SIMULTANEOUS DETERMINATION OF 23 VETERINARY DRUGS IN POULTRY FEED USING QuEChERS METHOD BY ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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ABSTRACT. An analytical method was developed and validated for simultaneous analysis of 23 veterinary drugs belonging to 7 different classes. The method was based on QuEChERS (quick, easy, cheap, effective, rugged, and safe) extraction. The sample preparation included ultrasonication with a mixture of acetonitrile, methanol and McIlvaine buffer followed by phase separation with magnesium sulphate and sodium chloride addition. The supernatant was extracted with ethyl acetate and mixed with hexane to ensure fat removal from the matrix. It was then cleaned by using a primary-secondary amine, octadecylsilane (C_{18}) and graphitised carbon black. Detection and quantification was by a single analytical run using ultra-performance liquid chromatography coupled with electrospray ionisation and tandem mass spectrometry (UPLC–ESI–MS/MS) operating in both positive and negative multiple reaction monitoring (MRM). Chromatographic separation was on a C_{18} column using methanol and 2 mM ammomium acetate in water (pH4) and 2 mM ammonium acetate in methanol as the mobile phase. Validation was in accordance with international guidelines. Good linearity was obtained for the analytes with correlation coefficients higher than 0.9735. The limit of detection and limit of quantification of all drugs were 0.05 to 0.93 mg/kg and 0.15 to 3.11 mg/kg, respectively. Average analyte recoveries ranged from 83.7 to 109.9%, and the repeatability was lower than 9.9%. The validation results demonstrate that the described LC-MS/MS method is sensitive, repeatable and reliable for safety monitoring and control of veterinary drug use in poultry feed.

Keywords: poultry feed, veterinary drugs, multi-drugs, QuEChERS, UPLC-MS/MS

INTRODUCTION

Veterinary drugs are a group of substances belonging to different chemical classes and therapeutic areas, e.g. antibiotics, antiparasitics, non-steroidal anti-inflammatory drugs (NSAIDs), hormones and β-agonists. They are generally used to prevent or cure diseases, to reduce the potential of diseases or as a growth promoter to increase feed conversion (Dugane, 2000). Veterinary drugs present in foodstuff are

potential risks to consumers e.g. multiplying into dangerous metabolites and influencing antibiotic resistance of pathogens (Apata, 2012).

The use of certain antibiotics and hormones are banned in Malaysia including β-agonist, nitrofuran, chloramphenicol, nitroimidazole and stilbenes (Feed Act 2009, 2012). Analytical methods for detection of very low limits are required to support the enforcement of laws and regulations. One of the methods is the use of ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) equipped with an electron spray ionisation system.

Feed analysis is important in assuring the safety and quality of feed, as well as enhancing productivity and ensuring animal welfare. It supports the National Surveillance of Antimicrobial Resistance, Malaysia as well as Global Antimicrobial Resistance Surveillance System by controlling the usage of drugs in poultry-based food to reduce antimicrobial resistance in human.

Consumers' awareness on the side-effects from feed additives in poultry production as a growth promoter and disease preventer has led to the urgency for poultry feed analysis.

Feed additives are used in enhancing feed efficiency to achieve normal poultry weight for meat production. Veterinary drugs that are administered to the food-producing animal may lead to antibiotic residues in the edible tissues or eggs and it will affect consumer health. There is a need to create an effective and comprehensive screening method for monitoring the contamination levels of drugs due to the lack of improved surveillance of antibiotic (Al-Mogbel *et al*., 2015).

An easy and rapid analytical method for multiclass screening that is capable of detecting selected multi-type compounds in samples is important in the present research.

The ability of QuEChERS (quick, easy, cheap, effective, rugged and safe) method for extraction of multiclass residue has been used previously for analysis of 13 sulfonamides in poultry and swine feed (Lopes *et al.,* 2012). It was primarily developed in 2003, and involved extraction using buffered mixture of water and a water-miscible solvent in the presence of sodium chloride for salting-out effect and magnesium sulphate as a drying agent (Schenck *et al*., 2002). The QuEChERS method usually used dispersive solid phase extraction (dSPE) for sample clean up. Primary secondary amine (PSA), octadecylsilane (C_{18}) and graphitised carbon black (GCB) were used in the form of powder for dSPE to provide an extract suitable for chromatographic analysis. As a weak anion exchanger, PSA traps different types of polar organic acids in the extract (Maštovská *et al*., 2005). C_{18} was used to remove lipophilic compounds such as fats (Dagnac *et al*., 2009) while GCB was used to absorb pigments and sterol, for instance, chlorophyll (Lehotay *et al*., 2010).

The purpose of this study was to develop and validate the UPLC-MS/MS method using a modified QuEChERS sample preparation procedure for the confirmation of 23 veterinary drug in poultry feed.

MATERIALS AND METHOD

Standards and reagents

All reagents were of LC-MS grade. Acetonitrile (ACN) and methanol (MeOH) together with mobile phase buffer ammonium acetate (NH_4OAc) were purchased from Merck (Darmstadt, Germany). Formic acid (FA) was supplied by Fisher Scientific (Hampton, NH, USA). Water Optima™ LC/MS Grade, was used for mobile phase and purified water (Integral 5 A10 Pure Purification System, Millipore, Bedford, MA) was used for sample extraction.

