5 eV; sampling cone voltage: 20 V; mass range 50–950 Da; Resolution: 15000 FWHM (measured with [Glu^1]-fibrinopeptide B at $[M+2H]^{2+} = 785.8426$ in W-mode); lock mass reference: leucine enkephalin, $[M+H]^+ = 556.2771$ Da. For analytes, scan time: 0.2 s; inter-scan delay: 0.05 s. For lock mass reference, scan time: 0.2 s; frequency: 1/10. Data were acquired in centroid format with programmable Dynamic Range Enhancement (pDRE) enabled for a wide dynamic range for quantification. As the QqTOF was operated in TOFMS mode, the quadrupole was set in RF mode only. With a certain MS profile defined for the quadrupole, a wide range of ions passes through to the TOF section of the MS. Q-TOF parameters were set up generically to detect all ions in a mass range from 50 to 950 in W-mode.

Experimental design and method validation

The method was validated with a nested experimental design, which was described elsewhere.[16,17] The main factors of variances associated with the method performance or measurement uncertainties of an in-house validated method using the spiked samples were concentrations or spike levels of analytes, matrix effects, day-to-day variation and within-day variation of the method. The last two factors were designated as the intermediate precision. In this study, there were a total of two different matrices per honey or milk sample. For each matrix, samples were spiked at four levels, i.e. 10.0, 25.0, 50.0, and 80.0 µg/kg, in triplicate. Spike experiments were repeated by two analysts. Overall recovery, intermediate precision and measurement uncertainty were calculated using a combined computer program that consisted of SAS codes (SAS Software Release 9.1, SAS Institute Inc., USA) along with a Microsoft Excel (Microsoft Office 2002) workbook.[17]

Results and discussion

Chromatography

Veterinary drugs or antibiotics included in this study were aminoglycosides (a total of 15), endectocides (7), fluoroquinolones (17), ionophores (6), β-Lactams (17) or penicillins (7), macrolides (7), NSAIDs (2), phenicols (2), sulfonamides (26), and tetracyclines (5). QqTOF data were acquired in TOFMS mode with a set of generic MS parameters that were applied to all analytes of interests. Veterinary drugs were ionized in form of $[M+H]^+$ or $[M+NH_4]^+$ (Table 1, column 3) in the positive electrospray mode. UHPLC was achieved using the Acquity UPLC BEH C_{18} column with a total analytical runtime of 14 min at the conditions described in the Experimental section. Good chromatographic retentions were observed for all drugs except for aminoglycosides under the chromatographic conditions above, and the retention times proved to be very reproducible under ±0.2 min within- and between-batches. The use of ion-pairing reagents in the mobile phases[18] or HILIC (hydrophilic interaction liquid chromatography) column[19] are possible solutions to the poor chromatographic retention of aminoglycosides. Because they can lead to ion suppression of other analytes, especially for the nitrogen atom containing compounds, no ion pairing reagents were utilized in the current study. Consequently, aminoglycosides were not included in the study.

Extraction

The buffered QuEChERS method or AOAC Official Method 2007.01[14,15] that included both extraction and/or clean-up was explored for its applicability for extraction of veterinary drug residues from honey and milk samples. The extraction step entailed the addition of 10 ml of a mixture of acetonitrile and acetic acid (99 + 1, *v/v*) to a sample (10 g), which required a 45 s shake before 1 g sodium acetate and 4.0 g $MgSO_4$ were added. For honey samples, 10 ml of water was added to the sample (10 g) which was shaken on a mechanical shaker to dissolve the honey in water prior to the extraction. Veterinary drug residues were partitioned into the acetonitrile layer by the salt-out effect of anhydrous $MgSO_4$. The pH was about 5 in the mixture. Clean-up or dispersive solid-phase extraction (*d*-SPE) step was performed by adding 1.2 g $MgSO_4$ and 0.4 g PSA to 6–8 ml of the supernatant obtained from the extraction step. Overall, there were four experiments performed that included Experiment I: extraction and clean-up; II: extraction and no clean-up; III: extraction without 1% acetic acid and no clean-up; IV: extraction plus EDTA and no clean-up. Because β-lactams, penicillins and tetracyclines were not recovered in Experiment I, Experiment II was performed to examine whether eliminating the clean-up step would help on recovering any analytes from those three groups. The experiment results indicated that the clean-up step was not the cause for the loss in these three groups, and in addition, it did not help to improve the method performance in terms of sensitivity, chromatographic peak shape, etc. Therefore, the clean-up step was not adopted in the QuEChERs procedure afterwards in this study. Experiment III was performed to investigate whether the acidic condition was the cause for the loss of β-lactams and penicillins because there was a concern that some of them might not be stable under acidic condition.[20] Once again, it was found that the elimination of 1% acetic acid did not help to regain β-lactams and penicillins and led to the low recovery for fluoroquinolones. Therefore, the buffered condition was kept in the procedure. Experiment IV was performed to look into whether EDTA helped to recover tetracyclines because they tended to form strong complex with Ca^{2+} and Mg^{2+} ions. Therefore, EDTA was added to the extraction step but it did not result in the recovery of tetracyclines. Nevertheless, the EDTA improved the extraction efficiency of fluoroquinolones slightly, some of which were rarely recovered in the first three experiments. Consequently, the method

