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Multiresidue method for the simultaneous analysis of antibiotics and mycotoxins in feeds by ultra-high performance liquid chromatography coupled to tandem mass spectrometry

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ABSTRACT

Residues in animal feeds and foods of animal origin have been important safety issue concerning both human and animal health. A multiresidue method for determination of eight mycotoxins and ten antibiotics was developed and validated in animal feeds by using QuEChERS (quick, easy, cheap, effective, rugged, and safe) extraction followed by UHPLC-MS/MS. Optimisation of UHPLC-MS/MS parameters was performed to achieve good separation and resolution. The method was validated according to the European Commission Decision 2002/657/EC. Matrix matched calibration curves showed good r^2 ($\geq$0.995) values, and limit of quantification (LOQ) values varied between 1.2 and 5.2 μg kg^{-1}. Average recoveries ranged from 60 to 102% with relative standard deviations of 2.2 and 15.6% for all type of feed samples except for tetracyclines, lincomycin, tylosin, ochratoxin A, and fumonisin (B_1 and B_2).

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KEYWORDS

multiresidue, feed, QuEChERS, UHPLC-MS/MS, validation

1. INTRODUCTION

Antibiotics are used regularly for the treatment of diseases in animals. Additionally, they are applied to animals for enhancing feed efficiency and growth rate (Greenless et al., 2012). Residues can occur in the edible tissues of the animals due to antibiotic usage in food producing animals. These residues can be toxic for humans and may develop allergic reactions or produce antibiotic-resistance pathogens in humans. Furthermore, they can even cause death (Gentili et al., 2005; Wang et al., 2006). Mycotoxins are small and toxic chemical products formed by different fungal species. These species can contaminate feedstuff with toxins during cultivation or after harvest and cause a toxic response when ingested by vertebrate species (Turner et al., 2009; Wang et al., 2015). Humans can be exposed to mycotoxins through the consumption of contaminated agricultural products or their metabolites in animal-derived products such as milk and egg. Additionally, exposure can also occur via dermal contact (Capriotti et al., 2012).

QuEChERS method consists of two steps: extraction with acetonitrile followed by liquid-liquid partitioning and purification with dispersive solid-phase extraction (d-SPE). The method was introduced as multiresidue analysis for pesticide residues in high moisture fruits and vegetables in 2002 (Rejczak and Tuzimski, 2015). However, applications of this method have been reported to additionally detect residues of antibiotics (Lopes et al., 2012; Robert et al., 2015) and mycotoxins (Dzuman et al., 2014; Qian et al., 2018) in feeds. UHPLC-MS/MS device has been extensively used for detection, identification, and quantification of multiclass antibiotic residues (Boscher et al., 2010; Zhang et al., 2013; Qian et al., 2019) or multiclass mycotoxin residues (Streit et al., 2013; Dzuman et al., 2014; Wang et al., 2015), individually. The objective of the present work was to develop a multi-residue UHPLC-MS/MS method using QuEChERS extraction to simultaneously detect and quantify antibiotics and mycotoxins in different types of animal feeds. The antibiotic and mycotoxin standards were selected according to their use in all food producing animal species (Ronquillo and Hernandez, 2017) and the degree of contamination in feed (Streit et al., 2013), respectively.

2. MATERIALS AND METHODS

2.1. Samples and chemicals

Twenty-seven different feed samples for poultry, cattle, and fish were collected from local feed markets in Antalya, Turkey and stored at 4 °C prior to analysis. All standards and chemicals were of high purity grade and supplied by Sigma-Aldrich (Steinheim, Germany). QuEChERS extraction-dispersive kits (Bond Elut) were supplied by Agilent (CA, USA). Individual stock solutions were prepared at 1,000 mg L^{-1} in methanol. A working mix from the standard solution of 5 mg L^{-1} was prepared by transferring an appropriate aliquot of each stock solution into methanol. All stocks and working solutions were stored at −18 °C in the dark.

2.2. QuEChERS extraction

The QuEChERS extraction method was performed according to AOAC Official Method (AOAC, 2007).