Chemical used for sample extraction such as disodium hydrogen phosphate, citric acid monohydrate, magnesium sulphate anhydrous (MgSO4) and sodium chloride (NaCl) were purchased from Merck. Ethylenediaminetetraacetic acid disodium (EDTA) salt was supplied by Classic Chemicals (Selangor, Malaysia) while graphitised carbon black (GCB), primary secondary amine (PSA) and C_{18} end-capped were purchased from Agilent Technologies (Santa Clara, CA, USA). Nitrogen (99.999%) (for desolvation and nebuliser gas) was generated through a nitrogen generator from Peak Scientific, NM32LA. Argon (99.999%) (MS-MS collision gas) were obtained from Malaysian Oxygen (Kuala Lumpur, Malaysia).

All standards (a total of 23 veterinary drugs) were of high purity grade (>99.0%). The following veterinary drugs were purchased: erythromycin (ERY) and sulfaquinoxaline (SQX) from Sigma-Aldrich (St. Louis, MO, USA), sulfamethazine (SMZ), sulfadiazine (SDZ), sulfamethazine-$13C_{6,}$ tylosin (TYL), tilmicosin (TIL), chloramphenicol (CAP), florfenicol (FF), thiamphenicol (TAF), norfloxacin-D5 and roxithromycin (ROX) from Dr. Ehrenstorfer GmbH (Augsburg, Germany), ciprofloxacin (CIP), sulfadimethoxine (SDMO), dimetridazole (DMZ) and ronidazole (RNZ) from Fluka (St. Gallen, Switzerland), enrofloxacin (ENR), ipronidazole (IPZ), dimetridazole-D3, ipronidazole-D3, enrofloxacin-D5, furazolidone (FZD), furaltadone (FTD), nifuroxazide (NXZ), clenbuterol (CBR), salbutamol (SBM), terbutaline (TBL), ractopamine (RPM), metronidazole (MNZ) and norfloxacin (NOR) from Witega (Berlin, Germany), and Clenbuterol-D9, ractopamine-D3, and chloramphenicol-D5 from Cambridge Isotopes Laboratories (Andover, MA, USA).

Preparation of standard solutions and reagents

Each stock solution of all 23 compounds was prepared at 1000 µg/ml by dissolving 10 mg of standard in 10 ml volumetric flask using methanol. Intermediate standards solutions of 100 µg/ml were prepared by mixing the compounds based on seven different groups. The dilution was made by dissolving 1 ml (1000 µL) of standard in 10 ml volumetric flask using methanol. Both stock and intermediate solutions were stored at -20 °C. Working standard solutions were prepared by mixing 1 ml of intermediate standard solution for every group and diluting in a 10 ml volumetric flask using methanol. The working solutions were stored at 4 °C. McIlvaine buffer was prepared by mixing of 192.8 ml solution of 0.25 M disodium hydrogen phosphate with 307.3 ml

solution of 0.1 M citric acid and adjusted to pH4. A 18.61 g of EDTA was dissolved in the mixed solution.

Sample extraction and clean up

Sample extraction

The poultry's feed samples used in this study were supplied by the Department of Veterinary Services, Malaysia (DVS). Each sample of finely ground poultry feed (0.50±0.05 g) was weighed and transferred to a 50 ml Falcon™ polypropylene tube. The tube was homogenised for 15 minutes. 20 ml of methanol, acetonitrile and 0.1 M EDTA-McIlvaine buffer (20:50:30, v/v/v) was added to the tube and vortexed for 60 s before being placed into an ultrasonic bath for 10 minutes. The buffer was prepared in between pH4 to pH4.5. The mixture was centrifuged for 10 min at 4000 rpm at 5±1 °C.

Sample clean up

After centrifuging, the supernatant was subjected to liquid-liquid partitioning by transferring into 50 ml Falcon™ polypropylene tube containing 1g sodium chloride and 4g of magnesium sulphate.

To prevent formation of bulk magnesium sulphate crystals during the hydration process, the tube was sealed quickly and vigorously shaken for 1 minute. A 10 ml of ethyl acetate and n-heptane (50:50) was added into the tube. The tube was centrifuged for 10 min at 4000 rpm for 5±1 °C. The organic phase of the supernatant on the second layer (7 ml) was then transferred into a 15 ml boiling tube.

Under a stream of nitrogen, the sample was evaporated to dryness on a sample concentrator at 45 °C. The residue was reconstituted in 1.5 ml 14% ACN in 0.1% formic acid in water and vortexed for 30 s before the extract was transferred into 2 ml micro test tubes containing 15 mg of GCB, 40 mg of PSA and 10 mg C_{18} for the d-SPE cleanup method.

The micro test tube was vortexed for 30 s before centrifuging at 15,000 rpm and 5 °C in a microcentrifuge. The aqueous phase was filtered through a 0.45 µm PTFE filter directly into HPLC vial. The vial was capped and arranged on the LC autosampler. A 10 µl of the supernatant was injected into the LC system.

UPLC–MS/MS Determination

UPLC was performed on an Acquity UPLC™ I-Class FTN system (Waters, Manchester, UK), equipped with an electrospray ionisation interface (ESI). Chromatographic separation was carried out using an Acquity UPLC BEH C_{18} 1.7 µm particle size analytical column 100 mm × 2.1 mm with a mobile phase containing 2 mM NH4Ac in (A) water, and (B) methanol. The following gradient programme was used: A (90%) (0.25 min) and A (10%) (7.25 min). This was followed by a re-equilibration time of 2.50 min, to give a total run time of 10 min. The flow rate used for full analysis was 0.35 ml/min under column pressure less than 18,000 psi. The column temperature was kept at 45 °C and the sample manager temperature was maintained at 15 °C. The injection volume of sample per analysis was 10 µL.