of Experiment IV was adopted and validated for the determination of the veterinary drugs that are listed in Table 1 in honey and milk. It should be noted that sulfonamides present in honey bind to the sugars, and the samples were typically subjected to acid hydrolysis, for example in 2 M HCl for 1 h at 50 °C,[21] to liberate them and to detect the free form. In the current study, the acid hydrolysis was not applied and it was assumed that the QuEChERs method only extracted free sulfonamides present in honey samples.

Method validation and method performance

QqTOF MS data processing, i.e. post-target analysis, was based on accurate masses with mass error ±50 mDa. The target ions or exact masses listed in Table 1 (column 4) were used to extract chromatograms for quantification. Mass accuracy, isotopic pattern and/or retention time were utilized for confirmation. The elemental compositions, mass errors and isotopical pattern (or i-FIT) of any ions were determined using the MassLynx Elemental Composition as described elsewhere.[22] To compensate for the matrix effects, matrix-matched standard calibration curves were used, especially for quantification.

The method was validated according to a statistical experimental design or the nested design, which included four factors, i.e. veterinary drug concentrations or spike levels, matrix effects, day-to-day variation and within-day variation. The designed experiment provided validation data to study and evaluate method performance parameters that covered accuracy expressed as overall recovery, intermediate precision and measurement uncertainty. Veterinary drugs were spiked into two matrices per honey and milk samples at 10, 25, 50, and 80 μg/kg in triplicate, and each experiment was repeated by different analysts or on a separate day. The performance parameters were calculated using a combined SAS statistical program. Detailed calculations and equations were described elsewhere.[16,17] The method performance results are summarized in Table 1 (honey, table columns 8–10; milk, table columns 14–16), and are depicted in Figure 1. When meeting the criteria of overall recovery between 50–120%, and intermediate precision ≤30%, veterinary drugs were considered as quantifiable by the method. Otherwise, they were accounted as qualitative screening only. Table 1 highlighted the veterinary drugs (columns 1 and 11) whose overall recoveries or intermediate precisions that were not in the range or not within the limit. Of 59 veterinary drugs listed in Table 1, 31, and 34 were able to be quantified, and 23 and 25 were only qualitatively determined in honey and milk respectively. The method demonstrated better performance towards milk matrices than for honey samples because of tighter overall recoveries, intermediate precision and measurement uncertainty (Figure 1B).

In milk, there was significant matrix enhancement for endectocides and ionophores in one of the samples, and their quantitative data were not able to obtain. As described in the Experimental section, matrix-matched calibration standard curves were prepared by adding the standards to the matrix extracts after the extraction while fortification experiments were performed by putting in the standards prior to the extraction. The extraction process might cause the enhancement of the responses of endectocides and ionophores significantly that could not be explained easily. However, this kind of enhancement was not observed in honey matrices. The method also had low recoveries and less repeatability for fluoroquinolones from both honey and milk samples. Nevertheless, they tended to show high responses, especially in honey.