2.3. UHPLC-MS/MS

UHPLC-MS/MS analyses were performed with a triple quadrupole TSQ Quantum Access Max (Thermo Fisher Scientific, CA, USA) equipped with Accela UHPLC system (Thermo Fisher Scientific, CA, USA). Chromatographic separations were performed with a Hypersil Gold^{TM} aQ column (100 × 2.1 mm, 1.9 µm, Thermo Fisher Scientific, CA, USA). The mobile phase A consisted of water with 0.5 mM oxalic acid and 1 mM ammonium formate, and the mobile phase B consisted of methanol with 0.2 mM oxalic acid. The gradient elution was: 90% A and 10% B for 2.5 min, then linearly changed to 0% A and 100% B in 1.5 min, and held constant for 2 min; then linearly changed 90% and 10% in 1 min, and finally held constant for 2 min. The total run time was 9 min. The flow rate of the mobile phase was 400 µL min^{-1}, the column temperature was set at 40 °C, and the injection volume was 10 µL. Electrospray ionisation was performed in the positive ion mode (ESI+), and the mass spectrometer was operated in a multiple-reaction monitoring (MRM) mode. The ion spray voltage was set at 3.2 kV, capillary voltage at 35 V, and the tube lens voltage at 82 V. The capillary temperature and vaporiser temperature were set at 250 °C and 350 °C, respectively. Sheath and auxiliary gas flow rates were 40 and 10 units, respectively. Data acquisition was done with Xcalibur 2.1 software with Qual and Quanbrowser.

2.4. Method validation

The following parameters were evaluated during method validation within laboratory: selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy and precision (repeatability and reproducibility) (Commission Decision, 2002).

2.5. Statistical analysis

Significant differences among UHPLC parameters were evaluated with analysis of variance (ANOVA) by using SAS System Software (SAS Institue Inc., Cary, NC, USA). Duncan's Multiple Range test ($P < 0.05$) was used to compare significant differences observed in mean values of the results.

3. RESULTS AND DISCUSSION

3.1. UHPLC-MS/MS parameters

Hypersil Gold^{TM} aQ column was selected to ensure good peak shape and resolution when using gradient elution with aqueous mobile phase (Konak et al., 2017). Therefore, UHPLC application was performed with reversed-phase chromatography by using Hypersil Gold^{TM} aQ column in this study. In order to optimise the chromatographic conditions for antibiotics and mycotoxins, effects of different mobile phases (methanol and acetonitrile) and mobile phase additives (formic acid, acetic acid, and oxalic acid) on signal intensity were studied first.

In general, better separation in UHPLC was obtained with methanol than acetonitrile. In addition, the intensity of the target analytes was also higher in MS with methanol ($P < 0.05$). Different concentrations of formic acid (0.05 and 0.1%), acetic acid (0.1 and 0.2%), and oxalic acid (0.25 and 0.5 mM) were used to enhance ionisation of the analytes. The results showed that signal intensities of the analytes were higher at the concentrations of 0.05% for formic acid, 0.1% for acetic acid, and 0.5 mM for oxalic acid ($P < 0.05$). Furthermore, the highest signal intensity of the analytes was obtained by using oxalic acid instead of formic or acetic acid ($P < 0.05$). Moreover, optimum peak shapes and reproducible analyte signals were achieved by using oxalic acid with ammonium formate (1 mM) in methanol. The use of an acidic mobile phase with salt promoted positive ionisation and provided the stability of the analysis.

Different gradient elution programs were tested to provide the desired separation of the target analytes. Additionally, other parameters such as flow rate (400, 500, and 600 μL min^{-1}), column temperature (20, 30, and 40 °C), and injection volume (10 and 20 μL) were also tested to get a fast separation and good peak shape. The best result was obtained when the flow rate was 400 μL min^{-1}, column temperature was 40 °C, and injection volume was 10 μL ($P < 0.05$).

Mass spectrometry parameters were optimised to achieve the highest signal intensity for the target analytes (Fig. 1). Each standard solution was infused directly into the mass analyser in order to determine the precursor ion and two product ions of the target analytes in scan mode.