MS/MS detection was performed using an Acquity TQS tandem quadrupole mass spectrometer (Waters, Manchester, UK). The ESI interface was used in positive ion (ESI+) and negative ion (ESI-) mode with the following settings: capillary voltage of 1.0 kV; source temperature of 150 °C; desolvation temperature of 600 °C; cone gas flow of 150 L/h; and desolvation gas flow of 1000 L/h. The transitions ion for quantification and qualifier ion of each analyte were determined by directly infusing the respective solutions (at 0.005 mg/L in water and acetonitrile (50:50) into the mass spectrometer ion source at a flow rate of 0.8 ml/min. Two multiple reaction monitoring (MRM) transitions were recorded for the 23 compounds. The most important parameters of MRM transitions of UPLC–ESI–MS/MS system for the acquisition and identification are summarised in Table 1. Dwell times were automatically selected to obtain enough points per peak. Data analysis and quantification were performed using the Waters MassLynx and TargetLynx software, respectively.

Method Validation

Method validation was carried out for the 23 veterinary drugs according to the procedures described in the Commission Decision (European Commission, 2002), ICH (2005) and other authors (Pietro *et al.*, 2014;

Table 1. Optimized MS/MS parameters using the UPLC–MS/MS method operating in ESI+ and ES- mode

Analyte	Electrospray ionization	Retention time window[a] (min)	Transition (*m/z*)	Cone voltage (V)	Collision energy (eV)
Roxithromycin	ESI+	5.42-5.52	837.5>158.1	36	34
Erythromycin	ESI+	4.89-4.99	734.5>158.1[b] 734.5>576.5	8 8	28 16
Tilmicosin	ESI+	5.05-5.15	869.5>174.2[b] 869.5>696.5	68 68	38 40
Tylosin	ESI+	5.12-5.22	916.5>174.1[b] 916.5>101.1	98 98	36 46
Nifuroxazide	ESI+	3.43-3.53	276.1>121.0	14	20
Furaltadone	ESI+	2.88-2.98	325.1>281.0[b] 325.1>252.2	8 8	14 14
Furazolidone	ESI+	2.28-2.38	226.1>139.1[b] 226.1>122.1	52 52	16 22
Clenbuterol-D6	ESI+	3.12-3.22	286.1>203.9	74	16
Ractopamine-D3	ESI+	2.72-2.82	305.1>167.1	78	14
Clenbuterol	ESI+	3.14-3.24	277.1>203.0[b] 277.1>132.0	64 64	14 28
Ractopamine	ESI+	2.73-2.83	302.2>164.1[b] 302.2>284.2	78 78	16 22

Table 1 continued next page

Table 1. (continued)

Analyte	Electrospray ionization	Retention time window[a] (min)	Transition (*m/z*)	Cone voltage (V)	Collision energy (eV)
Salbutamol	ESI+	1.68-1.78	240.2>148.1[b] 240.2>222.1	70 70	18 28
Terbutaline	ESI+	1.60-1.70	226.1>152.0[b] 226.1>107.0	66 66	16 28
Dimetridazole-D3	ESI+	2.15-2.25	145.0>99.0	2	22
Dimetridazole	ESI+	2.17-2.27	142.0>96.0[b] 142.0>81.0	2 2	22 22
Ipronidazole	ESI+	3.40-3.50	170.0>124.0[b] 170.0>109.0	2 2	18 24
Metronidazole	ESI+	1.82-1.92	172.0>128.0[b] 172.0>111.0	2 2	18 22
Ronidazole	ESI+	1.74-1.84	201.0>140.0[b] 201.0>55.0	32 32	10 20
Enrofloxacin-D5	ESI+	3.71-3.81	365.1>321.6	16	26
Norfloxacin-D5	ESI+	2.63-2.73	325.1>238.1	52	24
Ciprofloxacin	ESI+	2.73-2.83	332.1>314.1[b] 332.1>288.1	92 92	22 20
Enrofloxacin	ESI+	3.73-3.83	360.3>316.3[b] 360.3>342.3	100 100	22 20
Norfloxacin	ESI+	2.65-2.75	320.1>276.1[b] 320.1>233.0	90 90	18 24
Sulfamethazine-13C6	ESI+	2.52-2.62	285.0>186.0	46	16
Sulfadiazine	ESI+	1.72-1.82	251.0>156.0[b] 251.0>92.0	32 32	14 16
Sulfadimethoxine	ESI+	3.43-3.53	311.1>156.0[b] 311.1>92.0	38 38	20 24
Sulfamethazine	ESI+	2.54-2.64	279.1>186.0[b] 279.1>124.1	46 46	16 20
Sulfaquinoxaline	ESI+	3.52-3.62	301.1>156.1[b] 301.1>92.2	54 54	16 28
Chloramphenicol-D5	ESI-	3.47-3.57	320.9>152	2	18
Chloramphenicol	ESI-	3.49-3.59	321.2>152.2[b] 321.2>257.2	2 2	18 12
Florfenicol	ESI-	2.84-2.94	356.0>336.0[b] 356.0>185.0	56 56	10 22

Notes: a = retention time ± 0.05 b = *m/z* transition for quantification ion

Patyra *et al*., 2018). The performance criteria in terms of specificity, linearity, recovery and precision, analytical limits were conducted by spiking blank feed samples at different fortification levels. The method applies internal standard for quantification.

Twenty blank samples of six different types of poultry feed collected from several regions in the peninsula of Malaysia were evaluated to determine the selectivity of the method. The absence of interfering peaks was observed in the 5% range of the retention time window for each analyte peak.