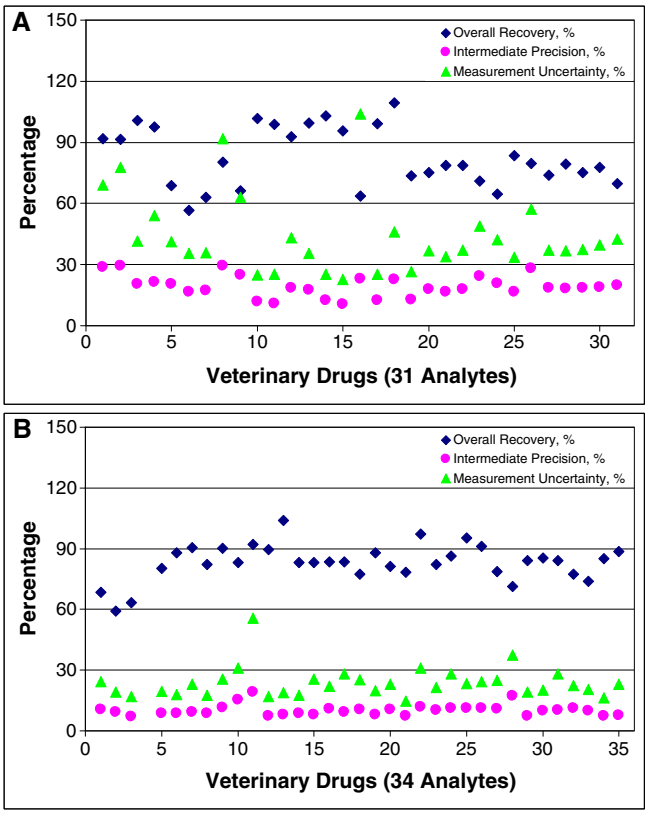

Figure 1. UHPLC QqTOF MS method performance. (A) Honey matrix. (B) Milk matrix.

The method could serve as a valuable tool to screen or confirm those groups of compounds.

For 59 veterinary drugs, the method was generally able to be detected at low parts-per-billion level such as 1 or 5 μg/kg (Table 1, columns 6 and 12) with signal-to-noise ratio >3:1 and mass error <8 ppm. Although the UHPLC QqTOF instrument is one-to-two orders of magnitude lower than that of a QqQ mass spectrometer in the MRM mode, this study proved that TOF instrument along with a generic extraction procedure could serve as a fast and simple tool to determine some veterinary drug residues in food at trace residue levels.

Confirmation and unknown identification

QqTOF mass spectrometers, such as used in this study, offer medium-range high resolution, accurate mass measurement, excellent full-scan sensitivity, and complete mass spectral information. Its full-scan data allowed retrospective processing to determine accurate mass for confirmation and unknown identification. Figure 2 shows one of examples that used the developed method, i.e. UHPLC QqTOF MS along with QuEChERs, to confirm the presence of incurred sulfathiazole in one honey sample. The mass error was −1.6 ppm (Figure 2C) and the retention time (1.80 min, Figure 2B) was almost the same as those from the reference standard (1.80 or 1.81 min). Since there was no acid hydrolysis step, it was assumed the detected sulfathiazole was unbound residue in this sample.

Figures 3 and 4 give another good example that utilized the developed method to investigate the degradation of erythromycin A in sample or as a result of extraction. Macrolides are not stable under acidic condition. For example, tylosin A