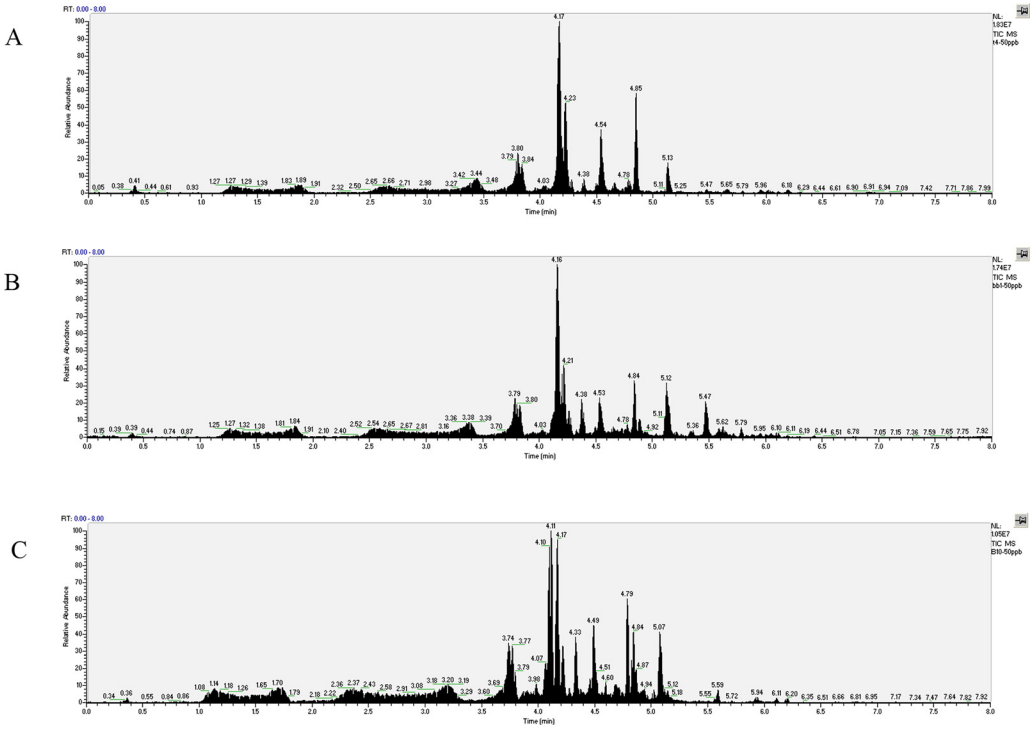

Fig. 1. UHPLC-MS/MS chromatograms for poultry (A), cattle (B), and fish (C) feed spiked with 50 μg kg^{-1}

Table 1. Retention time (RT) and multiple reaction monitoring conditions for each compound

Compound	RT (min)	Precursor ion (m/z)	Quantifier ion (m/z)	Qualifier ion (m/z)	CE (eV)
Sulfamerazine	1.15–2.05	265	156	172	20
Lincomycin	2.30–3.30	407	126.2	359	25
Sulfamethazine	2.40–3.63	279	186	156	20
Tetracycline	3.80	445	410	427	15
Oxytetracycline	3.84	461	426	443	15
Sulfadimethoxine	4.17	311	156	245	20
Chlortetracycline	4.18	479	444	462	20
Sulfaquinoxaline	4.23	301	156	108.2	18
Aflatoxin G1	4.28	329	243	215	35
Aflatoxin B2	4.34	315	259	287	35
Aflatoxin B1	4.38	313	241	269	35
Tylosin	4.50	917	174	773.1	35
Erythromycin	4.54	734	576	558	20
Aflatoxin G2	4.54	331	313	285	35
Fumonisin B1	4.61	723	353	705.1	35
Ochratoxin A	4.76	426	261	279	25
Fumonisin B2	4.78	706	336	354	35
Sterigmatocystin	4.85	325	310	281	25

The collision energy was optimised individually for each analyte. Retention time (RT) and MS/MS transitions for quantification and confirmation of the analytes are shown in Table 1.

3.2. Method validation

Performance characteristics of the method were established by spiked blank feed samples selected from 27 samples. The linearity was evaluated by using six calibration points (0, 10, 25, 50, 75, and 100 μg kg^{-1}) in the range of 0–100 μg kg^{-1}. Peak area was selected as response and a coefficient of determination (r^2) higher than 0.995 was obtained for all analytes. The low LOD and LOQ values of the developed method had the advantages of high selectivity, accuracy, and precision in simultaneous determination of the analytes.

Recovery experiments for the entire QuEChERS extraction and UHPLC-MS/MS procedure were carried out using blank matrix fortified at 10 and 100 μg kg^{-1} with six replicates for each fortification level. The obtained results showed that the extraction procedure was suitable for most of the analytes except for tetracycline, oxytetracycline, chlortetracycline, ochratoxin A, and fumonisin (B_1 and B_2). Likewise, lower recovery values were observed for tetracycline groups during acetonitrile extraction performed on animal feed. Researchers noticed that the high volume of acetonitrile employed caused co-precipitation of the analytes with the proteins (Aguilera-Luiz et al., 2013). On the other hand, another study showed that clean-up step after extraction led to lower recoveries for tetracyclines (Bourdat-Deschamps et al., 2014). However, a clean-up step was essential to obtain accurate results, because interfering compounds could be co-extracted with the target compounds during extraction and caused a reduction in the lifetime of the chromatographic column. Recovery, repeatability (intraday precision), and reproducibility

(interday precision) values belonging to the analytes used in the validation procedure within the laboratory were calculated for each matrix and summarised in Tables 2–4. According to the results, the average recovery values (between 60 and 102%) were acceptable for most target compounds at two concentration levels, except for aflatoxin G2 with recovery of 58.3–59.2% in poultry feed. Additionally, RSD values were within the acceptable limit of 20%. However, low recovery values of lincomycin and tylosin were obtained, especially at the lowest concentration level. It could be due to high polarity of the analytes, which resulted in low extraction with