The matrix-matched calibration curves were constructed by spiking feed samples with the working standard solution at five different levels in the following ranges: 0.75 to 3.75 mg/kg for ERY, 0.82 to 4.1 for TIL, 0.31 to 1.55 for TYL, 0.35 to 1.75 for FTD, 0.40 to 2.00 for FZD, 0.09 to 0.45 for CBR, 0.24 to 1.20 for RPM, 0.36 to 1.80 for SBM, 0.39 to 1.95 for TBL, 0.11 to 0.55 for DMZ, 0.09 to 0.45 for IPZ, 0.22 to 1.10 for MNZ, 0.08 to 0.40 for RNZ, 0.14 to 0.70 for CIP, 0.06 to 0.30 for ENR, 0.13 to 0.65 for NOR, 0.22 to 1.10 for SDZ, 0.36 to 1.80 for SDMO, 0.05 to 0.25 for SMZ, 0.46 to 2.30 for SQX, 0.14 to 0.70 for CAP, 0.56 to 2.80 for FF and 0.56 to 2.80 for TAF. Assessment of linearity was by determining the regression line via least square method followed by calculating the coefficient of determination (R^2).

The accuracy was expressed in terms of recovery, which was assessed by spiking blank samples at three concentration levels (lowest, medium and highest concentration). Each three-fortification level was analysed in three replicates for one day. Calculation of the recoveries was conducted by comparing the measured concentrations with the spiked concentrations of the samples. The precision was represented by the intra-day relative standard deviation (RSD). Ten replicates of three-fortification level were analysed to calculate the standard deviation (SD) and coefficient of variation (CV, %) for each level.

The limit of detection (LOD) and limit of quantification (LOQ) were estimated by analysing the spiked samples at five concentration levels: 0.15, 0.25, 0.50, 0.75 and 1.00 mg/kg. LOD and LOQ were calculated based on equation LOD = 3*Sa/r and LOQ = 10*Sa/r, where Sa/r is the standard deviation of residuals.

The stability of the working standard solution was tested monthly over a period of three months. Testing on stock solution was performed by analysing freshly prepared dilutions. Stability of active ingredients in the matrix feed was examined by spiking with 1000 mg/kg and stored in a refrigerator. Duplicate samples were taken from the fortified feed and analysed monthly for three months.

RESULTS AND DISCUSSION

Sample preparation

Components of poultry feed are varied based on energy requirement and category of poultry (starter, grower, finisher and layer). A complex matrix of poultry feed are normally made up of either cereal grains (wheat, barley, sorghum, and rye) (Singh and Kent-Jones, 2010) and oilseed meals (soybean and canola meal) (Khajali and Slominski, 2012; Dei, 2011) including many other additives such as amino acids, vitamins

or minerals (Tona, 2018). A method based on QuEChERS technique was used in this study to extract a wide range of different antimicrobial substances and hormone from a complex matrix sample of poultry feed. QuEChERS technique was modified based on sample extraction in milk (Olgun, Kaplan and Nazli, 2016; Arias *et al*., 2018), manure (Guo *et al*., 2016) and water (Cerqueira *et al*., 2014).

Method extraction was established by optimising the following factor: extraction solvent (methanol, acetonitrile and buffer), addition of ethyl acetate and n-heptane, concentration temperature (35, 45, and 55 °C), and an amount of graphitised carbon black (5, 15, 25, and 35 mg). Optimisation was by taking into account the method with low-cost, rapid and simple extraction.

Combination of 20 ml pH4, 0.1 M EDTA-McIlvaine buffer, acetonitrile and methanol were used as an extraction solvent in this method. EDTA and McIlvaine buffer were reported as a suitable chelating agent for sulfonamides due to the possible formation of chelate complexes with metal ions (Solliec *et al*., 2014). The buffer was prepared slightly higher than pH4 (between pH4 to pH4.5). A pH less than 4 is fragile for tylosin and leads to the formation of tylosin B metabolite (Pietroń *et al*., 2011). Protonation occurs only when pK_a of analytes is higher than the pH solution.

The best extraction solvents composition used were 20 ml methanol-acetonitrile-buffer (20:50:30, v/v/v). The extraction process showed that 50% and 70% buffer compositions resulted a long evaporation process. Analysis of individual analyte in combination with 30% buffer was favourable with methanol-acetonitril composition (10:60, v/v) for IPZ; MNZ, RNZ, DMZ, TIL, FZD and FF (20:50, v/v) for ERY; TYL, CAP and TAF (35:35, v/v) for CBR; NOR and CIP (50:20, v/v) for RPM; and TBL, SBM, SDZ, SMZ, SDMO, SQX and ENR (60:10, v/v) for FTD. The composition of methanol-acetonitrile (50:20, v/v) was chosen based on optimum recovery of all the 23 analytes.

The extraction procedure with and without addition of ethyl acetate and n-heptane showed a great difference. Addition of ethyl acetate gave a constant clear colour for all extracts and reduction of matrix interference in the analyses. Oil precipitate observed in the final extract was eliminated by adding the n-heptane before the concentration process.

Samples were concentrated under a stream of nitrogen at 45 °C. Degradation of analyte occurred under 55 °C as no peak was observed. Testing was conducted on different graphitised carbon black weight to remove the colour and to produce a higher recovery for the extracted sample. A constant clear extract was obtained by using graphitised carbon black at 15 mg or higher. An amount of 15 mg GBC produced optimum recovery for all the analytes.