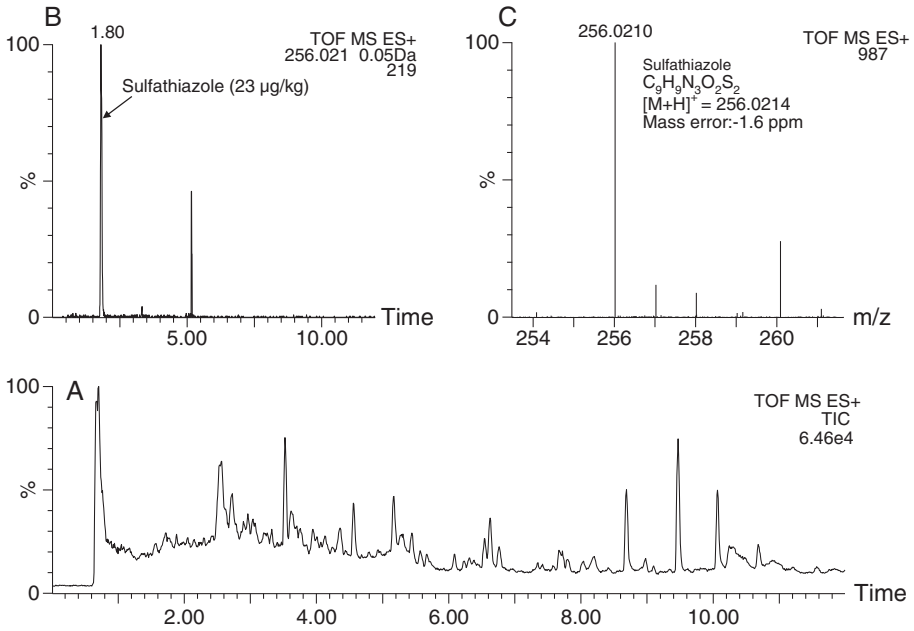

Figure 2. UHPLC QqTOF MS confirmation of sulfathiazole in honey. (A) Total ion current chromatogram. (B) Extracted ion chromatogram of exact mass of 256.0214 (sulfathiazole). (C) Mass spectrum of UHPLC peak at 1.80 min. The spectrum was obtained by 2 to 3 scans combined across the UHPLC peak with a number of background scans taken on each side of the peak subtracted.

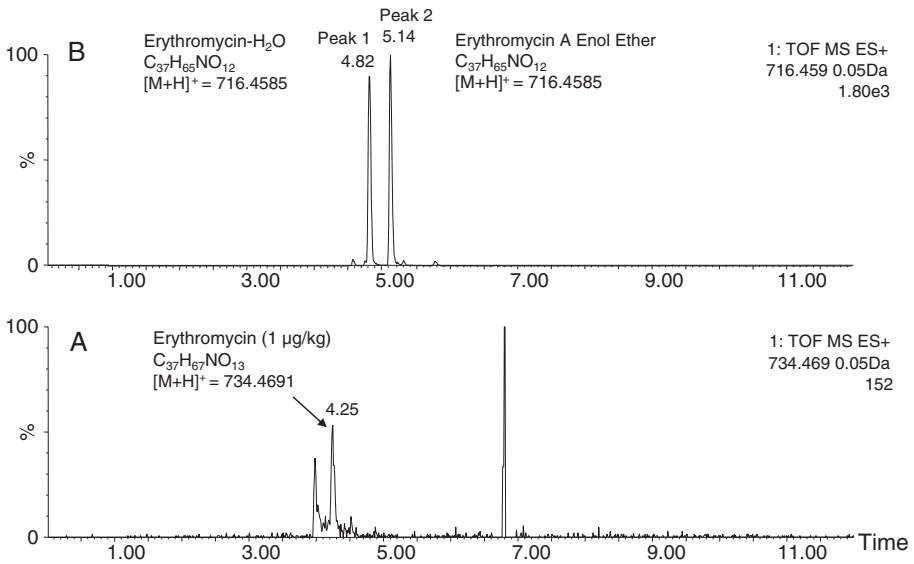

Figure 3. UHPLC QqTOF chromatograms of erythromycin A and its degradation products. (A) Extracted ion chromatogram of the exact mass of 734.4691 (erythromycin A). (B) Extracted ion chromatogram of the exact mass of 716.4585 (erythromycin A-H_2O or erythromycin A enol ether).

degrades gradually to tylosin B (desmycosin) under acidic conditions such as in honey. Previously we identified tylosin B in honey based on accurate mass measurement from the QqTOF full-scan data.[23] It has been reported that erythromycin A degraded to erythromycin A-H_2O in a mild acidic ambient condition such as that of yogurt[24]. Honey has an average pH of 3.9 with a typical range of 3.4–6.1,[25] which can possibly lead to erythromycin A breaking down as well. In acidic condition, erythromycin A reached equilibrium with erythromycin A-6,9-hemiketal. Erythromycin A-H_2O appeared to be produced directly through an internal dehydration of erythromycin A-6,9-hemiketal which simultaneously established an equilibrium with erythromycin A enol ether (Figure 5).[26] The determination of erythromycin A-H_2O and its related degradation products (i.e. erythromycin A enol ether) in food could be of importance because the toxicity of the erythromycin A enol ether could be 5-fold higher than that of the parent compound[25,27] Therefore, the data from one of the incurred erythromycin A honey sample was examined based on the exact masses of 734.4691 (erythromycin A) and 716.4585 (erythromycin A-H_2O or erythromycin A enol ether) and the extracted ion chromatograms were generated (Figure 3). The mass spectra at the retention times 4.25, 4.82, and 5.14 min (Figure 4) provided the accurate masses of precursor or fragment ions that were critical to the identification of those compounds