Table 2. Validation parameters of the method in poultry feed

Compound	10 μg kg^{-1}			100 μg kg^{-1}		
	Recovery (%)	Intraday precision (%)	Interday precision (%)	Recovery (%)	Intraday precision	Interday precision (%)
Tylosin	12.96	13.81	13.72	27.90	19.61	21.03
Lincomycin	20.01	4.33	1.28	23.96	10.38	11.72
Aflatoxin G2	59.15	5.92	5.38	58.29	14.97	17.60
Aflatoxin B1	71.11	7.35	4.21	75.43	10.60	5.18
Aflatoxin G1	73.51	6.55	2.70	64.01	14.09	14.03
Sulfadimethoxine	73.80	3.35	2.10	76.45	4.94	2.58
Sulfamethazine	75.86	5.54	3.68	89.89	5.40	5.14
Aflatoxin B2	76.49	5.21	1.52	80.33	14.56	6.22
Sulfaquinoxaline	77.13	7.75	6.0	72.73	4.45	3.43
Erythromycin	84.04	5.76	6.62	69.40	11.03	8.07
Sulfamerazine	90.67	4.35	3.47	78.54	6.69	4.15
Sterigmatocystin	95.05	3.99	4.42	82.95	4.99	4.36

Table 3. Validation parameters of the method in cattle feed

Compound	10 μg kg^{-1}			100 μg kg^{-1}		
	Recovery (%)	Intraday precision (%)	Interday precision (%)	Recovery (%)	Intraday precision (%)	Interday precision (%)
Tylosin	13.82	16.60	9.23	20.70	15.88	17.97
Lincomycin	14.96	10.55	13.22	16.30	7.46	6.89
Aflatoxin G2	69.87	3.93	4.26	91.59	11.14	3.14
Aflatoxin B1	71.02	5.28	4.42	72.45	7.27	10.38
Aflatoxin G1	75.31	9.74	4.57	73.50	8.85	6.33
Sulfadimethoxine	75.60	3.43	2.35	68.16	8.53	6.86
Sulfamethazine	76.44	8.38	9.74	69.43	2.24	1.61
Aflatoxin B2	77.88	7.26	8.21	65.14	5.64	5.82
Sulfaquinoxaline	78.39	9.08	5.60	76.50	4.76	2.95
Erythromycin	78.41	4.08	1.90	79.07	7.18	6.50
Sulfamerazine	84.34	4.01	2.77	85.38	4.53	2.76
Sterigmatocystin	89.27	5.29	6.07	72.29	3.26	1.62

Table 4. Validation parameters of the method in fish feed

Compound	10 μg kg^{-1}			100 μg kg^{-1}		
	Recovery (%)	Intraday precision (%)	Interday precision (%)	Recovery (%)	Intraday precision (%)	Interday precision (%)
Lincomycin	25.57	11.23	10.08	45.01	7.25	2.99
Tylosin	29.27	6.59	6.09	43.06	16.73	18.41
Aflatoxin G1	62.12	11.81	11.86	68.04	10.83	7.40
Aflatoxin B2	66.81	9.46	11.25	69.69	10.93	8.46
Sulfadimethoxine	67.81	6.80	4.99	65.79	4.23	2.84
Aflatoxin B1	70.03	15.60	10.68	61.37	6.52	3.09
Sulfamethazine	72.84	5.50	5.12	86.01	8.50	11.04
Sulfaquinoxaline	74.18	3.92	3.13	66.10	4.33	3.63
Sulfamerazine	78.90	4.71	3.70	92.55	7.00	8.74
Aflatoxin G2	79.47	6.69	5.39	66.55	9.29	6.44
Erythromycin	80.35	10.26	10.26	101.50	10.41	13.11
Sterigmatocystin	84.74	4.51	2.87	68.76	5.41	2.36

acetonitrile. In addition, differences between the recovery values of some analytes in different matrices could be due to the matrix effect resulting from the matrix complexity.

4. CONCLUSIONS

A multiresidue method was developed for rapid and simultaneous determination of antibiotics and mycotoxins in animal feeds. Chromatographic separation and detection of the target analytes were achieved in a single run via UHPLC-MS/MS conditions. The developed method was applied to the analysis of 27 animal feed samples (9 for poultry, 8 for cattle, and 10 for fish) collected from local feed markets in Antalya, Turkey, and no positive results were observed in feed samples. Standard QuEChERS extraction method can be improved by applying different extraction solvents or acidifying agents to increase the extraction yield and also the number of extracted analytes. Recovery values can be increased with changing the polarity of the extraction solvents.

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