The developed method was compared with two other methods: (1) using PRiME SPE as a clean-up process for the developed method, and (2) was adopted from a developed method using PRiME SPE for poultry egg analysis (Wang *et al*., 2017). Results from the analyses showed that the recovery for all drugs by using a developed method were between 18.70% to 135.54%. Recoveries for developed method equipped with PRiME SPE were 0.66% to 89.29% for 16 drugs except IPZ, MNZ, RPM, TBL, SBM,

Table 2 Validation parameter data for LOD, LOQ, linearity and R^2

Analyte	LOD (mg/kg)	LOQ (mg/kg)	Linearity (mg/kg)	R2
Erythromycin	0.63	2.10	0.75-3.75	0.9896
Tilmicosin	0.68	2.28	0.82-4.10	0.9986
Tylosin	0.29	0.96	0.31-1.55	0.9993
Furaltadone	0.30	1.01	0.35-1.75	0.9924
Furazolidone	0.36	1.18	0.40-2.00	0.9856
Clenbuterol	0.08	0.25	0.09-0.45	0.9921
Ractopamine	0.22	0.73	0.24-1.20	0.9843
Salbutamol	0.33	1.11	0.36-1.89	0.9884
Terbutaline	0.36	1.21	0.39-1.95	0.9859
Dimetridazole	0.10	0.33	0.35-1.75	0.9924
Ipronidazole	0.08	0.27	0.09-0.45	0.9921
Metronidazole	0.20	0.66	0.22-1.10	0.9947
Ronidazole	0.07	0.24	0.08-0.40	0.9812
Ciprofloxacin	0.13	0.42	0.14-0.70	0.9808
Enrofloxacin	0.05	0.17	0.06-0.30	0.9929
Norfloxacin	0.12	0.39	0.13-0.65	0.9915
Sulfadiazine	0.20	0.67	0.22-1.10	0.9882
Sulfadimethoxine	0.31	1.02	0.36-1.80	0.9874
Sulfamethazine	0.05	0.15	0.05-0.25	0.9735
Sulfaquinoxaline	0.39	1.31	0.46-2.32	0.9974
Chloramphenicol	0.12	0.41	0.14-0.70	0.9793
Florfenicol	0.51	1.70	0.56-2.80	0.9947
Thiamphenicol	0.93	3.11	1.12-7.84	0.9905

Table 3 Average RSD and recovery for obtained for 23 compounds in poultry feed

Analyte	Concentration (mg/kg)			Repeatibility[a] (%)			Recovery[b] (%)		
Erythromycin	0.75	2.25	3.75	7.70	8.40	2.26	87.0	107.5	96.7
Tilmicosin	0.82	2.46	4.10	7.83	8.15	3.10	83.7	109.8	94.0
Tylosin	0.31	0.93	1.55	8.86	3.28	4.33	93.7	109.9	100.8
Furaltadone	0.35	1.05	1.75	2.89	5.80	2.52	92.4	108.7	104.0
Furazolidone	0.40	1.20	2.00	3.36	4.64	6.06	89.6	108.3	99.0
Clenbuterol	0.09	0.27	0.45	5.52	1.52	3.46	105.4	100.6	103.5
Ractopamine	0.24	0.72	1.20	4.33	8.10	2.80	98.8	103.3	100.5
Salbutamol	0.36	1.08	1.89	9.91	8.44	3.24	99.5	100.9	99.8
Terbutaline	0.39	1.17	1.95	6.36	6.63	7.92	103.8	108.0	103.1
Dimetridazole	0.35	1.05	1.75	2.51	2.24	2.16	102.1	101.7	101.0
Ipronidazole	0.09	0.27	0.45	1.73	2.37	2.72	95.9	101.1	100.4
Metronidazole	0.22	0.66	1.10	7.86	1.76	1.84	97.7	104.3	105.5
Ronidazole	0.08	0.24	0.40	2.02	1.59	2.56	98.8	101.0	102.8
Ciprofloxacin	0.14	0.42	0.70	3.92	5.15	5.75	91.4	106.9	96.1
Enrofloxacin	0.06	0.18	0.30	1.47	3.92	1.65	98.1	105.0	100.7
Norfloxacin	0.13	0.39	0.65	9.77	0.43	4.92	91.9	108.5	95.1
Sulfadiazine	0.22	0.66	1.10	9.75	5.90	3.83	85.1	109.3	101.2
Sulfadimethoxine	0.36	1.08	1.80	9.76	5.89	2.46	96.0	104.4	104.1
Sulfamethazine	0.05	0.15	0.25	9.48	7.74	0.74	89.7	106.1	104.9
Sulfaquinoxaline	0.46	1.380	2.32	9.67	6.16	1.82	88.4	107.0	105.6
Chloramphenicol	0.14	0.42	0.70	9.60	9.36	6.53	108.1	90.1	107.7
Florfenicol	0.56	1.68	2.80	7.01	8.85	8.17	102.9	105.8	100.4
Thiamphenicol	1.12	3.36	7.84	9.77	8.09	4.00	103.3	108.3	91.1