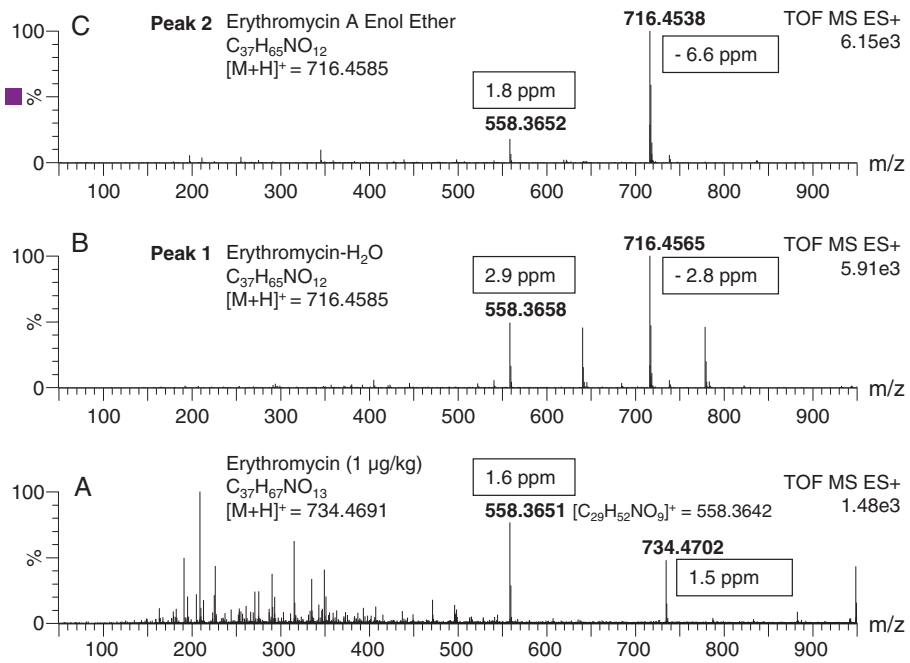

Figure 4. UHPLC QqTOF MS confirmation and identification of erythromycin A and its degradation products. (A) Mass spectrum of UHPLC peak at 4.25 min (erythromycin A). (B) Mass spectrum of UHPLC peak at 4.82 min (erythromycin A-H_2O or erythromycin A enol ether). (C) Mass spectrum of UHPLC peak at 5.14 min (erythromycin A-H_2O or erythromycin A enol ether). The spectra were obtained by 2 to 3 scans combined across the UHPLC peak with a number of background scans taken on each side of the peak subtracted.

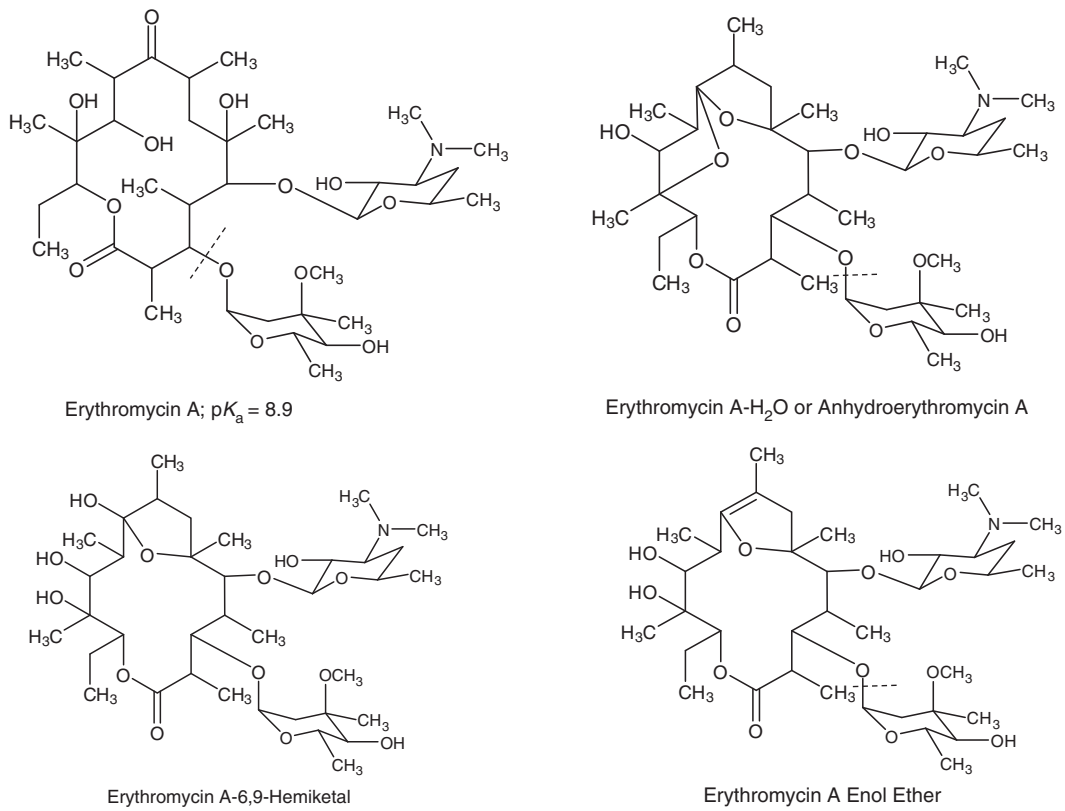

Figure 5. Chemical structures of erythromycin A and its degradation products.

and to propose a fragmentation pattern or a cleavage site along with the nitrogen rule (Figure 5). Peak 1 or 2 was tentatively identified as either erythromycin A-H_2O or erythromycin A enol ether. Apparently there was a significant amount of erythromycin A and erythromycin A enol ether in the sample extract. Based on our previous experience, the quantitative result of erythromycin A by SPE under a slight basic condition such as 0.1 M phosphate buffer (pH 8.0) was very reproducible with intermediate precision 3.4% in milk and 5.2% in eggs according to the same nested experimental design.[16,28] However, the intermediate precision was 39.4% in honey and 34.6% in milk in the current study. Apparently the extraction procedure, especially the acidic pH condition which could cause erythromycin A to degrade, contributed to the large variations. For the incurred sample, erythromycin A had broken down to its degradation products in honey as evidenced from the ratio between erythromycin A and its degradation products. The peak height ratio of erythromycin A-H_2O or erythromycin A enol ether from the incurred or spike samples was about 1:1 (e.g. in Figure 3B). The ratio of erythromycin A-H_2O or erythromycin A enol ether to erythromycin A was $>$20:1 in the incurred sample, whereas it was about 2:1 in spike samples. Therefore, erythromycin A-H_2O and erythromycin A enol ether detected in the incurred sample seemed mainly as a result of hydrolysis in the honey sample. To fully investigate the degradation of erythromycin A in food such as honey, there should be further studies to gain a better understanding of how the extraction conditions lead to the breakdown.

Conclusion

To develop a multi-class method to include all veterinary drugs in one single analytical run, challenges remain. Aminoglycosides were very polar compounds that were hardly retained on a reverse-phased column, which was often used for most of veterinary drug analysis. β-lactams, penicillins and tetracyclines were likely not recovered from the QuEChERs procedure. However, among the veterinary drugs investigated, 7 groups or classes were able to be determined in honey and milk samples using the modified QuEChERs without *d*-SPE. The method quantified 31 or screened up to 54 drugs (unbound) in honey, and quantified 34 or screened up to 59 drugs in milk at low trace level. UHPLC QqTOF along with the QuEChERs served as a generic method, which may be used for routine screening depending on the testing scope and be utilized for confirmation and metabolites investigation. The degradation of erythromycin A to its metabolites such as erythromycin A-H_2O or erythromycin A enol ether in food should be further investigated to determine their impact on food safety.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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