Notes: a RSD%, n=10 b n=3

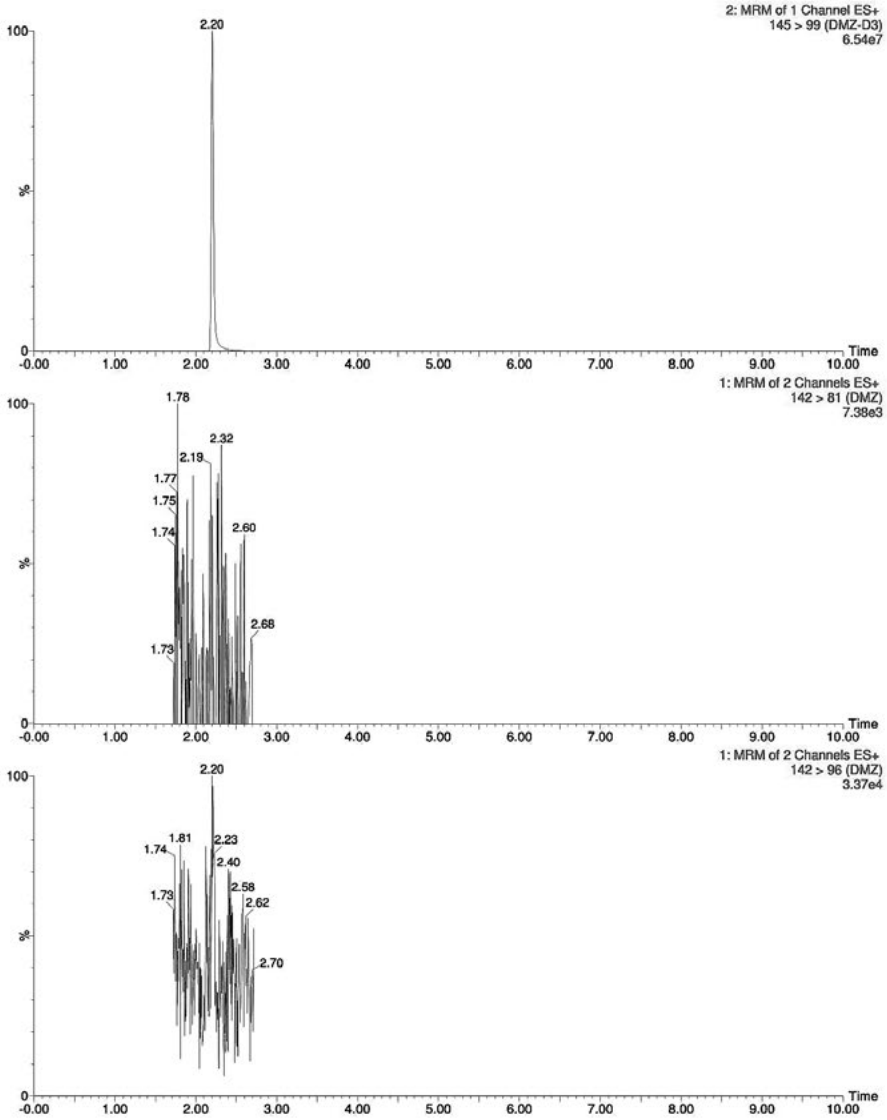

Figure 1a. Chromatogram for blank feed sample

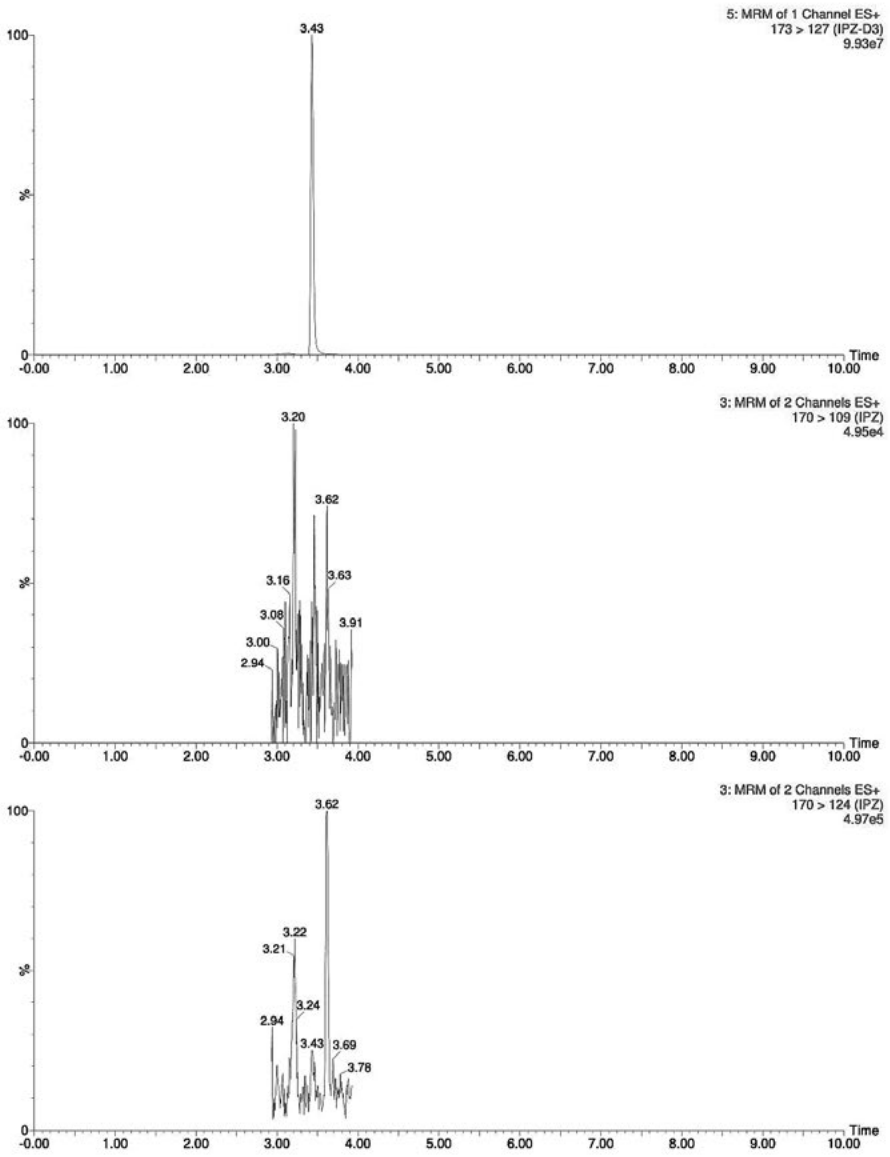

Figure 1b. Chromatogram for blank feed sample

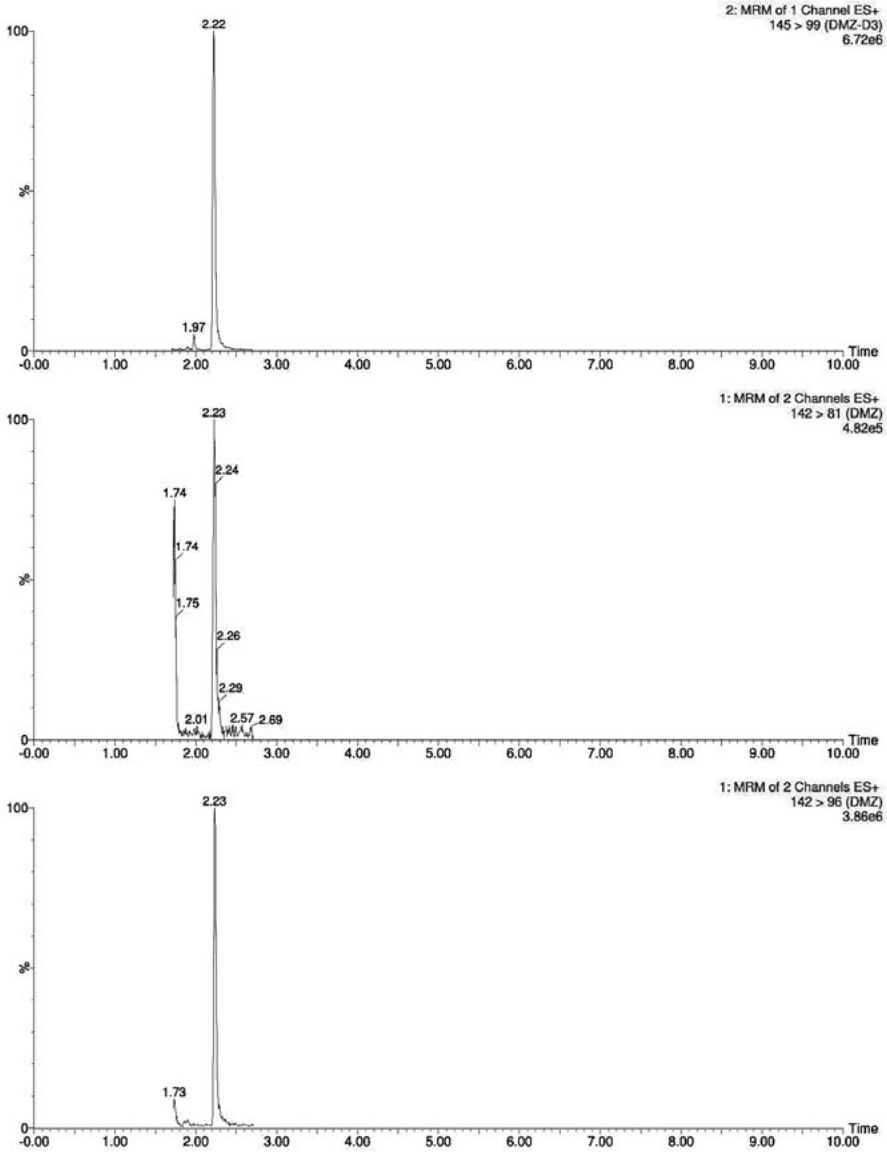

Figure 2a. Chromatogram for spiked feed sample at 0.55 µg/kg

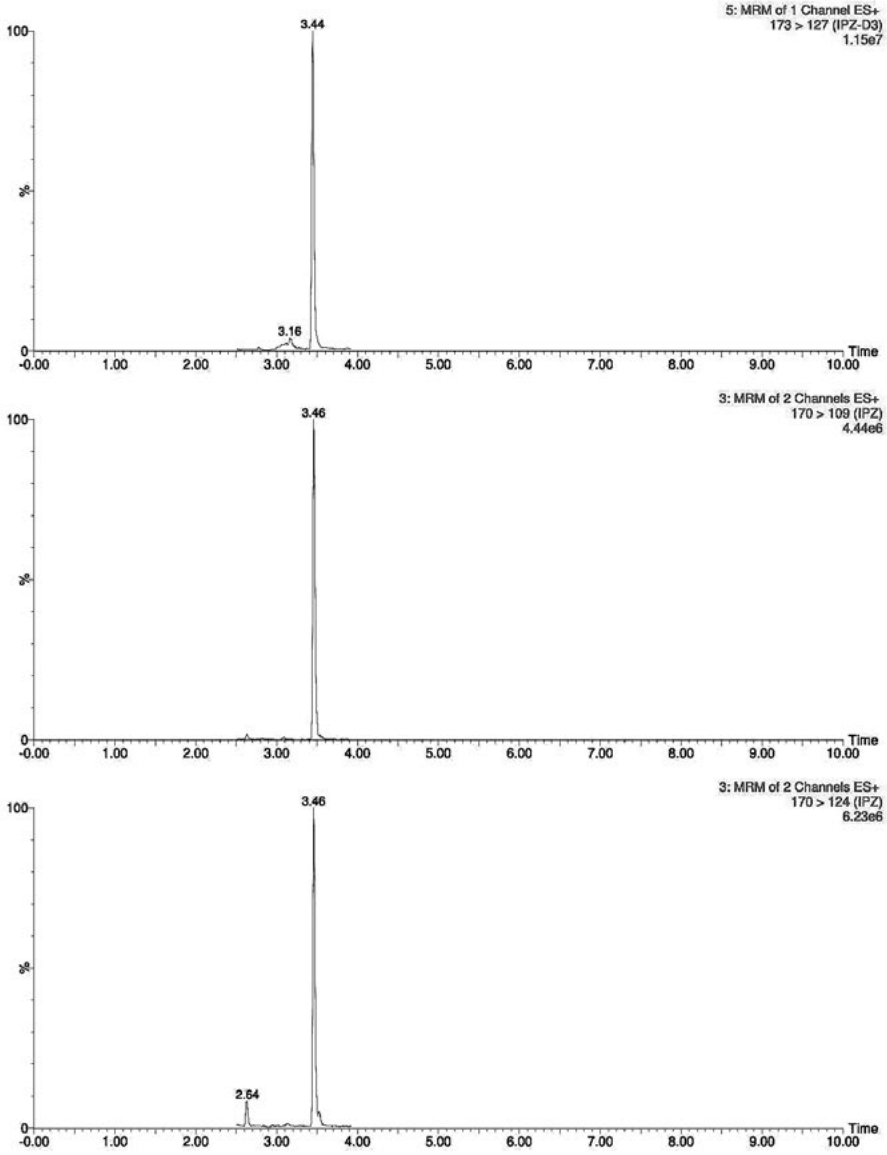

Figure 2b. Chromatogram for spiked feed sample at 0.55 µg/kg

CBR and FZD while the other method eluted 17 drugs except IPZ, MNZ, RPM, CBR, FZD and SDMO with recovery between 0.28% to 119.68%. Recovery for the drugs of interest were then enhanced by optimising the UPLC-MS/MS.

UPLC-MS/MS Optimisation

The detection of all 23 veterinary drugs in poultry feed was carried out using a reverse-phase LC-ESI-MS/MS. Individual standard solutions of 1 µg/ml in methanol were directly infused in the MS/MS system at a flow rate of 1.0 ml/min. Experiments were carried out with ESI ionisation in positive and negative mode. The majority of the compounds (20 out of 23) were determined with ESI operating in positive ionisation mode. CAP, TAF and FF were determined in negative ionisation using $[M-H]^-$ as precursor ion. Full-scan mass spectra were acquired to obtain at least one precursor ion and the optimum cone voltage. Furthermore, product ion scan at different collision energies was carried out to determine the most abundant product ion for each compound for quantification and identification purposes, according to the European Commission criteria (European Commission, 2002). In the MRM mode, the transition of the most abundant product transition (quantitative) ion was selected for quantification, while the second least abundant transition (target or confirmatory) ion was used for identification by calculating the ratio of the target ion to the quantification ion. The MRM parameters (transitions, cone voltages and collision energies) obtained in positive and negative mode are listed in Table 1. For legal compound identification and confirmation, at least three identification points (IPs) are required while four or more IPs are needed for prohibited compound (European Commission, 2002).

In this study, different mobile phases (acetonitrile and methanol) with different compositions (FA and ammonium acetate at various concentrations) were tested. The effects of pH and ionic strength of the mobile phase on the peak shape, resolution and efficiencies were evaluated by varying the buffer concentration. A gradient consisting of 2 mM ammonium acetate at pH 5 in water (mobile phase A) and 2 mM ammonium acetate in methanol (mobile phase B) was chosen as an appropriate mobile phase it produces a better peak shape and resolution than formic acid.

Resolution of nitrogen-containing compound on reverse-phase column can be enhanced by using the mobile phase of ammonium acetate and at the same time support the deprotonation of ESI- mode analyte by reducing the composition of H^+ proton in the environment. Formation of ammonium adduct on ESI+ mode analyte is a cutback for this mobile phase usage. Ammonium adduct formation can be overcome by adding formic acid for protonation before introducing to UPLC, therefore formic acid was used as a mixture of solution to reconstitute the sample. Figures 1a and 1b, and Figures 2a and 2b, respectively, show the chromatogram peak for blank feed and spiked feed with all selected analysed compounds.

Method validation

This study introduces a simple and rapid sample preparation based on QuEChERS method for the determination of 23 multi veterinary drugs in a single analysis. European Union regulation 2002/657/EC was used as guidance to validate this procedure. The accuracy and precision for each analyte in terms of trueness spiking and repeatability (CV %, intra-day precision) were determined using known spiked blank feed sample due to lack of certified reference material.

The relative recoveries for all analyte using this method ranged from 83.7 to 109.9%. The repeatability for all 23 analytes were lower than 9.91%. Summary of all analyte LOD and LOQ values ranged from 0.05 to 0.93 mg/kg and 0.15 to 3.11 mg/kg respectively. From the analysis we can conclude that calculation of detection limit and quantification limit for compounds that have identical internal standard are low for instance CAP with CAP-D_5, IPZ with IPZ-D_3 and CBR with CBR-D_9. The selectivity of the method was evaluated by the analysis of blank samples. The absence of any chromatographic signal at the same retention time as the target compounds indicated the absence of veterinary drugs or matrix interferences. The parameter for all analytes is shown in Table 2 and Table 3.

CONCLUSION

In this study, a modified QuEChERS method was successfully developed, optimized and validated for extraction of 23 types of veterinary drugs from poultry feed. The validation results showed that the method fit the purpose and was satisfactory in terms of linearity, selectivity and accuracy. The method was simple, cost-effective and can be used as a safety monitoring and controlling veterinary drug use in poultry feed.

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